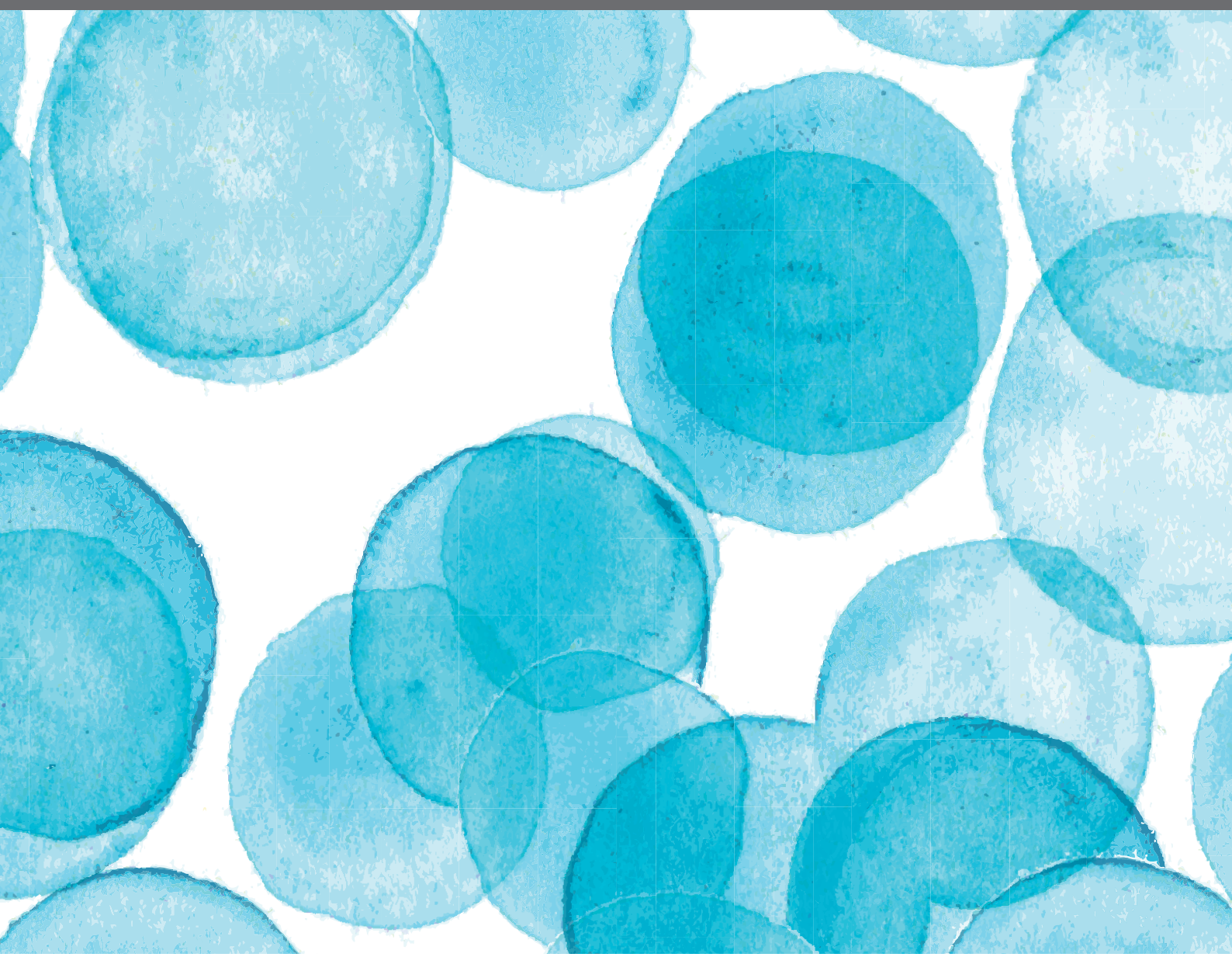
HARNESSING USEFUL RHIZOSPHERE MICROORGANISMS FOR PATHOGEN AND PEST BIOCONTROL, VOLUME II

EDITED BY: Aurelio Ciancio, Corné M. J. Pieterse and Jesús Mercado-Blanco

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HARNESSING USEFUL RHIZOSPHERE MICROORGANISMS FOR PATHOGEN AND PEST BIOCONTROL, VOLUME II

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Image: Aurelio Ciancio.

The use of biocontrol agents and beneficial organisms for management of plant and pest diseases appears as an environment-friendly and economic procedure. However, this option is not always available, depending on the lack of knowledge on the mechanisms of natural regulation, locally effective. In this view, this eBook considers studies and experimental works illustrating a range of problems and solutions based on microbial resources, suitable for management of biotic stress factors. These examples show how detailed data and knowledge on the organisms involved are of paramount importance to achieve a sustainable and durable management capability.

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Table of Contents

07 ***Editorial: Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol - Second Edition***
Aurelio Ciancio, Corné M. J. Pieterse and Jesús Mercado-Blanco

SECTION 1
PHYTOPHTHORA

12 ***Combining Different Potato-Associated* Pseudomonas *Strains for Improved Biocontrol of* Phytophthora infestans**
Mout De Vrieze, Fanny Germanier, Nicolas Vuille and Laure Weisskopf

25 ***Versatile Antagonistic Activities of Soil-Borne* Bacillus *spp. and* Pseudomonas *spp. Against* Phytophthora infestans *and Other Potato Pathogens***
Simon Caulier, Annika Gillis, Gil Colau, Florent Licciardi, Maxime Liépin, Nicolas Desoignies, Pauline Modrie, Anne Legrève, Jacques Mahillon and Claude Bragard

SECTION 2
BIOCONTROL OF FUNGAL DISEASES

40 ***Involvement of the Transcriptional Coactivator ThMBF1 in the Biocontrol Activity of* Trichoderma harzianum**
M. Belén Rubio, Alonso J. Pardal, Rosa E. Cardoza, Santiago Gutiérrez, Enrique Monte and Rosa Hermosa

52 ***Modulation of Tomato Response to* Rhizoctonia solani *by* Trichoderma harzianum *and its Secondary Metabolite Harzianic Acid***
Gelsomina Manganiello, Adriana Sacco, Maria R. Ercolano, Francesco Vinale, Stefania Lanzuise, Alberto Pascale, Mauro Napolitano, Nadia Lombardi, Matteo Lorito and Sheridan L. Woo

71 ***Rhizosphere Competence and Biocontrol Effect of* Pseudomonas *sp. RU47 Independent From Plant Species and Soil Type at the Field Scale***
Susanne Schreiter, Doreen Babin, Kornelia Smalla and Rita Grosch

81 ***Relationships Between Root Pathogen Resistance, Abundance and Expression of* Pseudomonas *Antimicrobial Genes, and Soil Properties in Representative Swiss Agricultural Soils***
Nicola Imperiali, Francesca Dennert, Jana Schneider, Titouan Laessle, Christelle Velatta, Marie Fesselet, Michele Wyler, Fabio Mascher, Olga Mavrodi, Dmitri Mavrodi, Monika Maurhofer and Christoph Keel

103 ***Potential of Finger Millet Indigenous Rhizobacterium* Pseudomonas *sp. MSSRFD41 in Blast Disease Management—Growth Promotion and Compatibility With the Resident Rhizomicrobiome***
Jegan Sekar, Kathiravan Raju, Purushothaman Duraisamy and Prabavathy Ramalingam Vaiyapuri

119 *Indigenous* Pseudomonas *spp. Strains From the Olive (*Olea europaea *L.) Rhizosphere as Effective Biocontrol Agents Against* Verticillium dahliae*: From the Host Roots to the Bacterial Genomes*
Carmen Gómez-Lama Cabanás, Garikoitz Legarda, David Ruano-Rosa, Paloma Pizarro-Tobías, Antonio Valverde-Corredor, José L. Niqui, Juan C. Triviño, Amalia Roca and Jesús Mercado-Blanco

138 Bacillus velezensis *FZB42 in 2018: The Gram-Positive Model Strain for Plant Growth Promotion and Biocontrol*
Ben Fan, Cong Wang, Xiaofeng Song, Xiaolei Ding, Liming Wu, Huijun Wu, Xuewen Gao and Rainer Borriss

152 *Corrigendum:* Bacillus velezensis *FZB42 in 2018: The Gram-Positive Model Strain for Plant Growth Promotion and Biocontrol*
Ben Fan, Cong Wang, Xiaofeng Song, Xiaolei Ding, Liming Wu, Huijun Wu, Xuewen Gao and Rainer Borriss

153 *Chemosensitization of* Fusarium graminearum *to Chemical Fungicides Using Cyclic Lipopeptides Produced by* Bacillus amyloliquefaciens *Strain JCK-12*
Kihyun Kim, Yoonji Lee, Areum Ha, Ji-In Kim, Ae Ran Park, Nan Hee Yu, Hokyoung Son, Gyung Ja Choi, Hae Woong Park, Chul Won Lee, Theresa Lee, Yin-Won Lee and Jin-Cheol Kim

169 *Antifungal Activity of Lipopeptides From* Bacillus *XT1 CECT 8661 Against* Botrytis cinerea
Laura Toral, Miguel Rodríguez, Victoria Béjar and Inmaculada Sampedro

181 *Complete Genome Sequence of Industrial Biocontrol Strain* Paenibacillus polymyxa *HY96-2 and Further Analysis of its Biocontrol Mechanism*
Yuanchan Luo, Yuejuan Cheng, Jincui Yi, Zhijun Zhang, Qian Luo, Daojing Zhang and Yuanguang Li

195 Streptomyces globosus *UAE1, a Potential Effective Biocontrol Agent for Black Scorch Disease in Date Palm Plantations*
Esam E. Saeed, Arjun Sham, Zeinab Salmin, Yasmeen Abdelmowla, Rabah Iratni, Khaled El-Tarabily and Synan AbuQamar

211 *Biological Control of Mango Dieback Disease Caused by* Lasiodiplodia theobromae *Using Streptomycete and Non-streptomycete Actinobacteria in the United Arab Emirates*
Fatima H. Kamil, Esam E. Saeed, Khaled A. El-Tarabily and Synan F. AbuQamar

230 *How* Streptomyces anulatus *Primes Grapevine Defenses to Cope With Gray Mold: A Study of the Early Responses of Cell Suspensions*
Parul Vatsa-Portugal, Aziz Aziz, Marine Rondeau, Sandra Villaume, Hamid Morjani, Christophe Clément and Essaid Ait Barka

SECTION 3
BACTERIAL DISEASES

242 *Biocontrol Rhizobacterium* Pseudomonas sp. *23S Induces Systemic Resistance in Tomato (*Solanum lycopersicum *L.) Against Bacterial Canker* Clavibacter michiganensis *subsp.* michiganensis
Yoko Takishita, Jean-Benoit Charron and Donald L. Smith

256 *Biocontrol of Bacterial Leaf Blight of Rice and Profiling of Secondary Metabolites Produced by Rhizospheric* Pseudomonas aeruginosa *BRp3*
Sumera Yasmin, Fauzia Y. Hafeez, Muhammad S. Mirza, Maria Rasul, Hafiz M. I. Arshad, Muhammad Zubair and Mazhar Iqbal

SECTION 4
MANAGEMENT OF NEMATODE AND INSECT PESTS

279 ***Rhizosphere Microbiomes Modulated by Pre-crops Assisted Plants in Defense Against Plant-Parasitic Nematodes***
Ahmed Elhady, Shimaa Adss, Johannes Hallmann and Holger Heuer

288 **Klebsiella pneumoniae *SnebYK Mediates Resistance Against* Heterodera glycines *and Promotes Soybean Growth***
Dan Liu, Le Chen, Xiaofeng Zhu, Yuanyuan Wang, Yuanhu Xuan, Xiaoyu Liu, Lijie Chen and Yuxi Duan

301 ***Chitosan Increases Tomato Root Colonization by* Pochonia chlamydosporia *and Their Combination Reduces Root-Knot Nematode Damage***
Nuria Escudero, Federico Lopez-Moya, Zahra Ghahremani, Ernesto A. Zavala-Gonzalez, Aurora Alaguero-Cordovilla, Caridad Ros-Ibañez, Alfredo Lacasa, Francisco J. Sorribas and Luis V. Lopez-Llorca

311 ***Combined Field Inoculations of* Pseudomonas *Bacteria, Arbuscular Mycorrhizal Fungi, and Entomopathogenic Nematodes and Their Effects on Wheat Performance***
Nicola Imperiali, Xavier Chiriboga, Klaus Schlaeppi, Marie Fesselet, Daniela Villacrés, Geoffrey Jaffuel, S. Franz Bender, Francesca Dennert, Ruben Blanco-Pérez, Marcel G. A. van der Heijden, Monika Maurhofer, Fabio Mascher, Ted C. J. Turlings, Christoph J. Keel and Raquel Campos-Herrera

SECTION 5
SUPPRESSIVE SOILS

328 ***Screening and Characterization of Potentially Suppressive Soils Against* Gaeumannomyces graminis *Under Extensive Wheat Cropping by Chilean Indigenous Communities***
Paola Durán, Milko Jorquera, Sharon Viscardi, Victor J. Carrion, María de la Luz Mora and María J. Pozo

344 ***Desirable Traits of a Good Biocontrol Agent Against Verticillium Wilt***
Silke Deketelaere, Lien Tyvaert, Soraya C. França and Monica Höfte

367 ***Assessment of the Diversity of* Pseudomonas *spp. and* Fusarium *spp. in* Radix pseudostellariae *Rhizosphere Under Monoculture by Combining DGGE and Quantitative PCR***
Jun Chen, Linkun Wu, Zhigang Xiao, Yanhong Wu, Hongmiao Wu, Xianjin Qin, Juanying Wang, Xiaoya Wei, Muhammad U. Khan, Sheng Lin and Wenxiong Lin

SECTION 6
SOIL ENVIRONMENT

379 ***Long-Term Irrigation Affects the Dynamics and Activity of the Wheat Rhizosphere Microbiome***
Dmitri V. Mavrodi, Olga V. Mavrodi, Liam D. H. Elbourne, Sasha Tetu, Robert F. Bonsall, James Parejko, Mingming Yang, Ian T. Paulsen, David M. Weller and Linda S. Thomashow

394 ***Impacts of Atmospheric CO_2 and Soil Nutritional Value on Plant Responses to Rhizosphere Colonization by Soil Bacteria***
Alex Williams, Pierre Pétriacq, David J. Beerling, T. E. Anne Cotton and Jurriaan Ton

402 ***Stereoisomers of the Bacterial Volatile Compound 2,3-Butanediol Differently Elicit Systemic Defense Responses of Pepper Against Multiple Viruses in the Field***
Hyun G. Kong, Teak S. Shin, Tae H. Kim and Choong-Min Ryu

SECTION 7
FUTURE CHALLENGES

415 ***Challenges and Approaches in Microbiome Research: From Fundamental to Applied***
Chrysi Sergaki, Beatriz Lagunas, Ian Lidbury, Miriam L. Gifford and Patrick Schäfer

427 ***Microbial Phosphorus Solubilization and its Potential for Use in Sustainable Agriculture***
Elizabeth T. Alori, Bernard R. Glick and Olubukola O. Babalola

435 ***The Chemistry of Plant–Microbe Interactions in the Rhizosphere and the Potential for Metabolomics to Reveal Signaling Related to Defense Priming and Induced Systemic Resistance***
Msizi I. Mhlongo, Lizelle A. Piater, Ntakadzeni E. Madala, Nico Labuschagne and Ian A. Dubery

452 ***Inner Plant Values: Diversity, Colonization and Benefits From Endophytic Bacteria***
Hongwei Liu, Lilia C. Carvalhais, Mark Crawford, Eugenie Singh, Paul G. Dennis, Corné M. J. Pieterse and Peer M. Schenk

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EDITORIAL
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Editorial: Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol - Second Edition

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Editorial on the Research Topic

Harnessing Useful Rhizosphere Microorganisms for Pathogen and Pest Biocontrol - Second Edition

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There is a worldwide interest in the exploitation of beneficial plant-associated microorganisms as an alternative to pesticides for pest and disease management. It is underpinned by practical and social reasons, including safety of consumers, farmers, and field workers, as well as the need for sustainable practices safeguarding the environment and protecting its biodiversity. Cost of conventional pesticides and the insurgence of resistance in pests also re-direct farmers' choice toward safer approaches. This trend is observed also in fast-growing population economies, propelling the global demand for eco-sustainable technologies.

Understanding the role of rhizosphere microorganisms in the control of pests and diseases appears as a growing research field, as shown by the sharp increase of studies carried out during the period 2000–2019. The number of records retrieved through a Google Scholar query with keywords "microorganisms," "control," "pest," and "diseases" increased from around 5000 (2000–2005) to ∼8500 and >20,000 (2006–2010 and 2011–2019, respectively), when the term "rhizosphere" was added. Without the latter the records instead lowered from around 17,000 to ∼15,000 in the last period (interrogation dated August 2, 2019). However, in spite of this increased interest in rhizosphere ecology managing and exploiting living organisms to regulate or control other noxious species still remains a complex task. Detailed data on interacting variables and processes are needed, as their final result often differs significantly from the simple sum of effects. Any information boosting our capacity to solve problems related to safer plant protection is, therefore, more than welcome.

BIOCONTROL OF *PHYTOPHTHORA*

The Oomycete *Phytophthora infestans*, the most widespread and severe pathogen of potato, is a global threat due to its capacity to develop new genotypes overcoming the resistance present in many cultivars (Fry, 2008; Cooke et al., 2011). Rhizo/phyllosphere *Pseudomonas* strains have been tested against this pathogen. The potato cultivar to which bacteria were originally associated, and the combined use of strains were found to underpin beneficial effects, including a higher disease inhibition (De Vrieze et al.). These authors tested the rhizosphere complexity, with combinations of native bacteria yielding benefits, not previously inferred from *in vitro* data. This approach showed that multiple strains can be exploited in combination, using poorly competitive bacteria that do not

interfere each other. Results stressed the potential of communities based on native microorganisms rather than single isolates, inhibiting *P. infestans* or halting its progression.

In a similar approach, Caulier et al. screened *Bacillus* and *Pseudomonas* spp. strains against potato pathogens including *P. infestans*. The most significant antagonistic activity appeared to be related to production of metabolites such as bacilysin, biosurfactants, and siderophores, characterizing some *Bacillus* spp. strains. Data indicated that indigenous rhizosphere microflora may yield effective bio-management tools.

BIOCONTROL OF FUNGAL DISEASES

The exploitation of rhizosphere microorganisms for management of fungal diseases is a fertile research field (Toyota and Shirai, 2018). Species mostly include antagonistic fungi such as *Trichoderma* spp. and bacteria of genera *Pseudomonas*, *Bacillus,* and *Streptomyces*. As assays showed biocontrol efficacy for many isolates, there is a need for characterizing metabolic and molecular mechanisms underpinning these activities.

Trichoderma

The role of gene regulatory elements may reveal key clues in biocontrol agents. In *Trichoderma harzianum* the transcription factor THCTF1 is involved in the production of 6-pentyl-pyrone, a volatile secondary metabolite active in interspecies cross-talk. THCTF1A is a homolog of the multiprotein bridging factor 1 (*mbf1*). Expression and regulation of both genes appeared to be important in modulating the activity of the fungus. Transformants overexpressing *Thmbf1* were used to investigate the effect of this gene in biocontrol of *Fusarium oxysporum* f. sp. *lycopersici* race 2 (FO) and *Botrytis cinerea*. *Thmbf1* affected production of volatile organic compounds (VOC) and low molecular weight molecules by *T. harzianum* with antifungal activity against FO. *Thmbf1* overexpression negatively affected *T. harzianum* biocontrol efficacy against both pathogens on susceptible tomato (Rubio et al.).

Manganiello et al. investigated transcriptomic and metabolomic changes induced by a *T. harzianum* strain, or its secondary metabolite harzianic acid, in Micro-Tom tomato infected by the soil-borne pathogen *Rhizoctonia solani*. Genes involved in resistance to biotic stress were differentially expressed, confirming *T. harzianum* ability to activate a defense response in plants. Genes of the ethylene/jasmonate and salicylic acid signaling pathways were up-regulated, as well as genes involved in reactive oxygen species (ROS) detoxification. Harzianic acid induced expression of defense genes such as protease inhibitors, CC-NBS-LRR resistance proteins, and hormone interplay. Both treatments increased steroidal glycoalkaloids, confirming activation of metabolic chemical defenses.

Pseudomonas

Rhizosphere competence is a key factor for a successful biocontrol agent, as plant species, soil type, and pathogen types affect the microbial community composition in the rhizosphere (Schreiter et al., 2014). The biocontrol efficacy of a *Pseudomonas* sp. isolate against *R. solani* on potato and lettuce was tested in three soil types. The antagonist colonized the rhizosphere of both crops and reduced the severity of black scurf on potato and bottom rot on lettuce in all soils. The *Pseudomonas* sp. isolate differently affected the bacterial community composition, depending on the host plant, as its effect was more evident in lettuce than potato (Schreiter et al.).

Pseudomonas spp. and antimicrobial metabolites such as 2,4-diacetylphloroglucinol (DAPG) or phenazines (PHZ), are likely involved in the insurgence of disease-suppressive soils, inhibiting soilborne plant diseases. However, antimicrobial-producing species can be found also in disease-conducive soils. Imperiali et al. compared soils from Swiss cereal farms, considering their resistance against two soilborne pathogens, the abundance of *Pseudomonas* spp. the biosynthesis DAPG, PHZ, and pyrrolnitrin, and the ability to support their production on roots. All these traits did not correlate to soil disease resistance. Data showed that this capacity depended on the pathogen species, as shown by soils exclusively not conducive for *Gaeumannomyces tritici* or *Pythium ultimum*. Factors related to soil structure and availability of nutrients were found to affect abundance and production of antimicrobial compounds.

A *Pseudomonas* spp. strain was tested for management of blast disease, caused by *Pyricularia grisea*, on the finger millet *Eleusine coracona* "Ragi," a cereal used in semi-arid regions of Asia and Africa. It inhibited *P. grisea*, solubilized phosphate and produced antifungal metabolites, siderophores, hydrolytic enzymes, and indolacetic acid. The strain had a direct effect on the pathogen, causing hyphal deformation, with a minimum impact on root microbial biomass and other rhizobacteria. It also reduced blast infestation and enhanced plant vigor and growth, showing a prolonged shelf-life when stored as a liquid formulation (Sekar et al.).

Similarly, several isolates collected by Gómez-Lama Cabanás et al. from olive rhizosphere showed potential against Verticillium wilt of olive, caused by *Verticillium dahliae*. Three *Pseudomonas* spp. strains were selected (PIC25, PIC105, and PICF141) with high *in vitro* inhibition against the pathogen. Effectiveness against the defoliating pathotype of *V. dahliae* was demonstrated, in particular for PICF141. Molecular analyses showed PICF141 as closest to *P. lini* ("*Pseudomonas mandelii* subgroup," within the "*Pseudomonas fluorescens* group"). Strains PIC25 and PIC105 were affiliated to the "*Pseudomonas aeruginosa* group," with *P. indica* as the closest relative. The isolates showed genotypic and phenotypic traits associated with plant growth promotion and/or biocontrol, including production of phytase, xylanase, catalase, cellulase, chitinase, glucanase activities, siderophores, and HCN. *Pseudomonas indica* PIC105 was identified for the first time as a biocontrol agent of fungi. Root colonization was useful for their application in biocontrol formulations.

Bacillales

Several *Bacillus* species induce beneficial effects on plants through the production of antimicrobial compounds, as shown by species with growth-promotion and/or biocontrol activities such as the model strain *Bacillus velezensis* FZB42. Genes involved in synthesis of secondary metabolites, suppressing the

growth of pathogens and inducing systemic resistance, were found together with VOC involved in biocontrol (Fan et al.).

Bacillus amyloliquefaciens strain JCK-12, isolated from soil, showed a significant antifungal activity vs. *F. graminearum*, the causal agent of Fusarium head blight. The fungus dramatically affects yields, with high risks to human and animal health due to its mycotoxins. JCK-12 fermentation broth and formulations reduced the disease incidence in wheat, likely related to production of cyclic lipopeptides including iturin A, fengycin, and surfactin. Iturin A inhibited *F. graminearum* spore germination, whereas the other lipopeptides showed, when mixed with iturin A, only a synergistic inhibitory effect on germination. Strain JCK-12 also affected production of trichothecenes. Moreover, it showed synergistic antifungal effects with synthetic fungicides *in vitro*, controlling the disease in greenhouse and field conditions (Kim et al.).

Lipopeptides production is a beneficial trait displayed by some biocontrol bacteria (Velho et al., 2011). Surfactin, bacillomycin, and fengycin, detected in cultures of *B. methylotrophicus* (syn. of *B. velezensis*) by mass spectrometry, inhibited *in vitro* growth of the necrotrophic plant pathogen *B. cinerea*. Electron microscopy observations confirmed this effect showing an alteration of the fungus morphology upon the interaction with lipopeptides, with degeneration of cell organelles and production of early stage resistance structures. Assays with tomatoes, grapes, and strawberries confirmed the lipopeptides efficacy against *B. cinerea*, with the induction of an antioxidant activity in fruit (Toral et al.).

Paenibacillus polymyxa (former *B. polymyxa*) strain HY96-2 is a plant-growth-promoting rhizobacterium and a biocontrol agent, commercialized in China as a microbial biopesticide. Comparison of complete genome sequence data with other *P. polymyxa* strains indicated the potential to antagonize plant diseases by biofilm formation, antibiotic production, and induced systemic resistance (Luo et al.).

Streptomyces

Several *Streptomyces* spp. are effective biocontrol agents in different climatic conditions and agroecosystems. A *S. globosus* isolate, originating from healthy date palm rhizosphere in the United Arab Emirates, showed antagonism vs. *Thielaviopsis punctulata*, the causal agent of black scorch disease. Its activity was associated *in vitro* with production of diffusible antifungal metabolites that inhibited *T. punctulata* mycelial growth. Greenhouse and pathogenicity tests showed that the isolate suppressed black scorch disease and prevented its spread at a level comparable to that of a synthetic fungicide (Saeed et al.). In the same country, a selective *in vitro* screening showed two *Streptomyces* spp. and one *Micromonospora* sp. isolates with a strong inhibitory effect against mango dieback, caused by the fungus *Lasiodiplodia theobromae*. In particular, activity of *S. samsunensis* was related to antibiosis and production of cell-wall-degrading enzymes (CWDE, i.e., chitinase) and siderophores, whereas *S. cavourensis* and *M. tulbaghiae* were associated with antibiotic and CWDE production, respectively. In greenhouse experiments the isolates showed high levels of disease protection in pre-inoculated mango seedlings, with a reduced number of defoliated leaves and lower counts of *L. theobromae* conidia (Kamil et al.).

Isolate S37 of *S. anulatus* from the rhizosphere of healthy *Vitis vinifera* promoted grapevine growth and induced resistance against *B. cinerea*, one of its most severe diseases. Interactions with S37, before and after the challenge with *B. cinerea*, induced local defense events including early responses such as oxidative burst, extracellular alkalinization, and activation of protein kinases, with expression of defense genes and phytoalexin accumulation. Moreover, Ca^{2+} also appeared to contribute upstream to the induced reactions. S37 primed grapevine cells for enhanced defense, with a decline in cell death rates. Grapevine cells also showed a distinct perception of beneficial and pathogenic microbes, as shown by desensitization assays based on alkalinization of extracellular pH. In fact, once the pH was increased by *S. anulatus* S37, the cells became refractory to further stimulation by *B. cinerea* (Vatsa-Portugal et al.).

BACTERIAL DISEASES

Takishita et al. tested a *Pseudomonas* sp. strain against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomato bacterial canker. The strain activated a systemic resistance reaction in treated plants, as shown by marker genes such as PR1a and ACO. The observed reduction of *C. michiganensis* subsp. *michiganensis* cells was also linked to a better plant performance. The antagonist showed ability to solubilize inorganic phosphorus, and to produce siderophores, indole acetic acid, and hydrogen cyanide. These metabolic profiles, but hydrogen cyanide production, were also found in a strain of *Pseudomonas aeruginosa* active against *Xanthomonas oryzae* pv. *oryzae*, the causal agent of the bacterial leaf blight of Basmati rice, a severe disease in Pakistan. Yasmin et al. showed a significant pathogen suppression by the tested strain, likely mediated by secondary metabolites such as siderophores, rhamnolipids, and hydroxy-quinolines, underpinning an antibacterial activity. Moreover, the bacterium was capable of colonizing the rice rhizosphere, inducing the production of defense-related enzymes in treated plants, eventually increasing grain and straw yields by $>50\%$.

MANAGEMENT OF NEMATODE AND INSECT PESTS

Being one of the most severe crop limiting factors present in soil, the interaction of plant-parasitic nematodes with the rhizosphere microbiome has been the object of many studies. Several microorganisms both affect and are influenced by soil and rhizosphere food webs, of which nematodes represent a key component. Rhizosphere microorganisms may indeed counterbalance the reproduction of many nematodes, and in some cases they are determinant in soil suppressiveness (Westphal and Xing, 2011; Giné et al., 2016). However, cropping practices, and in particular monoculture, may dramatically shift this balance toward nematodes, due to their high reproductive rates in the presence of a suitable host. Elhady et al. investigated

how the soil microbiome affects invasion and reproduction of plant-parasitic nematodes, studying the root knot nematode *Meloidogyne incognita* and the root lesion nematode *Pratylenchus penetrans*. They tested the effect of a transplanted rhizosphere microbiome from other crops (i.e., soybean, maize, or tomato), and the impact of a plant's own microbiome, in comparison to that from a fallow soil. The microbiome from maize or soybean significantly reduced root invasion by *P. penetrans*. Similarly, maize and tomato rhizosphere microbiomes affected the invasion of tomato roots by both *P. penetrans* and *M. incognita*, compared to soybean or bulk soil. Moreover, *M. incognita* was best suppressed on tomato by the own tomato rhizosphere microbiome. Data showed that the microbiome species composition, as selected by the previous cropping cycle, affect the subsequent development of a parasitic nematode population in soil.

In root-nematode interactions, the effect of soil bacteria may be deployed at multiple levels, depending on their metabolism and rhizosphere colonizing capability. Liu et al. studied the regulation of the soybean cyst nematode *Heterodera glycines* by an isolate of *Klebsiella pneumoniae*, a bacterium otherwise known for its clinical importance. One isolate, however, acts as plant growth promoter and biocontrol agent of sheath and seedling rice blights. The isolate tested showed an effect on nematode parasitism and density, when used as a seed coating or in field assays. Pot assays with split roots showed systemic resistance induced by *K. pneumoniae* in treated plants. Increased expression for some defense genes such as *PR1*, *PR2,* and *PR5* (pathogenesis-related genes), as well as *PDF1.2* (plant defensin), was observed. Moreover, the isolate improved the development of roots through the combined action of nitrogen fixation, phosphate solubilization, and siderophore production.

The links between plants and soil microorganism likely arise as the result of functional, coevolutive adaptations beneficial for the organisms involved. In this perspective it is useful to examine factors such as nutrients or substrates, present in the rhizosphere. Escudero et al. investigated the interaction of the hyphomycete *Pochonia chlamydosporia* with chitosan, a chitin-derived product. The fungus has multiple behaviors in soil, as a specialized parasite of nematode eggs, a saprotroph, or a root endophyte (Manzanilla-López et al., 2013). Chitosan enhances *P. chlamydosporia* sporulation, production of extracellular enzymes and egg parasitism (Escudero et al., 2016). At low concentrations (up to 0.1 mg ml^{-1}) it improved the fungal growth and did not affect chlamydospores viability and germination. Treatments increased tomato root and plant development in presence of both *P. chlamydosporia* and *Meloidogyne javanica*, but did not affect eggs parasitism, unless a highly suppressive (>70% prevalence in eggs) sterilized soil was used.

As many antagonists occur in soil at the same time, it is worth checking whether their combined application may yield synergic effects. Experimental data indicated, however, that this situation is difficult to observe in natural conditions. Imperiali et al. studied field application of beneficial rhizosphere organisms, inoculated alone or in combination at wheat seeding. Tested antagonists included *Pseudomonas* spp. arbuscular mycorrhizal fungi (AMF), and entomopathogenic nematodes (EPN). The beneficial organisms persisted in soil after their introduction, as confirmed by molecular identification and increased plant health and productivity. However, no difference was observed between combined and single applications. Best wheat survival was reported on plants exposed to a biotic stress such as infestation by the frit fly, *Oscinella frit*, an important pest of cereals, which was reduced in treatments with *Pseudomonas* and AMF. Inoculations with EPN displaced some other native EPN, but only on the short-term.

SUPPRESSIVE SOILS

Soils showing presence of a pathogen with, however, a low disease insurgence on a susceptible crop, are known as suppressive. This trait is likely due to the beneficial, indigenous microorganisms (Schlatter et al., 2017). Suppressive soils may be found in extensive cropping systems or in areas with long-term cultivation. Durán et al. screened soils for suppressiveness against "take-all" disease caused by the fungus *Gaeumannomyces graminis* var. *tritici*, sampling extensive wheat fields managed by indigenous Mapuche communities in Southern Chile. *In vitro* growth inhibition tests allowed identification of putative soils suppressive for take-all disease. Suppressiveness was closely associated to the soil microbiome, being lost upon soil sterilization and then recovered by addition of a natural soil inoculum.

A review on Verticillium wilts, vascular diseases caused on annual crops and woody perennials by several species of the soil-borne fungus *Verticillium*, showed the potential of bacteria belonging to *Bacillus* and *Pseudomonas* and of non-pathogenic xylem-colonizing *Verticillium* and *Fusarium* isolates as biocontrol tools. Soils and composts suppressive to Verticillium wilt allowed isolation of biocontrol agents characterized by useful traits such as inhibition of primary inoculum germination, plant growth promotion, competition, and induced resistance. However, *in vitro* antibiosis and mycoparasitism did not correlate with *in vivo* or *in planta* activities. Most useful traits included activity against the pathogen microsclerotia, a significant xylem and/or cortex colonization with nutrients and/or space competition, the induction of resistance reactions, and growth promotion in treated plants. All these effects should be screened under field conditions for selection of biocontrol agents suitable for large scale production, stable formulation, and application (Deketelaere et al.).

However, the impact on soil microbial communities of external factors such as the farmers' technical decisions (i.e., selecting crop species with/without rotations) should also be evaluated. A long-term monoculture increased the diversity of *F. oxysporum* and had a negative effect on antagonistic *Pseudomonas* spp. as shown by a 3 years long cropping of the medicinal plant *Radix pseudostellariae* (Chen et al.).

SOIL ENVIRONMENT

Several physical and climatic factors affect the microbiome diversity and the interactions of soil antagonists with plants, pests, and pathogens (Lareen et al., 2016). Mavrodi et al. characterized the establishment and activity of microbial

communities in the rhizosphere of dryland wheat, and the microbiome response to irrigation. They also studied the population dynamics and activity of indigenous rhizobacteria producing antibiotics (phenazines), contributing to the natural suppression of wheat soilborne pathogens. Irrigation and soil moisture had a negative effect on plant colonization, density of bacteria and antibiotic levels. Although irrigation had a limited effect on the global microbiome diversity, differences were found for some groups such as the plant growth-promoting *Bacteroidetes* and *Proteobacteria*.

Atmospheric CO_2 is a further physical factor related to climate change. Williams et al. studied its effect on strains of *Pseudomonas simiae* and *P. putida* in the rhizosphere of *Arabidopsis*. Differences in rhizosphere colonization were observed for the two bacteria, with an increase linked to CO_2 levels (from 200 to 1,200 ppm), observed only for *P. simiae* in soil with relatively low C and N. Plant growth and systemic resistance to *P. simiae* varied in relation to the CO_2 concentrations and soil type. Growth promotion and induced susceptibility were observed at sub-ambient CO_2, whereas an opposite response was found at highest levels.

Stereoisomers of 2,3-butanediol, produced by some root-associated bacteria, were reported as triggering immunity of pepper plants against *Cucumber mosaic virus* and *Tobacco mosaic virus*. In a field trial, treatments with an enantiomer (2R,3R-butanediol) and a meso compound (2R,3S-butanediol) significantly reduced the prevalence of naturally occurring viruses, compared with a further enantiomer (2S,3S-butanediol) and control. Moreover, 2R,3R-butanediol induced the expression of plant defense genes in the ethylene/jasmonate and salicylic acid pathways at levels similar to those of benzothiadiazole-treated control (Kong et al.).

OUTLOOK AND FUTURE CHALLENGES

Soil microbial communities offer an unlimited potential to develop locally customized and innovative approaches, facing major threats for global food security (Sergaki et al.). Challenges mainly concern plant protection and soil fertility. Many microorganisms that mineralize insoluble phosphate hold potential to improve growth and yield of many crops, in a sustainable way. This technology appears ready for commercial exploitation in various regions of the world, but more detailed information is still needed to be considered as an effective replacement of conventional fertilizers (Alori et al.). Studies on induced resistance and chemical communication of roots with soil microorganisms open unlimited perspectives for development of sustainable management tools, as shown in the reviews by Mhlongo et al. and Liu et al. Due to the many links among below- and above-ground physiological processes, chemical communication has been only partially deciphered, thanks to advanced techniques such as mass spectrometric-based metabolomics. The complexity and stability of microbial communities also depend on the capacity of roots to screen and select rhizosphere/rhizoplane taxa, mostly dominated by Proteobacteria with favorable traits such as motility, plant cell-wall degradation and ROS scavenging, nutrients procurement, nitrogen fixation, and defense priming.

AUTHOR CONTRIBUTIONS

AC wrote the manuscript. JM-B and CP revised the manuscript, helped structure and edit it, and approved its final version for publication.

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Combining Different Potato-Associated *Pseudomonas* Strains for Improved Biocontrol of *Phytophthora infestans*

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Late blight caused by *Phytophthora infestans* is considered as the most devastating disease of potato and is a re-emerging problem worldwide. Current late blight control practices rely mostly on synthetic fungicides or copper-based products, but growing awareness of the negative impact of these compounds on the environment has led to the search for alternative control measures. A collection of *Pseudomonas* strains isolated from both the rhizosphere and the phyllosphere of potato was recently characterized for *in vitro* protective effects against *P. infestans.* In the present study, we used a leaf disk assay with three different potato cultivars to compare the disease inhibition capacity of nine selected *Pseudomonas* strains when applied alone or in all possible dual and triple combinations. Results showed a strong cultivar effect and identified strains previously thought to be inactive based on *in vitro* assays as the best biocontrol candidates. One strain was much more active alone than in combination with other strains, while two other strains provided significantly better protection in dual combination than when applied alone. A subset of five strains was then further selected to determine their mutual influence on each other's survival and growth, as well as to characterize their activity against *P. infestans* in more details. This revealed that the two strains whose dual combination was particularly efficient were only weakly interfering with each other's growth and had complementary modes of action. Our results highlight the potential to harness the crop's native rhizosphere and phyllosphere microbiome through re-assembling strains with differing modes of action into small communities, thereby providing more consistent protection than with the application of single strains. We consider this as a first step toward more elaborate microbiome management efforts, which shall be integrated into global strategies for sustainable control of potato late blight.

Keywords: late blight, pseudomonads, *Solanum tuberosum*, consortium, biocontrol, rhizosphere, phyllosphere

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INTRODUCTION

Sustainable crop production faces the challenge of maintaining high yields to meet the food requirements of an increasing world population while limiting its own environmental impact. In potato production, the major yield-threatening disease is the oomycete *Phytophthora infestans*, causing late blight (Fry, 2008). In Europe, late blight's costs, resulting both from yield loss and

disease control, have been estimated to over 1 billion Euros per year (Haverkort et al., 2008). In conventional agricultural management, potato late blight, as many other diseases, is controlled by multiple applications of fungicides of varying modes of action during the growing season (Haverkort et al., 2008; Cooke et al., 2011). However, increasing awareness of the negative side effects of synthetic pesticides on environmental and human health has led to growing interest in organically produced food. In organic potato production, growers use copper-based products as alternative to synthetic fungicides to protect their crops from late blight, but since copper is not degradable, it accumulates in the soil and is toxic to the soil fauna (Du Plessis et al., 2005; Eijsackers et al., 2005). Therefore, there is a need for alternative measures to control late blight in an environmentally friendly manner. One such alternative is the use of natural enemies of the disease-causing agent, also termed "biological control" or "biocontrol." Microbial biocontrol agents such as the bacterium *Bacillus thuringiensis* or the entomopathogenic fungus *Beauveria bassiana* have been successfully applied to control insect pests (Arthurs and Dara, 2018), but only few examples (mostly based on bacteria of the genera *Pseudomonas* or *Bacillus*) exist where such biocontrol strategy was efficient enough against fungal pathogens to lead to product commercialization (reviewed in Velivelli et al., 2014). Beyond these few examples, controlling an aggressive pathogen such as *P. infestans* is a significant challenge, as evidenced by the number of biocompatible treatments that have been tested but did not, or only partially, meet the required level of reproducible efficacy (Dorn et al., 2007; Axel et al., 2012; Alaux et al., 2018).

One possible factor underlying this lack of success might be that *P. infestans* can infect its host plants by different means, e.g., through direct germination of sporangia or release of motile zoospores, both leading to host tissue penetration and mycelial development (Fry, 2008). An efficient biocontrol agent should therefore either induce plant resistance (Syed Ab Rahman et al., 2018) or, when acting directly on the pathogen, inhibit both types of infection routes. Alternatively, using different biocontrol agents each targeting one of the pathogen's modes of infection could improve biocontrol efficiency. Such approaches based on strain combinations rather than on single strains could not only bring functional complementary as exemplified above for sporangia vs. zoospore-mediated infection, but they could also be useful in providing functional redundancy. Indeed, a key element in the success of a biocontrol agent is the ability to colonize its host, which in turn is influenced by many factors including the resident microbiome (Sylla et al., 2013). Using strain consortia might therefore lead to enhanced protection robustness in the face of varying environmental and genetic (e.g., different crop varieties) conditions. To date, few studies have addressed this question and tested the impact of mixed strains rather than single ones on plant protection against diseases. Among these, Niu and co-workers could protect maize against *Fusarium* by applying a consortium of seven different bacterial species (Niu et al., 2017), while Hu and co-workers obtained significantly better protection of tomato against *Ralstonia*-induced wilt when using a mixture of 8 *Pseudomonas* strains than when applying each strain individually (Hu et al., 2016).

We have previously isolated and characterized the protective potential of single *Pseudomonas* strains isolated from the rhizosphere and phyllosphere of potato against *P. infestans* (Guyer et al., 2015; Hunziker et al., 2015). In the present study, we hypothesized that mixing different *Pseudomonas* strains, which differed in their phylogenetic identity, in their origin of isolation (rhizosphere vs. phyllosphere) and in their emission of volatile (De Vrieze et al., 2015) and non-volatile anti-*Phytophthora* metabolites could increase the disease-inhibiting potential of these strains. Since the discrepancy between *in vitro* activity and *in planta* protection is usually large (Dorn et al., 2007), and since we had recently established a high-throughput assay to screen for inhibition of late blight development on leaf material (Guyer et al., 2015), we started with a leaf disk-based screening rather than an *in vitro* screening procedure. To this end, all possible twofold and threefold combinations of nine selected *Pseudomonas* strains were tested for late blight inhibition on leaf disks of three potato cultivars of varying late blight susceptibility. This first experiment enabled to select five promising strains, which were further analyzed for better understanding of the mechanisms underlying the observed synergetic effects. This was done by characterizing their effect as single strains vs. in dual and triple combinations on the pathogen's development (both mycelial growth and zoospore release), as well as their growth behavior when inoculated alone or in dual and triple combinations, in order to identify putative synergetic or antagonistic effects.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Nine *Pseudomonas* strains were selected from a collection of strains isolated from the rhizosphere (R) and from shoots (S) of field grown potato plants, based on their *in vitro* activity against *P. infestans* (Guyer et al., 2015; Hunziker et al., 2015). The bacteria were routinely grown on Luria Bertani medium (LB), which was prepared by dissolving 20 g L^{-1} of Difco LB broth (Lennox, United States) in distilled water supplemented with 15 g L^{-1} of agar (Agar-agar, ERNE surface AG, Switzerland). For bacterial cell suspensions, cultures were prepared by suspending single colonies from 2-day-old plates in 0.9% NaCl and streaking the obtained suspensions on LB agar medium. After overnight incubation at 20°C, bacterial cells were resuspended in 0.9% NaCl. For bacterial competition assays, rifampicin-resistant derivative strains obtained as described in Guyer et al. (2015) were used. LB and Pseudomonas Isolation Agar (PIA), supplemented or not with rifampicin (50 μg mL^{-1}) and nystatin (500,000 UL^{-1}) were used for these experiments. PIA medium was prepared by dissolving 45 g L^{-1} of Pseudomonas Isolation Agar (Fluka) in distilled water, to which 20 mL L^{-1} of glycerol (Sigma–Aldrich) was added. Optical density (OD) measured at 570 nm was used to quantify and adjust bacterial density. The nine strains showed slightly different cell numbers/OD_{570} unit but these differences were within the same order of magnitude: most strains had between 1.4×10^8 and 2×10^8 cells per mL for an OD_{570} of 1, while slightly higher cell numbers were observed

for R32, S34 and S35 (between 4.5×10^8 and 5.5×10^8 cells per mL at OD_{570} of 1).

Phytophthora infestans and Culture Media

Phytophthora infestans isolate Rec01 (originally isolated by H. Krebs, Agroscope) was used for all inhibition assays and grown on unclarified V8 (10%) medium for collection of sporangia and zoospores, or on Pea agar medium for mycelial growth assays. Unclarified V8 medium was prepared by diluting V8 juice (100 mL L^{-1}) amended with $CaCO_3$ (1 g L^{-1}) and 15 g L^{-1} of agar according to Miller (1955). Pea agar medium was prepared by skimming 120 g of autoclaved frozen peas in water and adding 15 g of agar. The isolate was regularly inoculated on potato slices for host passage. Sporangia suspensions were prepared by scraping off the mycelium of 14-day-old plates and suspending it in demineralized water. After vigorous shaking, the suspension was filtered using cloth to discard the mycelium. Concentration of sporangia was determined using a Thoma chamber. Sporangia suspensions were maintained in the dark until use. To obtain zoospore suspensions, sporangia were subjected to a cold shock by adding ice-cold water to sporangia suspensions in Eppendorf tubes, which were subsequently incubated at 4°C for 2 h and then left to rest at room temperature for 20 min to allow zoospore release.

Effects of Single Strains vs. Strain Combinations on Disease Protection in a Leaf Disk Assay

The 3rd and 4th leaves of greenhouse grown potato plants of the cultivars Bintje, Lady Claire and Victoria were harvested 7 weeks after emergence. Using a cork borer, leaf disks (1.8 cm diameter) were cut and positioned abaxial face up on 1% water agar plates. Droplets of 10 μL of a mixture of bacterial and sporangial suspensions were pipetted in the center of each leaf disk, at final concentrations of 125,000 sporangia/mL for *P. infestans* and of OD_{570} = 0.9 for single bacterial strains (simple), OD_{570} = 0.45 for combinations of two strains (double), and OD_{570} = 0.3 for combinations of three strains (triple). For negative control plates, bacterial suspensions were replaced with a 0.9% NaCl solution. The plates were stored at 18°C (the ideal growth temperature for *P. infestans*) and at high humidity in the dark. After seven days, the plates were photographed. Severity of infection was assessed through estimation of sporangiophore development using a macro-instruction in ImageJ as described previously (Guyer et al., 2015). Per experiment, each treatment was tested in five replicates consisting of five disks from five different plants in Lady Claire and Victoria, and in ten replicates consisting of ten leaf disks from ten different plants in Bintje (because we had more plants available for Bintje than for Lady Claire and Victoria). Each batch of five plants could be used to assess the efficiency of 27 different treatments by comparing infection severity in treated vs. untreated disks coming from the same plants. To enable comparison between different batches of plants, the infection severity quantified on untreated control disks was set to 100% and the infection severity of treated disks was expressed as percentage of the control (relative infection severity). Finally, treatment efficiency was calculated with the following formula: Treatment efficiency (%) = 100 – (relative infection severity of treatment). With this calculation, a treatment efficiency of 100% corresponds to no infection, an efficiency of 0% corresponds to the same infection as in untreated controls and a negative value indicates higher infection severity in treated disks compared to untreated controls.

Effects of Strain Combinations on *P. infestans* Mycelial Growth

Bacterial strains and *P. infestans* were co-inoculated on Pea agar plates. Three drops of 10 μL of bacterial suspensions (prepared as described above) were pipetted on the medium equidistantly and 10 mm from the border of the plates. One 5 mm plug of a 14-day-old *P. infestans* culture was placed in the center. Negative control plates contained 10 μL of NaCl 0.9% instead of bacterial cell suspension. The plates were prepared in three replicates and were incubated in the dark for 6 days at 18°C before being photographed. Mycelium growth was assessed by measuring the growth area of *P. infestans* using the ImageJ software. Relative mycelial growth was calculated by dividing the mycelial area quantified in treated plates by the mycelial area quantified in negative control plates (in the absence of bacteria). Treatment efficiency was then calculated as above (100 – relative mycelial growth).

Effects of Strain Combinations on *P. infestans* Zoospore Release

Cold shock was applied to sporangia suspensions freshly mixed with bacterial suspensions. Thirty μL of the mixture were pipetted onto a 24-well plate (Costar), which contained one well per treatment. Per well, one picture was taken at 4-fold magnification using a Cytation5 plate reader (Biotek, United States) once the zoospores had settled and once more after shaking the plate to insure even distribution of the zoospores for counting. The experiment was repeated three times. An average of the three replicate experiments was calculated for each treatment. Relative zoospore release was calculated by dividing the number of zoospores released in treated samples by the average number of zoospores released in the untreated samples (negative controls non-exposed to bacteria). Treatment efficiency was calculated as above (100 – relative zoospore release).

Growth/Survival of Bacteria as Single Strains vs. in Combinations With Other Strains

To find out whether the development of single bacterial strains was affected by the presence of other strains, the growth/survival of each bacterial strain was assessed alone and in combination. Rifampicin resistant and wild type bacteria were mixed in equal densities in NaCl (0.45%) and incubated at 18°C without shaking, to mimic the conditions of the leaf disk experiment. We used 0.45% NaCl to have the same concentration as in leaf disk experiments, where bacterial cells suspended in 0.9% NaCl were mixed with equal volumes of *P. infestans* sporangia suspended

in water. After 1 day and after 5 days, 10 μL of bacterial suspension were 10-fold serially diluted in NaCl (0.9%) and 15 μL of the diluted suspensions were plated onto differentially selective media to discriminate the two or three different strains, taking advantage of rifampicin resistance vs. sensitivity, of ability vs. inability to grow on PIA and of different colony morphology. Colony forming units (CFUs) were counted after two or three days depending on the medium.

Statistical Analyses

All statistical analyses were performed using R software (Sasaki et al., 2005). When possible, one-way or two-way ANOVA was performed followed by Dunnett's test or Tukey's HSD test using agricolae and multcomp packages. If needed, boxcox transformation computed via the MASS package was used to meet normality and homogeneity of variances. For effects on zoospore release and mycelial growth data, Kruskal–Wallis test was used to discriminate between treatments.

RESULTS

Disease-Inhibiting Effects of Nine *Pseudomonas* Strains in Single, Dual and Triple Combinations

Nine *Pseudomonas* strains previously isolated from the rhizosphere (R) or phyllosphere (S) of field-grown potatoes and displaying various levels of *Phytophthora*-inhibiting activity *in vitro* were selected for this experiment. To determine whether these strains would confer higher protection when applied in combinations than when applied as single strains, we carried out a leaf disk infection experiment with 129 treatments (9 single strains, their 36 dual and 84 triple combination possibilities) using three potato cultivars differing in late blight sensitivity. We selected Bintje as highly sensitive, Lady Claire as sensitive and Victoria as moderately tolerant. This experiment revealed a strong cultivar effect, with higher overall protection efficacy in Bintje than in Lady Claire and Victoria (**Figure 1**). Among the nine treatments with single strains, only one strain (S35) significantly reduced disease progression in all three varieties, while 7 offered protection on some but not all varieties and one (R84) was inefficient in all varieties. Among the dual combinations, six (out of 36 possible combinations) provided protection on all three cultivars, i.e., R32/S34, R76/S49, R84/S35, R84/S49, S04/S49, and S19/S49. This latter dual combination offered best protection in terms of quantitative disease inhibition (**Figure 1**). Interestingly, four out of six of these efficient combinations contained the strain S49, while only one contained the strain S35, which showed consistent protection when applied as single strain. Among the 84 possible triple combinations, only seven were able to significantly reduce disease progression in Lady Claire, among which two were also efficient on the two other varieties. When considering less stringent conditions, e.g., triple combinations able to reduce disease progression in at least two of the three varieties, 16 combinations were found to be efficient, among which seven contained strain S35 and six contained strain S49, indicating putative synergistic effects of these two strains when applied in combinations with two additional other strains. In addition to the overrepresentation of S35 and S49 in the efficient combinations, we noticed that the duo R47/S35 combined with either S19, S34, or R76, yielded significant protection against *P. infestans* infection.

To gain a general view on the performance of the strains in single, dual and triple combinations, we calculated the percentage of efficient treatments, i.e., those significantly reducing disease symptoms, in the three varieties and for each strain in its respective modes of application (single or combinations) (**Figure 2A**). The relative efficiency of the strains in the different modes of application strongly depended on the variety, yet some strains showed consistent differences between the three application modes: R84 was consistently more efficient when applied with one or two other strains than when applied alone. The same was observed for S49 on Bintje and Lady Claire. In Bintje, the strains performed generally better in triple combinations than in dual combinations in terms of percentage of efficient treatments, while the opposite trend was observed in Lady Claire and Victoria (**Figure 2A**). Because the total bacterial cell density was not the same in single, dual and triple treatments (OD of 0.9 for single, 0.45 for dual and 0.3 for triple treatments), we wondered whether the low percentage of efficient treatments in triple combinations on Lady Claire and Victoria could be explained by these differences in inoculum density. We therefore tested the protective effect of selected single strains applied in the three different densities. This revealed that in Lady Claire and Victoria, cell density in the range of OD = 0.3 – 0.9 did generally not influence the extent of protection conferred by the strains (**Supplementary Figure S1**). However, in Bintje, a dose-dependent protective effect was observed, with applications at OD = 0.3 generally being less efficient than applications at OD = 0.9, although both concentrations were able to significantly reduce disease symptoms (with the exception of strains R32 and S19) (**Supplementary Figure S1**). Therefore, the high percentage of efficient treatments in triple combinations in Bintje that were observed despite overall lower cell density were likely due to synergetic effects between the strains, that were able to compensate the overall lower cell density.

To better understand the mechanisms underlying disease inhibition by strain combinations, we selected a subset of five strains to compare their effect as single strains and as double/triple combinations on two main developmental stages of *P. infestans,* mycelial growth and zoospore release. The selection of these five strains was carried out such as to maximize the chance to see synergetic effects and was based on the number of efficient treatments per strain (**Figure 2B**). We therefore selected R47, S19, S35 and S49 based on the results on Bintje, and included R32 for its efficient protection of Victoria (**Figure 2B**). R32 was remarkably efficient in protecting Victoria when applied in dual combinations, where it led to significant disease reduction when combined with six of the eight other strains (**Figure 1**). The last criterion for strain selection was to ensure some diversity, both in terms of phylogeny and in terms of origin of isolation (rhizosphere vs. phyllosphere). The main properties of these five selected strains are listed in **Table 1**.



FIGURE 1 | *Phytophthora infestans* relative infection severity in leaf disks of three potato cultivars treated with nine *Pseudomonas* applied as single strains vs. in double or triple combinations. Results are expressed as treatment efficiency (100 – relative infection severity in each treatment compared with the untreated leaf disks), with means and standard errors of 10 replicates for Bintje and five replicates for Lady Claire and Victoria (see Materials and Methods for more details). Two-way ANOVA revealed interactions between cultivar and treatment effects, resulting in separate analysis of all three cultivars. Significantly efficient treatments (leading to significant infection reductions compared with the untreated control) are marked in green (Dunnett's test, $p < 0.05$). Inefficient treatments are marked in red (Dunnett's test, $p > 0.05$).

Mycelial Growth Inhibition by Five Selected Strains and Their Dual and Triple Combinations

When inoculated alone, three strains (R32, R47 and S49) were able to inhibit fully the mycelial growth of *P. infestans,* while the two others (S19 and S35) induced more moderate, but still significant growth inhibition. The dual combination of the two "weaker" strains did not result in stronger mycelial growth reduction, nor did the "stronger" strains lose their activity when mixed with other strong strains (**Figure 3**). Interestingly, when either S19 or S35 were mixed with R32, the same complete inhibition of mycelial growth was observed as when R32 was inoculated alone. However, this was not the case with either R47 or S49, whose effects were weakened when mixed with S19 or S35, especially in the S49/S19 and in the R47/S35 combinations, and to a lesser extent in the S35/S49 combination. Concerning the triple combinations, the beneficial impact of R32 was also clearly visible, since its addition conferred strong activity to any couple of strains, including the inactive S19/S35 but also the moderately active R47/S35 and S19/S49 (**Figure 3**). In contrast, adding the active R47 to the "inactive" couple S19/S35 did not improve mycelial inhibition efficiency. From this experiment, it appeared that (i) R32 was the best helper in dual and triple combinations, (ii) S49 only improved the efficiency of S19/S35 but had no positive effect on the other dual combinations, and (iii) R47 was not able to increase the efficiency of S19/S35 and had generally little positive influence in triple combinations (**Figure 3**). Here as well as in the leaf disk experiments, it should be noted that single strains were applied with an optical density of 0.9, against 0.45 for the double and 0.3 for the triple combinations. We therefore tested whether cell density changes at the start of the experiment would influence the extent of mycelial growth inhibition and this was not the case for any of the strains (**Supplementary Figure S2**).

Zoospore Release Affected by Five Selected Strains and Their Dual and Triple Combinations

Preliminary experiments revealed that the total cell density of the strains in single or mixed applications influenced their effect on *P. infestans* zoospores (**Supplementary Figure S3**), therefore this assay was carried out with two different optical

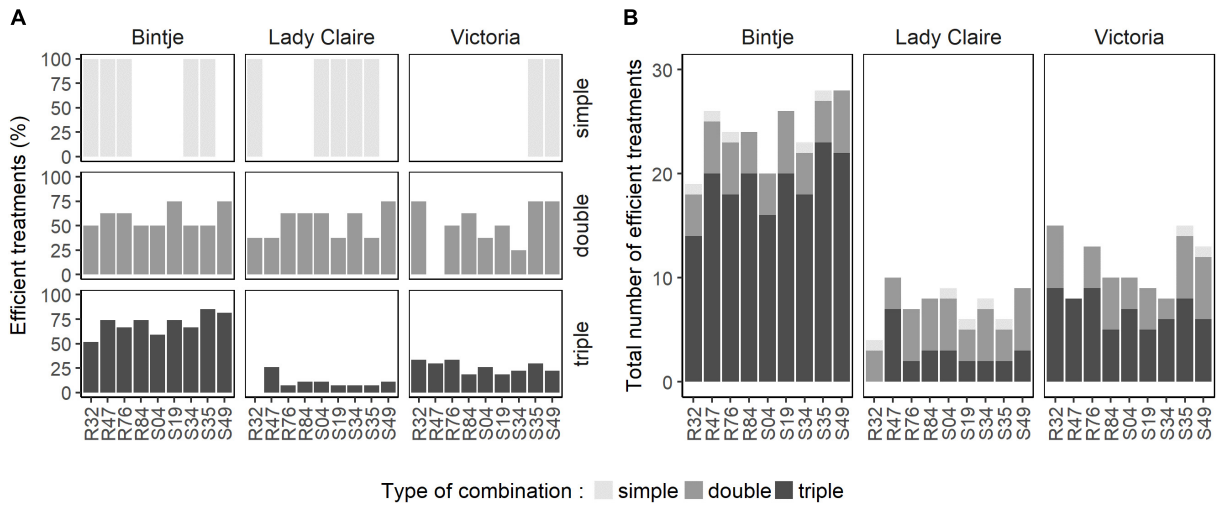


FIGURE 2 | Evaluation of combinatory competence of individual *Pseudomonas* strains when enlisted in single, double or triple combination treatments. For each *Pseudomonas* strain, all efficient treatments featuring the respective strain were counted per cultivar. Treatments were considered efficient if they significantly reduced sporangiophore development on leaf disks (based on data from **Figure 1**). **(A)** Percentage of efficient treatments obtained per bacteria per treatment type (8 possible treatments per strain in double and 26 possible treatments in triple combinations). **(B)** Overall number of efficient treatments per bacteria in single use or in combination with one or two other strains.

densities, a high one (OD = 0.9) and a lower one (OD = 0.3). When bacteria were applied at an OD of 0.9, all treatments significantly and drastically inhibited zoospore release (**Figure 4**). Interestingly, the dual combination of S19/S49 caused lesser reduction in zoospore release than each of the strains applied individually, although it was still significant compared with the control. This decrease in activity was also observed in the more diluted applications (OD = 0.3) and was even more pronounced when S19 was mixed with R32, R47 or S35, where it led to complete loss of activity. Adding any other strain to the couples S19/R32, S19/R47 or S19/S35 could not restore the significant activity observed with the strains applied alone (**Figure 4**). In contrast to the observed antagonistic effects between S19 and other strains, some synergetic effects could also be seen in the experiment carried out with lower cell density: mixing S49 with R32 resulted in almost total inhibition of zoospore release, while the single strains still allowed ca. 20% of the sporangia to release the zoospores. Likewise, mixing S49 with S35 resulted in more consistent (less variable) inhibition of zoospore release than either of the strains applied alone. Remarkably, all combinations of strains containing this couple (S35/S49) significantly reduced zoospore release, which was not the case for any other couple. When comparing the total number of efficient combinatory treatments for each strain (out of 10 possible dual and triple combinations), we observed that S49 scored best (7/10), followed by S35 and R32 (6/10), while combinations containing R47 (4/10) and especially S19 (2/10) were much less efficient. This suggests that in the experimental setup used to assess zoospore release, S49, S35 and R32 had a beneficial effect on other strains present in the respective mixtures, while R47 and especially S19 had deleterious effects on the same strains (**Figure 4**).

Survival and Growth of Five Selected Strains Alone and in Their Dual and Triple Combinations

Some of the results described above hinted at possible direct stimulating or inhibiting effects of strains on each other. To assess whether co-incubation in conditions similar to those applied in the leaf disk assay would lead to preferential survival/growth of specific strains, we incubated the five selected strains alone,

TABLE 1 | Properties of the five selected *Pseudomonas* strains.

Strain	Origin of isolation	Phylogeny	HCN	Phenazines
R32	Rhizosphere	*P. putida*	Yes	No
R47	Rhizosphere	*P. chlororaphis*	Yes	Yes
S19	Phyllosphere	*P. frederiksbergensis*	No	No
S35	Phyllosphere	*P. fluorescens*	No	No
S49	Phyllosphere	*P. fluorescens*	Yes	No

Phylogenetic analysis was based on (De Vrieze et al., 2015; Hunziker et al., 2015). HCN and Phenazines are listed as the two major known determinants of anti-Phytophthora activity (Hunziker et al., 2015; Morrison et al., 2016).

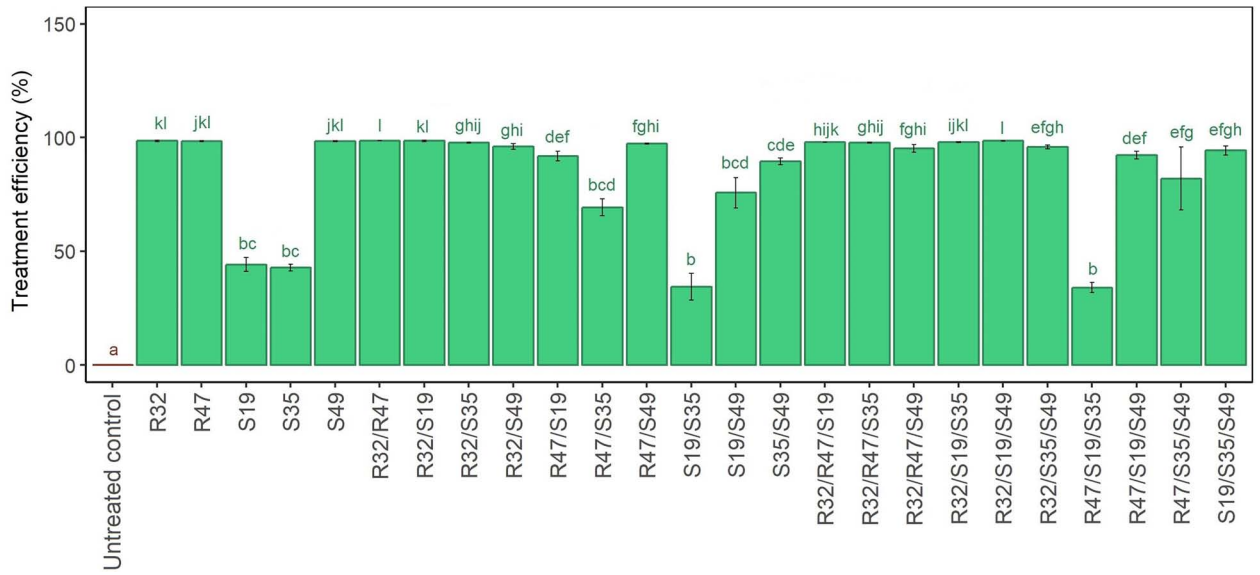


FIGURE 3 | Relative mycelial growth of *P. infestans* when exposed to single, double and triple combinations of five *Pseudomonas* strains in a dual culture Petri dish assay. Untreated controls represent *P. infestans* grown without bacteria. Relative mycelial growth was calculated by dividing the mycelial area obtained in the respective treatments with that obtained in the untreated controls (not exposed to bacteria). Results are means of three replicates from the same experiment. They are expressed as treatment efficiency and calculated as above (100 – relative mycelial growth). Letters indicate significant differences between treatments according to Kruskal–Wallis test ($p < 0.05$, $n = 3$).

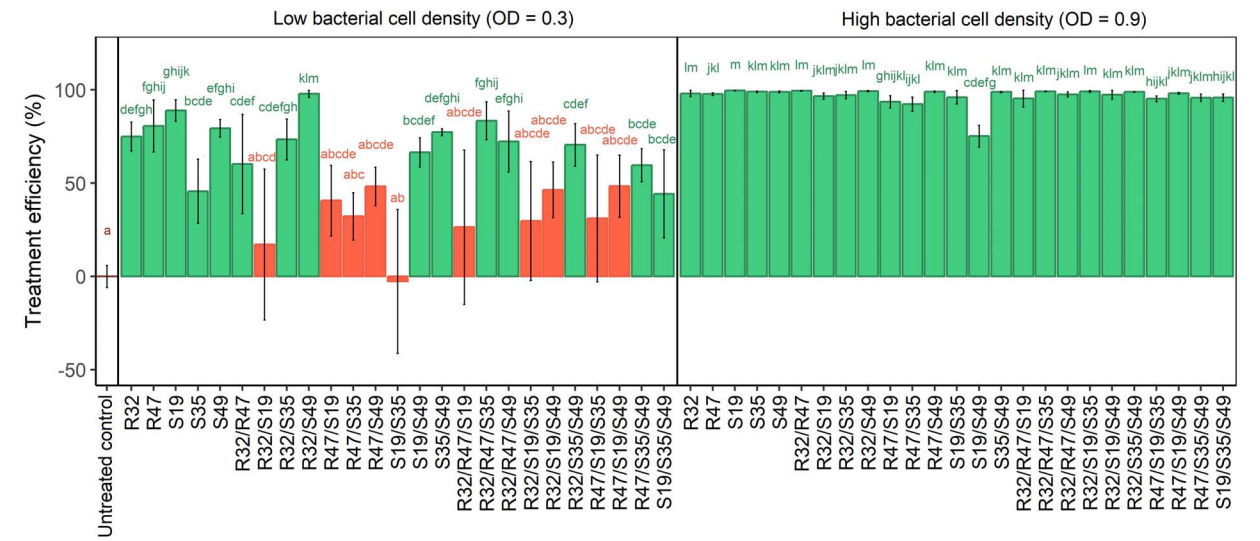


FIGURE 4 | Relative zoospore release from *P. infestans* sporangia exposed to single, double and triple combinations of five *Pseudomonas* strains applied at two different cell densities. Sporangia suspensions pre-mixed with saline instead of bacterial cell solution were used as untreated controls. Released zoospores were counted and the relative release rate was calculated by dividing values obtained for treatments by those obtained for the untreated controls. Results are means of three experiments with one sample per treatment. They are expressed as treatment efficiency and calculated as above (100 – relative zoospore release). Letters indicate significant differences between treatments according to Kruskal–Wallis test ($p < 0.05$, $n = 3$).

as well as in dual and triple combinations for five days in physiological solution and quantified their relative abundance after one day and at the end of the experiment. For this experiment, all treatments had the same global cell density at the start of the experiment (as estimated by optical density), meaning that each individual strain started with half the inoculum vs. 1/3 of the inoculum in dual vs. triple combinations compared with the treatment where it was inoculated alone. Despite these differences, the total CFU counts (taking all strains together) at the end of the experiment were much higher for dual and triple combinations compared to single inoculations (**Figure 5**), indicating that (i) strains were able to compensate the lower

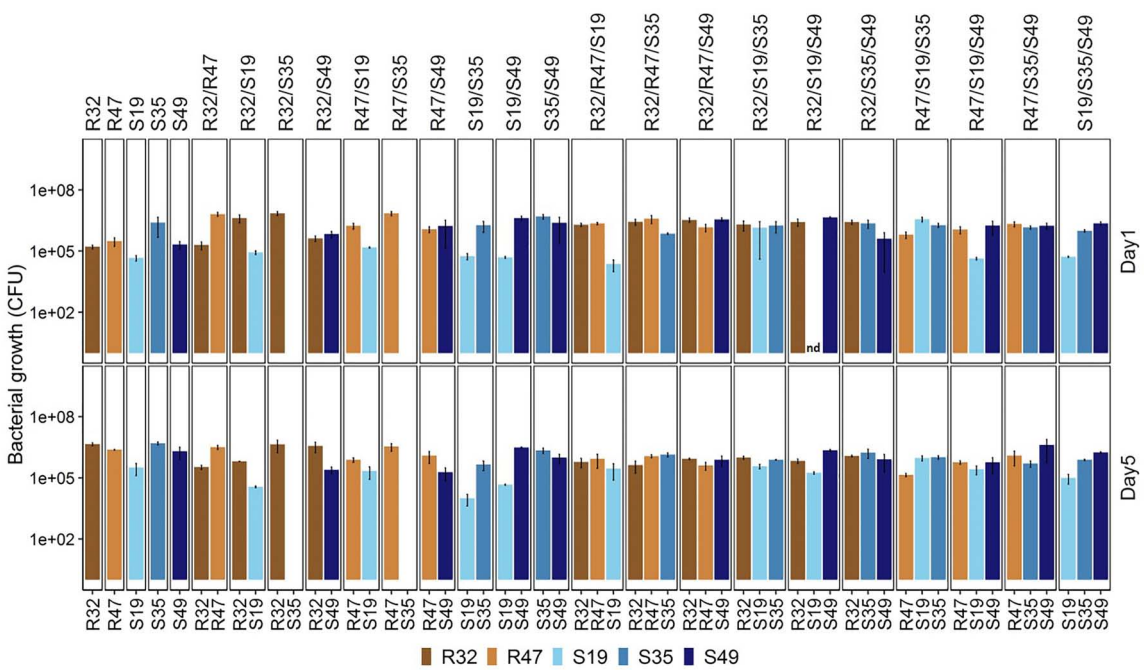


FIGURE 5 | Abundance of each bacterial strain (alone and in dual vs. triple combinations) after 1 **(top)** and 5 **(bottom)** days of incubation in NaCl (0.45%). Results are means of three replicates from the same experiment. nd, no data (this strain's CFUs could not be counted due to a technical problem but the strain was present, as indicated by its abundance after 5 days). Please refer to **Table 2** for statistical analysis of the data after 5 days.

initial cell density over the 5 days of growth, and (ii) strains generally did not grow at the expense of each other, although they were incubated in saline solution only (no nutrient supply). When inoculated alone, four of the five strains developed to a density of roughly a million CFU/mL after 5 days, while their abundance was slightly lower after 1 day, indicating mild growth from day 1 to day 5 even in these nutrient poor conditions. S19 was already less abundant than the others after 1 day, and this strain hardly grew or even decreased in abundance depending on the combinations during the four following days (**Figure 5** and **Table 2**). Overall, being incubated with different partners did not affect all strains in the same way (**Table 2**): S35 and, to a lesser extent, R32, were inhibited in their growth in many of the dual and triple combinations compared to when they were inoculated alone, while R47 grew less well mainly in triple combinations but was not affected by dual combinations. S19 was only growing less well in combinations than alone in three out of 10 possible combinations and S49 was only affected by the presence of R47 in dual combination, but otherwise grew as well with any partner as alone (**Table 2** and **Figure 5**). A striking fact was observed in the case of S35: beyond its general decrease in abundance when mixed with other strains, it appeared to be completely outcompeted or even killed when incubated with either of the two rhizosphere strains R32 and R47. However, these latter strains did not seem to profit from the presence of S35, since their abundance was not significantly higher in the presence of S35 than in its absence (**Figure 5** and **Table 2**). This inhibition of S35 in presence of either R32 or R47 was rescued when any additional strain was present, since S35 grew normally in all tripartite combinations tested, even in the combination with R32 and R47.

DISCUSSION

Efficient control of late blight by bacterial biocontrol agents has been observed in few cases in greenhouse or even field experiments (Puopolo et al., 2014; Caulier et al., 2018), but most of the studies reported lack of reproducibility in protection against this disease (reviewed in Dorn et al., 2007; Axel et al., 2012). Indeed, in contrast to a synthetic molecule acting directly on a specific target of the pathogen, biocontrol agents that are applied, e.g., on leaves need (i) to efficiently compete with the native microbiota to colonize this environment, and (ii) to survive there despite exposure to UV and to rapidly changing temperature and humidity. Once established, they can produce bioactive molecules that either trigger the host plant's immune defense or that directly inhibit the pathogen's development. *Phytophthora infestans,* as many other plant pathogens, undergoes different developmental stages during the infection season, such as producing/releasing spores (sporangia and zoospores) or growing mycelium to colonize the host tissues (Fry, 2008). Ideally, control measures should target as many of these stages as possible to maximize efficiency.

One possible way to increase the chances for biocontrol agents to overcome the above-mentioned hurdles consists in using mixtures of strains rather than single agents, to increase both functional polyvalence (targeting different stages of the pathogen

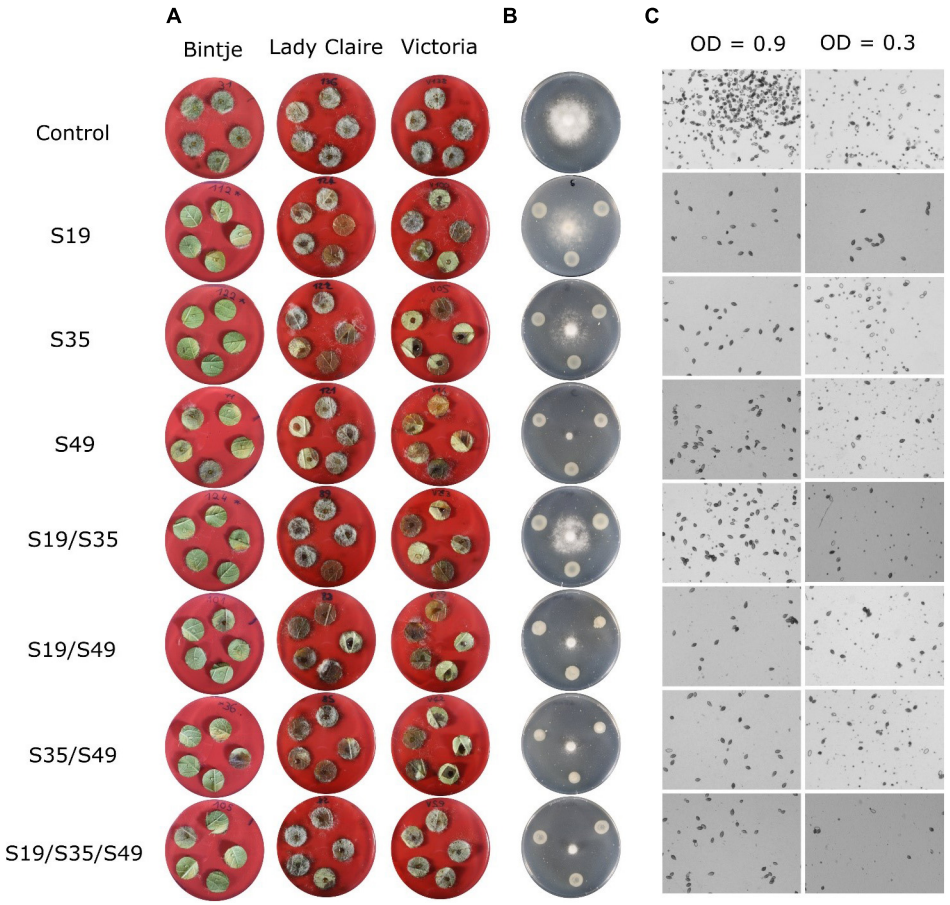


FIGURE 6 | Representative pictures of the effects of S19, S35, and S49 applied as single strains or in dual vs. triple combinations. **(A)** Leaf disk assay on the three cultivars; **(B)** mycelial growth; **(C)** zoospore release with total bacterial cell densities of 0.9 (left) and 0.3 (right). Please see Materials and Methods for details.

life cycle) and redundancy (maximizing the chances of successful host plant colonization in various environmental conditions). This "polymicrobial" approach has drawn considerable attention in recent years, although most studies have so-far focused on mixing well-known, commercially available microbial agents such as *Trichoderma* and *Bacillus/Pseudomonas* or mycorrhizal fungi and nitrogen-fixing bacteria (Xu et al., 2011; Reddy and Saravanan, 2013; Sarma et al., 2015; Parnell et al., 2016). Few

TABLE 2 | Statistical analysis of the abundance of the five selected strains alone or in dual vs. triple combinations.

R32			R47			S19			S35			S49		
R32	**30.0**	**a**	**R32/R47**	**28.7**	**a**	**R47/S19/S35**	**30.3**	**a**	**S35**	**32.0**	**a**	**S19/S49**	**30.0**	**a**
R32/S49	28.3	a	R47/S35	27.8	ab	R32/S19/S35	24.8	ab	S35/S49	26.3	ab	R32/S19/S49	25.7	ab
R32/S35	27.0	ab	R47	26.0	abc	S19	22.2	ab	R32/R47/S35	23.7	abc	S19/S35/S49	23.0	abc
R32/S35/S49	22.0	abc	R32/R47/S35	19.8	abcd	R32/S19/S49	20.2	abc	**R32/S35/S49**	**21.7**	**bc**	R47/S35/S49	21.3	abcd
R32/S19/S35	**18.5**	**bcd**	R47/S49	17.0	bcd	R47/S19	19.7	abc	**R47/S19/S35**	**20.0**	**bc**	S49	20.0	abcde
R32/R47/S49	**15.7**	**cde**	R47/S19	15.7	cd	R32/R47/S19	17.8	bcd	**R32/S19/S35**	**17.0**	**cd**	S35/S49	15.7	bcdef
R32/S19/S49	**11.7**	**de f**	R47/S35/S49	15.5	cd	R47/S19/S49	17.5	bcd	**S19/S35/S49**	**16.8**	**cd**	R32/R47/S49	14.7	bcdef
R32/S19	**11.2**	**de f**	**R32/R47/S19**	**12.3**	**de**	S19/S35/S49	14.8	bcd	**R47/S35/S49**	**11.3**	**de**	R32/S35/S49	12.5	cdef
R32/R47/S19	**11.0**	**de f**	**R47/S19/S49**	**12.3**	**de**	**S19/S49**	**10.3**	**cde**	**S19/S35**	**10.2**	**de**	R47/S19/S49	9.8	def
R32/R47/S35	**7.3**	**ef**	**R32/R47/S49**	**9.0**	**de**	**R32/S19**	**7.2**	**de**	**R32/S35**	**4.0**	**e**	R32/S49	8.5	ef
R32/R47	**4.3**	**f**	**R47/S19/S35**	**2.8**	**e**	**S19/S35**	**2.2**	**e**	**R47/S35**	**4.0**	**e**	**R47/S49**	**5.8**	**f**

Treatments are ordered from highest (top) to lowest (bottom) abundance. For each strain (column), different letters indicate statistically different values of the treatments according to Kruskal–Wallis test, $p < 0.05$, $n = 3$. Bold font indicates significantly lower abundance of the combined treatments compared with the control (single strain).

studies compared the effect of such strain combinations with that of the respective strains applied alone, and they came to divergent conclusions: Pertot et al. (2017) performed a 4-year field study on biological control of *Botrytis cinerea* in grapevine using a combination of two fungi (*Trichoderma*, *Aureobasidium*) and a *Bacillus*. They observed good efficacy for each of the antagonist but no additive value of combining the three (Pertot et al., 2017). Using five commercially available biocontrol agents (two based on *Bacillus,* one on *Streptomyces* and two on *Trichoderma* strains) against *Phytophthora ramorum* in a detached leaf assay, Elliott et al. (2009) observed lower efficacy of the mixture compared to some of its individual components, suggesting antagonistic effects between the different strains composing the mixture (Xu et al., 2011). The performance of strain combinations compared to individual strains might also depend on the targeted disease, as observed in rice for a dual treatment of *Trichoderma* and *Pseudomonas* strains, which was more effective than its single constituents against blast (caused by a fungus) but not against blight (caused by a bacterium) (Jambhulkar et al., 2018). In contrast, protection against *Ralstonia*-induced wilt in tomato was much higher when a mixture of eight *Pseudomonas* strains was applied than when the strains were applied individually (Hu et al., 2016).

Most of the above-mentioned studies used strains available as commercial products or in strain collections; however, these strains might not be adapted to the plant host and its pathogens, depending on their origin of isolation. Moreover, most screening efforts leading to the discovery (and putative registration) of antagonist strains have been done in *in vitro* experiments, which does not necessarily reflect the true antagonistic potential *in planta* or even in field conditions. In the present study, we investigated whether protective effects of *Pseudomonas* strains would be higher when applied in combinations than as single strains. Using nine potato-associated *Pseudomonas* strains, we performed a leaf disk infection assay with all 129 possible dual and triple combinations to circumvent the bias of the *in vitro* selection procedure. We performed this leaf disk screening on three different potato cultivars since we expected that the strain performance would vary according to the host plant genotype and sensitivity to late blight. As expected, a strong cultivar effect was observed, but surprisingly, best overall protection occurred on Bintje, which is most sensitive to late blight, while the two other cultivars were less efficiently protected by the strains (**Figure 2B**). This, however, might be at least partially because the screening on Bintje was carried out on ten leaf disks, while only five leaf disks per treatment were analyzed for the other two cultivars (see Material and Methods for the underlying reason). Only one strain, *P. fluorescens* S35, conferred significant protection on all three cultivars when applied alone, but this strain was less represented among efficient dual combinations than, e.g., *P. fluorescens* S49. This might be due to inability of S35 to compete with other *Pseudomonas* strains, as evidenced by the fact that in most combinations tested, S35 grew less well than when incubated alone (**Table 2**). In contrast, S49 could grow to the same level when co-incubated with any other strain we tested, except with R47, where it was slightly inhibited in its growth. Interestingly, when S35 was co-incubated with either R32 or R47, it could not be recovered after five days, suggesting strong inhibition or even killing of S35 by R32 and R47. This observation could explain the loss of activity of S35 when mixed with R47 on Lady Claire and Victoria, on which R47 was not active on its own, while R47/S35 was still active on Bintje, where R47 was active on its own (**Figure 1**). Likewise, S49, which was offering significant protection on Victoria when applied alone, lost its activity when mixed with R47, while it kept it when mixed with other strains that did not interfere with its growth. These results indicate that the mutual influence of strains on each other, when incubated in very low nutrient conditions, might be a useful parameter to investigate when designing microbial consortia for protection against diseases.

The screening of the 129 different treatments did not lead to an overall "champion" combination, but it highlighted the consistent protective activity of some strains, either when applied alone (S35), or in dual combinations (S19/S49). This latter combination was particularly interesting since it was efficient on all three cultivars, but when applied alone, neither strain was efficient on Bintje, only S19 was efficient on Lady Claire and only S49 was efficient on Victoria, thereby suggesting a synergetic effect between the two strains. Likewise, the dual combination S35/S49 was efficient on Bintje although only S35 was efficient on this cultivar when applied alone (**Figures 1**, **6A**). We wondered whether such synergetic effects could be due to differential modes of action of the different strains, e.g., inhibiting specifically the mycelial or spore stage of the pathogen, and tested these three potato phyllosphere isolates, together with two rhizosphere isolates previously shown to display strong anti-*Phytophthora* activity *in vitro* (Guyer et al., 2015; Hunziker et al., 2015).

We observed that the dual combination of S19/S49, which was particularly efficient on leaf disks, was only moderately inhibiting the mycelial growth of *P. infestans* in the *in vitro* assay, much less than when S49 was applied alone. However, all triple combinations containing S19/S49 (including that with the otherwise moderately active S35) were highly efficient in inhibiting mycelial growth, in contrast to those containing R47/S35 (**Figure 3**). In the natural leaf infection cycle, as well as in our leaf disk assays, the infection starts with a sporangium that, depending on temperature, can either directly germinate or release motile zoospores (Fry, 2008). Originally we aimed at investigating the effects of the five selected strains on these two processes but due to unknown reasons and despite repeated trials in different conditions, the harvested *P. infestans* sporangia did not germinate (even in the control) as they did previously in our hands, but consistently released zoospores. We therefore focused on analyzing how the five selected strains would affect this important route of infection in single, dual and triple combinations. Interestingly, the strain inhibiting this stage of *P. infestans* development in the strongest and most consistent way was S19, while S35 was the least active one (**Figure 4**). However, mixing S19 with any other strain but S49 led to loss of the activity. Among triple combinations, all those containing both S35 and S49 led to significant inhibition of zoospore release, suggesting good tolerance of these strains toward additional members of the tripartite consortium.

In summary, our screening of 129 different treatments of single, dual and triple strain combinations against *P. infestans* on three potato cultivars led to the observation that despite strong cultivar specificity, some strains showed strong and consistent protective effects, either when applied alone (S35) or in combination (S19/S49). The effects of these three phyllosphere strains on disease development, mycelial growth and zoospore release are shown through representative picture of the respective assays in **Figure 6**. When investigating the effect of these strains on each other's growth, we observed that S35 was less able to compete with other strains than S49 or even S19 (**Figure 5** and **Table 2**), possible explaining its better leaf disk performance when applied alone than in combination. The successful combination of S19 and S49 could be explained by their different mode of action: while S49 had much stronger inhibiting effect on mycelial growth than S19, S19 was a very efficient inhibitor of zoospore release. Despite their difference, S19 and S49 were able to maintain sufficient population densities when grown together (**Figure 5**), which is a prerequisite for synergetic effects.

In previous studies, we had considered S19, S35 and even S49 as among the lesser active strains, because our activity screening was performed mostly on *in vitro* tests assessing mycelial growth inhibition in dual assays (Guyer et al., 2015; Hunziker et al., 2015). Interestingly, when screening for protection using leaf disks rather than *in vitro* assays, these three phyllosphere strains turned out to be the most promising ones, which might be due to a particular ability to survive on leaf tissues or to cope with plant defenses. In dual and triple combinations, mixing of either S19/S49 (leaf disks) or S35/S49 (zoospore release assay) proved efficient in inhibiting *P. infestans* development. This good compatibility of strains sharing the same – phyllosphere – origin was not observed when mixing strains from the phyllosphere with strains from the rhizosphere, which might indicate that these strains have different requirements with respect to environmental conditions. In addition to leaf blight, *P. infestans* also causes tuber blight and it would be interesting to see whether rhizosphere isolates would prove more efficient than phyllosphere isolates for this particular form of the disease. In contrast to foliar blight, tuber blight was shown in an earlier study to be efficiently controlled by a mixture of four strains, among which three were fluorescent pseudomonads (Slininger et al., 2007). These strains were originally isolated from suppressive soils supplemented with tuber slices (Schisler and Slininger, 1994) and their protection efficacy was much higher in the mixture than with either of the strains applied alone, which highlights the potential of such host plant- or even host-tissue derived consortia to fight oomycete diseases.

Overall, our study clearly shows the potential added value of combining different, but compatible strains, with the example of a dual combination that led to stronger and more consistent protection than that obtained with the single strains. This study also highlights the complexity of interactions taking place even in such limited tripartite consortia. When increasing the number of partners, much higher complexity shall be expected, opening a wide range of fascinating questions related to the role of each strain in the consortium and the broader community (Lindemann et al., 2016), as recently exemplified by Niu and co-workers, with the identification of one "keystone" species in a 8-member consortium (Niu et al., 2017). Beyond the traditional way of systematically isolating strains to test them later in single or combined applications, future endeavors might rely on the plant's ability to specifically recruit beneficial microbes when facing a particular pathogen attack, as recently demonstrated in the model plant *Arabidopsis thaliana* (Berendsen et al., 2018). Such recruited microbes might then be assembled in synthetic communities and investigated for protective potential against the original pathogen, as well as for other desired features to be conferred to the plant. This new and booming field of microbiome management is likely to provide innovative alternatives to our current ways of protecting plants against diseases (Herrera Paredes et al., 2018; Syed Ab Rahman et al., 2018), which would ideally be combined with more traditional strategies such as adapted crop management or selection of resistant varieties to achieve more durable and sustainable crop protection.

AUTHOR CONTRIBUTIONS

LW and MDV designed the research. NV, FG, and MDV performed the experiments. MDV analyzed the data. LW and MDV wrote the manuscript with help from FG and NV.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02573/full#supplementary-material

FIGURE S1 | *Phytophthora infestans* relative infection severity in leaf disks of three potato cultivars treated with five *Pseudomonas* applied as single strains at low, medium and high cell density (OD_{570} = 0.3, OD_{570} = 0.45, OD_{570} = 0.9). Results are expressed as treatment efficiency (100 – relative infection severity in each treatment compared with the untreated leaf disks), with means and standard errors of 15 replicates for Bintje, Lady Claire and Victoria. Within each variety, different letters indicate significant differences between the treatments according to Tukey's HSD test ($P < 0.05$, $n = 15$).

FIGURE S2 | Relative mycelial growth of *P. infestans* when exposed to five *Pseudomonas* applied as single strains at three different bacterial cell densities in a dual culture Petri dish assay. Untreated controls represent *P. infestans* grown without bacteria (see Materials and Methods for details). Relative mycelial growth was calculated by dividing the mycelial area obtained in the respective treatments with that obtained in the untreated controls (not exposed to bacteria). Results are means of four replicates from the same experiment. They are expressed as treatment efficiency and calculated as above (100 – relative mycelial growth). Letters indicate significant differences between treatments according to Kruskal–Wallis test ($p < 0.05$, $n = 4$).

FIGURE S3 | Relative zoospore release from *P. infestans* sporangia exposed to five *Pseudomonas* strains applied at three different cell densities. Sporangia suspensions pre-mixed with saline instead of bacterial cell solution were used as untreated controls. Released zoospores were counted and the relative release rate was calculated by dividing values obtained for treatments by those obtained for the untreated controls (see Materials and Methods for details). Results are means of three experiments with one sample per treatment. They are expressed as treatment efficiency and calculated as above (100 – relative zoospore release). Letters indicate significant differences between treatments according to Kruskal–Wallis test ($p < 0.05$, $n = 3$).

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Versatile Antagonistic Activities of Soil-Borne *Bacillus* spp. and *Pseudomonas* spp. against *Phytophthora infestans* and Other Potato Pathogens

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The world potato is facing major economic losses due to disease pressure and environmental concerns regarding pesticides use. This work aims at addressing these two issues by isolating indigenous bacteria that can be integrated into pest management strategies. More than 2,800 strains of *Bacillus*-like and *Pseudomonas*-like were isolated from several soils and substrates associated with potato agro-systems in Belgium. Screenings for antagonistic activities against the potato pathogens *Alternaria solani*, *Fusarium solani* (BCCM-MUCL 5492), *Pectobacterium carotovorum* (ATCC 15713), *Phytophthora infestans* (CRA-W10022) and *Rhizoctonia solani* (BCCM-MUCL 51929) were performed, allowing the selection of 52 *Bacillus* spp. and eight *Pseudomonas* spp. displaying growth inhibition of at least 50% under *in vitro* conditions, particularly against *P. infestans*. All 60 bacterial isolates were identified based on 16S rRNA gene sequencing and further characterized for the production of potential bio-active secondary metabolites. The antagonistic activities displayed by the selected strains indicated that versatile metabolites can be produced by the strains. For instance, the detection of genes involved bacilysin biosynthesis was correlated with the strong antagonism of *Bacillus pumilus* strains toward *P. infestans*, whereas the production of both bio-surfactants and siderophores might explain the high antagonistic activities against late blight. Greenhouse assays with potato plants were performed with the most effective strains (seven *Bacillus* spp. and four *Pseudomonas* spp.) in order to evaluate their *in vivo* antagonistic effect against *P. infestans*. Based on these results, four strains (*Bacillus amyloliquefaciens* 17A-B3, *Bacillus subtilis* 30B-B6, *Pseudomonas brenneri* 43R-P1 and *Pseudomonas protegens* 44R-P8) were retained for further evaluation of their protection index against *P. infestans* in a pilot field trial. Interestingly, *B. subtilis* 30B-B6 was shown to significantly decrease late blight severity throughout the crop season. Overall, this study showed that antagonistic indigenous soil bacteria can offer an alternative to the indiscriminate use of pesticide in potato agro-systems.

Keywords: *Bacillus* spp., bacilysin, bio-control, lipopeptides, potato diseases, *Phytophthora infestans*, *Pseudomonas* spp., siderophores

INTRODUCTION

The increasing production of potato (*Solanum tuberosum* L.) is still facing important losses due to bacteria, fungi and fungus-like microorganisms, insects and viruses (Locke, 2002). Among these pathogens, the oomycete *Phytophthora infestans* (Mont.) de Bary, is responsible for causing late blight disease. The management of potato diseases, particularly late blight, is based on a massive use of chemical pesticides (i.e., fungicides such as mancozeb), causing tremendous costs, both direct and environmental (Cooke et al., 2011). The search for an environmental-friendlier pest management approach has led to study microbial agents with antagonistic capacities. Bacteria from both *Bacillus* and *Pseudomonas* genera are known to be appropriate candidates to be used in a bio-control approach due to their predominance in various environments, resilience and survival ability, but also for the number of bio-active molecules they are potentaially able to produce (Kloepper et al., 2004; Haas and Defago, 2005; Raaijmakers et al., 2010). Most of these bio-active compounds are secondary metabolites that exhibit direct and/or indirect antagonistic effects mediated through mechanisms such as antibiosis, competition, stimulation of plant growth and/or defenses (Emmert and Handelsman, 1999; Ongena and Jacques, 2008; Mavrodi et al., 2010).

Bacteria belonging to the genus *Bacillus*, particularly *Bacillus thuringiensis* strains with insecticidal properties, have been used in bio-control strategies since the mid-1930s (Bravo et al., 2011). Since then, the interest in *Bacillus* spp. has grown and numerous remarkable agricultural applications have been found. Some of them are currently exploited by the phytopharmaceutical industry which is commercializing some *Bacillus*-derived products: EcoGuard® by Novozymes (Gladsaxe, Hovedstaden, Denmark), Serenade® by Bayer (Leverkusen, North Rhine-Westphalia, Germany) or Kodiak® by Gustafson (Plano, TX, USA) as plant growth promoters and/or antagonists of phytopathogens with variable efficacy (Brannen and Kenney, 1997; Jacobsen et al., 2004; Kong et al., 2010; Lahlali et al., 2012). These claimed activities mostly rely on the enormous diversity of secondary metabolites produced by these bacteria, which can be divided into ribosomally synthesized peptides (e.g., bacteriocins), non-ribosomally synthesized peptides (e.g., lipopeptides, siderophores), polyketides (macrolides, polyenes) and volatile in-/organic compounds (hereinafter referred to as VIC and VOC). These compounds produced by *Bacillus* spp. are accompanied by various modes of action. Lipopeptides and VOCs, for instance, have direct antifungal activities as it is the case for the lipopeptide iturin A on *Rhizoctonia solani* (Yu et al., 2002) or volatiles pyrazine (2,5-dimethyl), benzothiazole and phenol-(4-chloro-3-methyl) against *Alternaria solani* and *Botrytis cinerea* on tomato plants (Gao et al., 2017). But other VOCs (2,3-butanediol, methyl jasmonate or methyl salicylate), as well as other lipopeptides (surfactin, fengycin) are also able to induce systemic resistance in plants through an ethylene-dependent pathway (Ryu et al., 2004; Ongena et al., 2007; Jourdan et al., 2009). Besides these activities, plant growth promotion was also reported using volatiles (2,3-butanediol, acetoin) on *Arabidopsis thaliana* (Ryu et al., 2003). Other compounds produced by *Bacillus* spp. like phytohormones (cytokinin, auxin) (Hussain and Hasnain, 2009; Lim and Kim, 2009) or siderophores (Yu et al., 2011) showed similar growth promoting effects on cucumber or red-pepper. In this last study, for example, the catecholic siderophore bacillibactin was proved to be involved in reducing the incidence of *Fusarium* wilt on pepper through induced systemic resistance, in addition to a direct bio-control effect.

Pseudomonas spp. are also known to produce a broad range of bio-active metabolites including lipopeptides, siderophores, polyketides, and volatile compounds with interesting activities against phytopathogens. It has been shown that these metabolites enable pseudomonads to directly compete with plant pathogens, promote plant growth or induce systemic plant resistance. The best example among these *Pseudomonas* spp. metabolites is the 2,4-diacetylphloroglucinol (DAPG). The antifungal activity of this polyketide has been extensively reviewed on damping-off, take-all or root rot diseases in various crops (Keel et al., 1992; Cronin et al., 1997; Huang et al., 2004; Ahmadzadeh and Sharifi Tehrani, 2009). Moreover, a clear link has been established between the disease-suppressiveness of some soils and the amount of DAPG producers (Weller et al., 2002) that include a large set of pseudomonads. But besides its direct antagonistic activity, DAPG is also involved in inducing systemic resistance through jasmonic acid and ethylene-dependent pathways (Iavicoli et al., 2003), highlighting its versatile mode of action. Lipopeptides are another example of the multiple antagonistic mechanisms used by pseudomonads. Some are known to lyse zoospores of *P. infestans* (de Souza et al., 2003; de Bruijn et al., 2007) and to induce resistance in tomato plants infected by this pathogen (Bakker et al., 2007; Tran et al., 2007). Pseudomonads siderophores (e.g. pseudobactin) are also able to induce systemic resistance in plants (Leeman et al., 1996; Meziane et al., 2005) and participate in direct competition with soil-borne plant pathogens for bio-available iron (Loper and Buyer, 1991). Siderophores produced by *Pseudomonas putida* or *Pseudomonas fluorescens*, for instance, are also able to promote plant growth and improve nutritional quality of major crops, such as rice, by enhancing the grain iron content (Sharma et al., 2013). Besides these soluble compounds, some VOCs produced by *Pseudomonas* spp. are also able to directly inhibit the development of *P. infestans* (de Vrieze et al., 2015) or *R. solani* (Kai et al., 2007).

In this study, we show the wealth of naturally occurring indigenous *Bacillus* spp. and *Pseudomonas* spp. strains associated with potato agro-systems that have the potential to be used at implementing biological control of potato diseases. For this purpose, a large collection of strains was isolated. Those displaying the highest *in vitro* antagonistic activity toward *P. infestans* were retained. An extensive characterization of these strains, including the detection of the genetic determinants of known antifungal metabolites, the *in vitro* assessment of lytic enzymes, the production of lipopeptides and siderophores and the *in vitro* activities against other potato pathogens, was assessed. The most interesting strains were tested on potato plants under greenhouse conditions. The implemented methodology allowed the selection of *Bacillus* spp. and

Pseudomonas spp. bacteria with versatile mode of actions and very effective against *P. infestans*. Because *in vitro* antagonistic activities have been shown to be rarely reproducible in field conditions (Ravensberg, 2011; Glare et al., 2012), a pilot field trial was also conducted with the most promising strains selected in this study. The results pointed out the attractive potential of *B. subtilis* 30B-B6 to be used as a bio-control agent against late blight in field conditions.

MATERIALS AND METHODS

Sampling Procedure and Bacteria Isolation

The sampling campaign was performed during the potato crop season from March to September 2012, mainly in loamy, shale-sandstone and sandy soils in Belgium. A total of 48 field soils from different locations and five soils from gardens were sampled from two soil horizons (A: 0–12 cm; B: 13–24 cm). Twenty-five other samples were also collected as follows: four from compost, eight from manure and 13 from potato plants divided in three parts (leaf, root and tuber) (Table S1). Plant samples were directly homogenized in 30 mL sterile Ringer's solution. Soil, compost and manure samples (25 g) were placed in blender bags with filters (pore size 330 μm; VWR) and mixed with 30 mL of sterile Ringer's solution in a Stomacher® machine (Seward) for 10 s. Samples were then collected through the blender bag filter and decanted overnight at 4°C. Each sample was then divided in aliquots supplemented with glycerol (3:1 vol/vol) and cryopreserved at −80°C for further bacterial selective isolation. *Bacillus*-like strains were isolated as follows: 1 mL of each sample was subjected to heat treatment (75°C, 20 min), 10-fold diluted with sterile Ringer's solution, plated on Lysogeny Broth (LB; also known as Luria-Bertani) (NaCl, 5 g L^{-1}; yeast extract, 5 g L^{-1}; tryptone, 10 g L^{-1}) solidified with 1.4% (wt/vol) agar and incubated at 30°C for 24 h. *Pseudomonas*-like selective isolation relied on growth (30°C, 24 h) on Cephalothin-Sodium Fusidate-Cetrimide (CFC) agar medium (Biokar Diagnostics®), combined with oxidase test for presumptive *Pseudomonas* strains. After selective isolation, nine strains of each genus were retained for each sample. The final collection of more than 2,800 isolates was then conserved in LB-glycerol (70:30 vol/vol) at −80°C.

Microorganisms, Culture Conditions and Potato Plant Varieties Used in this Study

Five potato pathogens were used in this work (**Table 1**): *A. solani* and *P. infestans* were cultivated on V8-agar ($CaCO_3$, 1 g L^{-1}; agar, 15 g L^{-1}; vegetable juice V8, 200 mL L^{-1}) in the dark, at room temperature and 18°C, respectively. *F. solani* and *R. solani* were cultivated on potato dextrose-agar (PDA, Oxoid) in the dark at 22°and 20°C, respectively. *Pectobacterium carotovorum* was grown overnight at 28°C on LB-agar. Bacteria belonging to the *Bacillus and Pseudomonas* genera were routinely grown on LB-agar (*Bacillus* spp. and *Pseudomonas* spp.) or PDA (*Pseudomonas* spp.). Certified tubers of *S. tuberosum* "Bintje" and "Challenger" varieties from CONDIPLANT® (Gembloux, Belgium) were used in greenhouse experiments and field trials. For greenhouse experiments, tubers were grown in pots containing twice-sterilized compost, daily watered and, maintained under controlled conditions with a photoperiod of 16:8 h at 25/15°C (day/night). For field trials, 10 acres of soil were supplemented with a mix of Nitrogen/Phosphorus/Potassium (NPK: 12-9-22, 1000 Kg ha^{-1}) and NH_4 (27%, 300 kg ha^{-1}). Prior to tuber plantation, an herbicide treatment was performed (2 Kg ha^{-1} Artist®, 1 L ha^{-1} Linuron®, 2 L ha^{-1} Challenge®; water 250 L ha^{-1}). No chemical weed control was performed before harvest and weather conditions were daily registered throughout the growing season.

TABLE 1 | Potato pathogens used in this study.

Pathogens	Remarks	Strain source
BACTERIA		
Pectobacterium carotovorum	Reference strain	ATCC 15713[a]
CHROMISTA		
Phytophthora infestans	Belgian field isolate, A2 mating type, race 1.3.4.5.7.10.11	CRA-W10022[b]
FUNGI		
Alternaria solani	Belgian field isolate	Not defined
Fusarium solani	Belgian field isolate	BCCM-MUCL 5492[c]
Rhizoctonia solani	Belgian field isolate	BCCM-MUCL 51929[c]

[a] *ATCC, American Type Culture Collection, Manassas, VA, United States.*
[b] *CRA-W, Walloon Agronomical Research Center, Libramont, Belgium.*
[c] *BBCM-MUCL, Microbial Collection of the Université catholique de Louvain, Louvain-la-Neuve, Belgium.*

Direct Antagonism Assays

Direct antagonism activities were assessed through confrontation on solid media in Petri dishes. For this purpose, bacterial cultures were prepared in 10 mL LB medium inoculated by a single colony and incubated 24 h at 30°C and 120 rpm. Antagonistic activities against *P. carotovorum* were evaluated by streaking the bacterial culture of interest in one third of the Petri dish containing LB-agar. Then, an overnight (O/N) broth culture of *P. carotovorum* (28°C, 120 rpm) was streaked three times perpendicularly to the bacterial inoculum (**Figure 1A**). LB medium was used as negative control. Petri dishes were scanned (HP Scanjet G4010) after 48 h of incubation. To evaluate the potential antagonistic activity against the other pathogens, two streaks of two cm of the bacterial culture of interest were made at each side of a piece of agar (Ø 5 mm) covered with the pathogen mycelium aged of 7–10 days (**Figures 1B–E**). As negative control, LB medium was streaked at each side of the piece of agar covered with the pathogen mycelium. Petri dishes were scanned (HP Scanjet G4010) after 7–10 days of incubation. Image analysis (Image J® software) was used to quantitatively determine the Growth Inhibition Percentage (GIP) by comparing the surface covered by the pathogen in the presence of bacterial isolate and the negative controls. Antagonistic activity was categorized in four different classes: (i) GIP $<$ 30%, (ii) GIP $\geq$ 30%, (iii) GIP $\geq$ 50% and GIP $\geq$ 70% (Figure S1). Each test was repeated twice with three technical replicas and statistically treated. At the end of those

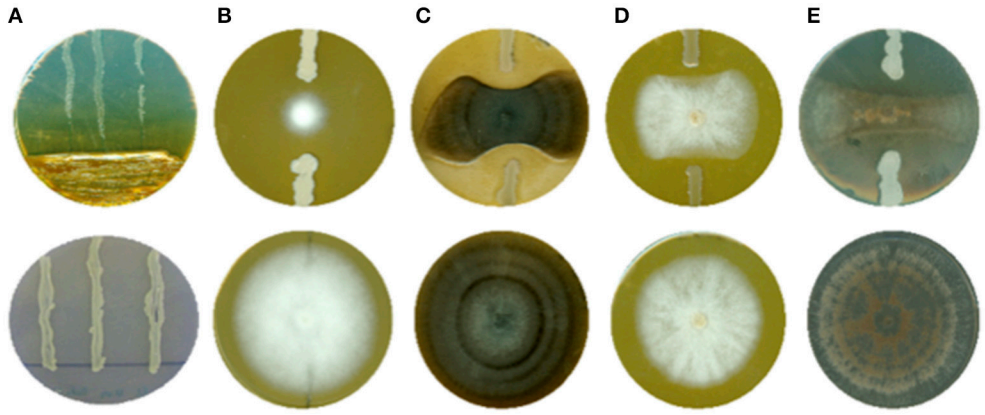


FIGURE 1 | *In vitro* confrontation (up) and negative control (down) of **(A)** *Pectobacterium carotovorum,* **(B)** *Phytophthora infestans,* **(C)** *Alternaria solani*, **(D)** *Fusarium solani*, and **(E)** *Rhizoctonia solani*. The percentage of growth inhibition was calculated through image analysis by comparing the area covered by tested pathogen with negative control. Each test was done three times, in triplicate.

tests, the 60 most active strains (GIP $\geq$ 50%) were selected for further characterization.

Bacterial Isolates Identification

Bacterial identification was performed through 16S rRNA gene sequencing. For this, bacterial isolates were grown overnight on LB-agar and one single colony was picked-up for "colony-PCR" using primers pair 27F (AGAGTTTGAT CCTGGCTCAG) and 1492F (GGTTACCTTGTTACGACTT). PCR reactions were performed using the GoTaq® G2 Flexi DNA Polymerase (Promega) with colorless buffer following the manufacturer's recommendations. Thermal cycling parameters were as follows: a denaturation step at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 55°C for 30 s and 72°C for 90 s. Finally, an elongation step at 72°C for 10 min. PCR amplicons were verified by gel electrophoresis, purified using the GenElute PCR cleanup kit (Sigma) and sequenced in both orientations at Macrogen Europe (Amsterdam, The Netherlands). For each bacterial isolate, nucleotide sequences were trimmed, aligned and compared with the BLASTn search available in GenBank database. Sequences were deposited in the NCBI database under GenBank accession numbers MF062580 to MF062639. Phylogenetic relationships based on partial 16S rRNA gene sequences were determined with MEGA 6.0 software (Tamura et al., 2013) using maximum likelihood (ML) method with the General Time-Reversible plus gamma model of nucleotide substitution and bootstrap values of 1,000 iterations.

PCR Screening for Genes Related to Known Antagonistic Metabolites and Virulence Factors

Total DNA from bacterial isolates were extracted by using the Wizard® Genomic DNA Purification Kit (Promega) following manufacturer's instructions. DNA extractions were PCR-screened for the presence of the genetic determinants of previously reported antagonistic metabolites and virulence factors (Table S2). All PCR reactions were performed using the One *Taq*® Quick-Load 2X Master Mix with standard buffer (New England BioLabs) following the manufacturer's recommendations. Thermal cycling parameters were 5 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at the annealing temperature, followed by 1 min/kb of the PCR amplicon (Table S2) at 68°C, and finally, 10 min at 68°C. Reference strains used as positive controls for each PCR are indicated in Table S2. PCR amplicons were analyzed by agarose gel electrophoresis.

Production of Enzymes, Siderophores and Bio-Surfactants by the Bacterial Isolates

The production of enzymes (Ariffin et al., 2006; Youcef-Ali et al., 2014), siderophores (Neilands, 1981) and bio-surfactants (Siegmund and Wagner, 1991; Youssef et al., 2004) were assessed for each selected bacterial isolate. Briefly, the production of proteolytic, cellulolytic and chitinolytic enzymes, along with the siderophore production, were respectively assessed throughout halo formation on specific solid media: Skimmed Milk (SM)-agar [Skimmed milk, 28g L^{-1}; casein hydrolysate, 5 g L^{-1}; yeast extract, 2 g L^{-1}; dextrose, 1 g L^{-1}; agar, 15 g L^{-1} at pH 7.0 $\pm$ 0.2]; Carboxy-methyl Cellulose (CMC)-agar [KH_2PO_4, 1 g L^{-1}; $MgSO_4.7H_2O$, 0.5 g L^{-1}; NaCl, 0.5 g L^{-1}; $FeSO_4.7H_2O$, 0.01 g L^{-1}; $MnSO_4.H_2O$, 0.01 g L^{-1}; NH_4NO_3, 0.3 g L^{-1}; CMC, 10 g L^{-1}; agar, 12 g L^{-1} at pH 7.0 $\pm$ 0.2]; Colloidal Chitin (CC)-agar [$NH_4H_2PO_4$, 1 g L^{-1}; KCl, 0.2 g L^{-1}; $MgSO_4.7H_2O$, 0.2 g L^{-1}; CC, 10 g L^{-1}; agar, 20 g L^{-1} at pH 6.0 $\pm$ 0.2]; and modified Chrome Azurol S (CAS)-agar [10 mL of $FeCl_3.6H_2O$, 27 mg/100 mL of HCl 10 mM; 50 mL of CAS, 1.2 g L^{-1}; 40 mL of HDTMA, 1.82 g L^{-1}; 900 mL of LB-agar for *Bacillus* spp. and King Broth agar for *Pseudomonas* spp. at pH 6.8 $\pm$ 0.2]. A 10 µL drop of a bacterial culture in LB (24 h, 30°C, 120 rpm) was spotted on the above-mentioned media and activity was observed after an incubation of 24 h at 30°C (proteolytic enzymes), 120 h at 37°C (cellulolytic enzymes), 120 h at 30°C (chitinolytic enzymes and siderophores). Each test was repeated twice with

three technical replicas. Bio-surfactant production was assessed via a "drop collapse test" based on amphiphilic properties of compounds like lipopeptides. Twenty-five µL drops of filtered supernatant (0.22 µm Minisart®, Sartorius Stedim) from a 48 h bacterial culture (*Bacillus* spp.: LB, 30°C, 120 rpm; *Pseudomonas* spp.: KB, 25°C, 120 rpm) were spotted on a hydrophobic surface (Petri dish Sarstedt®). Drops of sterile media (LB; KB) were used as negative control. After 5 min of incubation at room temperature, plates were scanned (HP Scanjet G4010) and the area covered by the drop was measured with an image analysis software (Image J®). The percentage of "spreading" was then calculated by comparison with the negative control and normalized with the optical density at 600 nm (OD_{600nm}) of the initial culture (Multiskan FC, Thermo Scientific). Each test was performed four times with three technical replicas. *Bacillus amyloliquefaciens* S499, *B. subtilis* GA1 and *Pseudomonas aeruginosa* PAO1 were used as positive controls, since they are known as bio-surfactant producers (de Souza et al., 2003; Touré et al., 2004; Arguelles-Arias et al., 2009; Nihorimbere et al., 2012). *Pseudomonas* spp. bio-surfactant production was also assessed via a culture of 5 days on Siegmund and Wagner (SW) medium [glycerol, 20 g L^{-1}; KH_2PO_4, 0.7 g L^{-1}; Na_2HPO_4, 0.9 g L^{-1}; $NaNO_3$, 2 g L^{-1}; $MgSO_4.7H_2O$, 0.4 g L^{-1}; $CaCl_2.2H_2O$, 0.1 g L^{-1}; 2 mL L^{-1} of trace elements containing $FeSO_4.7H_2O$, 2 g L^{-1}; $MnSO_4.H_2O$, 1.5 g L^{-1}; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.6 g L^{-1}; at pH 6.7 ± 0.2] supplemented with cetyltrimethylammoniumbromide (CTAB)-methylene blue agar [CTAB-methylene, 0.2 g L^{-1}; methylene blue, 0.005 g L^{-1}; agar, 15 g L^{-1}] where bio-surfactant producers are able to form a dark blue halo around the colony (Siegmund and Wagner, 1991).

Bio-Control Assays Against Late Blight in Greenhouse

Greenhouse experiments were performed in order to evaluate the antagonistic effect of selected bacteria against potato late blight. Assays were conducted with seven *Bacillus* spp. and four *Pseudomonas* spp. showing high *in vitro* antagonistic activities (GIP ≥ 70%) against *P. infestans*. Potato plants were obtained by planting potato tubers from sensitive "Bintje" variety and growing them for a month in a G2 greenhouse facility with cycles at 25/15°C (day/night) with 70% relative humidity (RH) and 16:8 photoperiod. Potato plants were arranged every 30 centimeters and were daily irrigated. Bacteria were grown in LB for 48 h at 30°C and 120 rpm. Bacterial cultures adjusted to an OD_{600nm} of 1 with LB were sprayed on potato leaves using 6 plants by treatment (2 mL/leaf and 3 leaves/potato plant) 4 h before pathogen inoculation. Sterile LB was used as negative control. Prior to the pathogen inoculation, *P. infestans* strain CRA-W10022 pathogenicity was restored *in vitro* on potato leaves as described by Clinckemaillie et al. (2016). Suspensions of *P. infestans* (ca. 15,000 sporangia mL^{-1}) were applied through pulverization (2 mL/leaf). Plants were placed in a humid chamber for 24 h at 90% RH to enable infection and then moved back to the G2 greenhouse. Quantitative scoring of the symptoms was done daily as described by Clinckemaillie et al. (2016) and the disease progression was followed for 7–10 days after inoculation. At the end of the experiment, the area under the disease progression curve (AUDPC) was calculated for each replica (Campbell and Madden, 1990) in order to determine a normalized protection index (PI) calculated as follows:

$$\mathbf{PI} = \left[\left(1 - \frac{\textit{AUDPC treatment}}{\textit{AUDPC control}}\right) * 100\right]$$

The greenhouse assays were repeated four times using 18 technical replicas (6 plants, 3 leaves/potato plant) each and were statistically treated.

Pilot Field Trial against Late Blight

A pilot field assay aiming at assessing selected bacteria effectiveness as bio-control agents of *P. infestans* was conducted at the Alphonse de Marbaix Research Center in Louvain-la-Neuve (Belgium) from April to September 2016. A randomized complete block assay was designed with 7 treatments (4 replicas/treatment, 4 lines/replica, 48 plants/replica) and interstices of sensitive "Bintje" potato variety distributed on a 10,000 m² field (15 m²/replica). 'Bintje' potato plants were used as reservoir to improve natural *P. infestans* infection in field. Bacterial strains of *Bacillus* spp. and *Pseudomonas* spp. were selected on the basis of *in vitro* and *in vivo* assays performed against *P. infestans*. Treatments applied were divided as follows: (i) plants treated with antagonistic strain *B. amyloliquefaciens* 17A-B3, (ii) plants treated with antagonistic strain *B. subtilis* 30B-B6, (iii) plants treated with antagonistic strain *Pseudomonas brenneri* 43R-P1, (iv) plants treated with antagonistic strain *Pseudomonas protegens* 44R-P8, (v) plants treated with chemical fungicide Revus® (250 g L^{-1} mandipropamid; 300 L ha^{-1}), (vi) plants treated with Cuprex® (300 L ha^{-1}), an authorized fungicide for organic production and (vii) untreated potato plants from late blight semi-resistant "Challenger" variety. Bacterial cultures suspensions were produced as described for greenhouse experiments and 2.5 L/replica were foliar-sprayed once a week from 8th June to 17th August 2016 (12 applications). Fungicides, supplemented with Trend 90® (1 mL L^{-1}), were applied six times in the potato crop growing season following Belgian warning prediction system (CARAH, PCA). Late blight disease progression was monitored as described for greenhouse assays, three times a week throughout the growing potato crop season, with a quantitative scoring of all leaves of eight plants/replica. AUDPC and normalized PI were calculated as described above.

Statistical Analysis

Data from antagonistic activity assays *in vitro*, greenhouse assays and pilot field trial were statistically treated using the one-way analysis of variance (ANOVA) using open source software R® (Ihaka and Gentleman, 1996). The Tukey HSD multiple-comparison test was used for comparison of the means, with confidence interval specified through *p*-value. The Pearson Correlation Index (PCI) was calculated with corrplot package in R® (Wei and Simko, 2016).

RESULTS

Isolation and *in Vitro* Screening of Bacteria Displaying Antagonistic Activities

A collection of 2,826 bacterial strains was isolated from a total of 157 collected samples (Table S1). Based on their *in vitro* antagonistic activities toward at least one of the five pathogens tested (*A. solani, F. solani, R. solani, P. carotovorum, P. infestans*) with a GIP $\geq$ 50%, 52 *Bacillus* spp. and eight *Pseudomonas* spp. strains were selected for further characterization (**Tables 2**, **3**). For this subset of 60 strains, 12 *Bacillus* spp. strains were shown to be active against *F. solani* (GIP $\geq$ 50%), whereas no *Pseudomonas* spp. showed a strong activity against this fungus. It is worth to note that due to the rhizoidal growth displayed by nine of the active *Bacillus* strains, their activity against *F. solani* might also be due to competition for surface colonization (Figure S2). Concerning the antagonism against late blight (*P. infestans*), 18 *Bacillus* spp. and seven *Pseudomonas* spp. strains displayed a strong inhibitory activity (GIP $\geq$ 70%). Also, 25 *Bacillus* spp. strains showed a good inhibition activity against *A. solani* (GIP $\geq$ 50%) and four against *P. carotovorum*, while none of the *Pseudomonas* strains were active against these two microbes. The assays against *R. solani* also revealed effective strains of *B. amyloliquefaciens* (one), *B. pumilus* (three) and *P. putida* (one). Taken together, a strong *in vitro* antagonistic activity (GIP $\geq$ 70%) against at least one of the pathogens tested was observed for 26 *Bacillus* spp. and five *Pseudomonas* spp. strains.

Identification of the Antagonistic Bacteria

BLAST and phylogenetic analyses based on 16S rRNA gene sequences revealed that the majority of the selected *Bacillus* spp. strains belong to the groups of *B. subtilis* (i.e., *B. amyloliquefaciens, B. licheniformis,* and *B. sonorensis*) and *B. cereus sensu lato (s.l.)* (i.e., *B. cereus* and *B. mycoides*) (**Table 2**, Figure S3). It is worth noting that the *B. mycoides* strains were further identified through their characteristic rhizoid growth on LB plates. Strains belonging to the *B. pumilus* complex, along with *B. megaterium* and *B. simplex*, were also identified among the selected strains as displaying remarkable antagonistic activities against the pathogens tested. It is interesting to note that the strains that showed strong competitive abilities, which are not necessary antagonistic activities, are all *B. mycoides* except for one *B. cereus* strain. Selected isolates from the genus *Pseudomonas* were identified through 16S rRNA gene sequences as *P. alkylphenolia*, *P. brenneri*, *P. chlororaphis*, *P. fluorescens, P. helmanticensis*, *P. protegens*, *P. putida,* and *P. salomonii* (**Table 3**, Figure S4).

PCR Screening for Known Antagonistic Metabolites

Using a PCR approach, the genetic determinants of potential antagonistic molecules screened for the collection of *Bacillus* strains were (Table S2): β-1,3-glucanase, AHL-lactonases, bacilysin, bacillomycin D, chitinases, difficidin, fengycin, iturin, macrolactin, plipastatin, surfactin and Zwittermicin A. As shown in **Table 2**, it seems evident that each species of *Bacillus* spp. present a specific pattern of detection associated with potential antagonistic metabolites. Indeed, *B. mycoides* strains were mostly positive to AHL-lactonases (PCI: 0.77), and exochitinases (PCI: 0.77), *B. licheniformis* strains to bacillomycin D (PCI: 0.47), *B. megaterium* strains to bacillomycin D (PCI: 0.48) and surfactin (PCI: 0.33), and *B. pumilus* strains to bacilysin (PCI: 0.61). Spectra of potential antagonistic metabolites were broader for *B. subtilis* strains (15A-B91, 15A-B92, 15A-B93, 27A-B4, 30B-B6) and even reached seven bio-active compounds for *B. amyloliquefaciens* strain (17A-B3). Bacilysin-related genes were the most commonly detected, in particular in all the strains displaying a strong inhibitory activity against *P. infestans* (GIP $\geq$ 70 %). These strains belong to the *B. amyloliquefaciens, B. pumilus,* and *B. subtilis* species (10 strains). Remarkably, five of these strains displayed positive PCR results only for bacilysin, which might indicate that this molecule was responsible for the strong antagonism observed toward *P. infestans*.

The genetic determinants associated with the production of phenazin, pyrrolnitrin, orfamid, syringopeptin, viscosin, 2,4-diacetylphloroglucinol, and pyoluteorin were detected by PCR in four strains of *Pseudomonas* spp. (**Table 3**) suggesting their potential implication in the *in vitro* antagonistic activities against the pathogens. In particular, *P. protegens* 44R-P8, which was positive for 5/10 molecules tested by PCR, showed an important *in vitro* antagonistic potential. Interestingly, although the *P. brenneri* 43R-P1 strain was negative to all tested genes, it displayed a strong antagonism toward *P. infestans* (GIP $\geq$ 70%; **Table 3**).

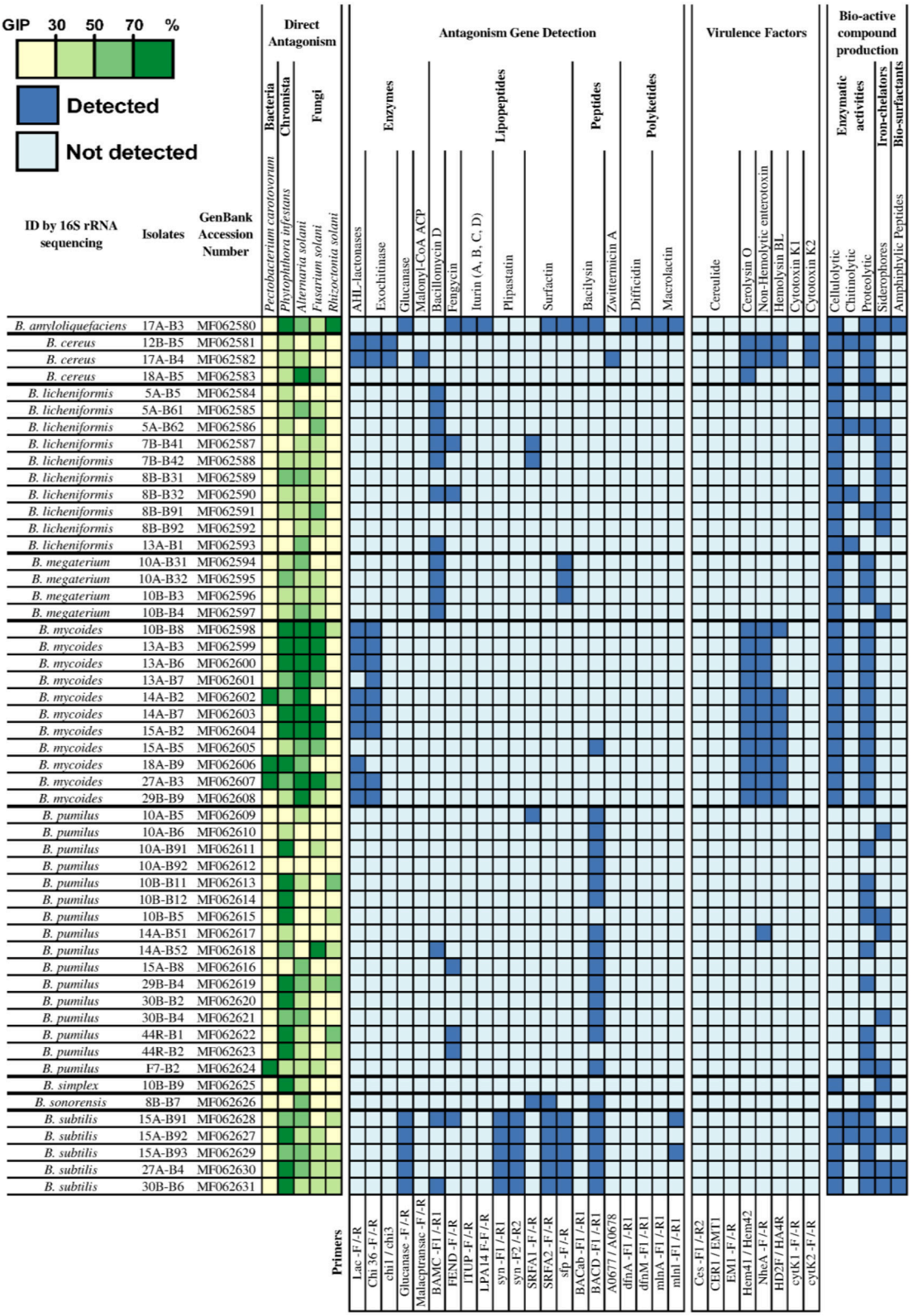
PCR Screening for Virulence Factors

The detection by PCR of the genetic determinants related to potential virulence factors of *B. cereus s.l.* group and *Pseudomonas aeruginosa* species was performed on all strains of the collection since some members of these groups may be potential human pathogens. Among the collection of *Bacillus* spp., the screening indicated that strains belonging to *B. mycoides*, *B. cereus* and one strain of *B. pumilus* were positive for at least one gene coding for a potential virulence factor, mainly the non-hemolytic enterotoxin (Nhe), Hemolysin BL (HBL) or Cerolysin O (CerO) (**Table 2**). However, none of the *Bacillus* spp. were positive to the genetic determinants coding for the lethal toxins cereulide and cytotoxin K1 (CytK1), both associated with severe foodborne toxi-infections in humans, including fatalities (Lund et al., 2000; Naranjo et al., 2011). Among the selected *Pseudomonas* spp. strains, none were positive to any tested genes related to the potential virulence factors (Finnan et al., 2004; Lanotte et al., 2004; Khalifa et al., 2011; Holban et al., 2013).

Evaluation of the Production of Enzymes, Siderophores, and Bio-Surfactants

The assays performed to evaluate enzymatic activities (i.e., proteolytic, cellulolytic and chitinolytic) of the selected strains indicated that the majority of the *Bacillus* spp. and *Pseudomonas* spp. were able to produce proteolytic and cellulolytic enzymes. On the contrary, very few *Bacillus* spp. and no *Pseudomonas* strains were able to display chitinolytic activity on colloidal chitin medium (**Tables 2**, **3**). No relation was found between any specific bacterial species, their enzymatic activities and

TABLE 2 | *Bacillus* spp. strains isolated in this study and selected for their statistically relevant *in vitro* antagonistic activities, evaluated through observed growth inhibition percentage (GIP), against *P. carotovorum*, *P. infestans*, *A. solani*, *F. solani*, and *R. solani*.



Genbank accession number, isolate code, 16S identification, antagonism exhibited toward pathogens tested (scaled as described in color legend at the top of the table), PCR detection of related genes to known bio-active metabolites and virulence factors, and bio-active compound production (enzymes, siderophores, bio-surfactants) are indicated.

TABLE 3 | *Pseudomonas* spp. strains isolated in this study and selected for their statistically relevant antagonistic activities, evaluated through observed growth inhibition percentage (GIP), against *P. carotovorum*, *P. infestans*, *A. solani*, *F. solani*, and *R. solani*.

Genbank accession number, isolate code, 16S identification, antagonism exhibited toward pathogens tested (scaled as described in color legend at the top of the table), PCR detection of related genes to known bio-active metabolites and virulence factors, and bio-active compound production (enzymes, siderophores, bio-surfactants) are indicated.

their antagonistic behavior (data not shown). Regarding the siderophore production, while all *Pseudomonas* spp. strains produced siderophores under the condition tested, only 19 *Bacillus* spp. strains did, mostly strains of *B. licheniformis*, *B. pumilus,* and *B. subtilis* (**Tables 2**, **3**). Drop collapse tests also showed that all the *Pseudomonas* spp. strains were able to produce bio-surfactants (**Table 3**), while only four strains of the *B. subtilis* group did (**Table 2**).

Greenhouse Assays against Late Blight

In vivo assays conducted under controlled greenhouse conditions were designed and tested beforehand to ensure a well-established and reliable pathosystem allowing the pathogen to grow under good conditions and perform a complete infection of inoculated leaves under 8 days. Results from statistical analysis (ANOVA) performed on 72 replicas for each tested strain, distributed among four assays, confirmed the strong antagonistic activities of seven *Bacillus* spp. and four *Pseudomonas* spp. against *P. infestans* on the sensitive potato variety "Bintje." As shown in **Figures 2**, **3**, three *Bacillus* spp. (30B-B6, PI: 67%; 15A-B2, PI: 65%; 17A-B3, PI: 62%) and two *Pseudomonas* spp. (44R-P8, PI: 83%; 43R-P1, PI: 64%) gave significant protection (PI $>$ 60 %) against *P. infestans* after disease developmental level normalization.

Pilot Field Trial against Late Blight

Based on greenhouse assays, *B. amyloliquefaciens* 17A-B3 and *B. subtilis* 30B-B6, along with *P. brenneri* 43R-P1 and *P. protegens* 44R-P8 were evaluated in a pilot field trial to assess their PI against *P. infestans*. Environmental conditions during field trial (April, 20 to September, 22, 2016) were appropriate for late blight infection and development. Potato plants of semi-resistant "Challenger" variety reached 99.8% of late blight infection at the end of evaluations, while the sensitive "Bintje" potato plants presented already 100% of late blight infection from the sixteenth day. During the assay, significant rainy events occurred and matched with temperatures favorable for the growth and transmission of *P. infestans* in the field (Figure S5). Disease progression was followed through quantitative evaluations from June, 27 (day 0) to August, 22 (day 56) (**Figure 4A**), allowing calculation of AUDPC and determination of a normalized PI (**Figures 4B–G**). The protection conferred by bacterial treatments was less effective than fungicide treatments at any time in the assay. As expected, the fungicide Cuprex and Revus treatments allowed a disease severity reduction of 83 and 98%, respectively. However, the bacterial treatments with *P. protegens* 44R-P8 strain provided a significant protection for 16 days after the first signs of symptoms (**Figures 4B–D**) and up to 45 days with *B. subtilis* 30B-B6 (**Figures 4B–F**). Moreover, treatment with 30B-B6 strain allowed shifts in time of 5 days to reach 25 and 50% of infection compared to controls. Interestingly, protection conferred by the treatment with *B. subtilis* 30B-B6 stabilized around 20% for nearly 30 days from day 14 to day 42 (**Figure 4G**). At the end of the trial, the treatment with *B. subtilis* 30B-B6 allowed a statistically significant reduction of late blight severity (PI: 22%; p-value $<$ 0.001). Remarkably, monitoring of late blight progress showed that each application of *B. subtilis* 30B-B6 allowed a decrease in the disease development rate until an infection rate of 90% (**Figure 4A**), even in rainy conditions. The other bacterial treatments did not allow a significant symptoms reduction up to the end of the trial (**Figure 4F**). Tubers were harvested on September, 22 (day 85) when the yield data (t ha^{-1}) were monitored. They showed a minor increase (not statistically significant) in yield of potato treated with *B. subtilis* 30B-B6 compared to the other bacterial treatments and control potato plants (**Figure 5**).

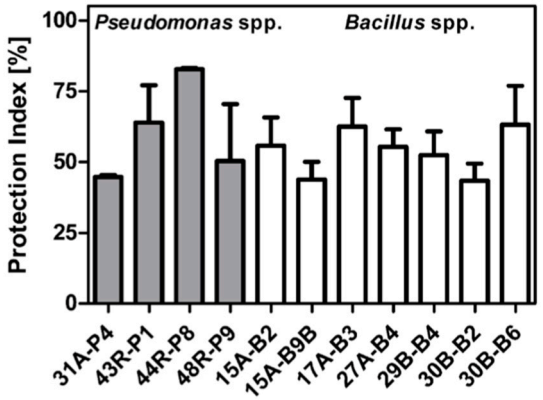


FIGURE 2 | Mean of normalized protection index (PI) against late blight observed after foliar spray on sensitive "Bintje" variety of potato plants with *Pseudomonas* spp. (gray) or *Bacillus* spp. strains (white), and standard deviation based on four greenhouse assays.

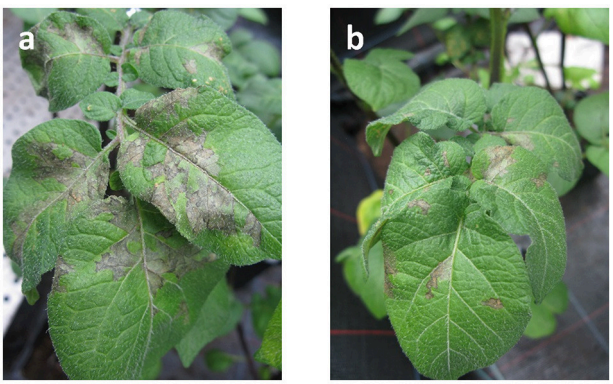


FIGURE 3 | Late blight progression observed in greenhouse assay after foliar spray of *P. infestants* (15,000 sporangia mL-1) on: **(a)** sensitive "Bintje" variety of potato plant and **(b)** potato plant previously treated with *P. protegens* 44R-P8 suspension.

DISCUSSION

In this study, we highlighted the fact that, in soil and/or on plants, there is a wealth of *Bacillus* and *Pseudomonas* bacteria present and displaying versatile antagonistic activities against potato pathogens. Starting from more than 2,800 environmental isolates, a core collection of 52 *Bacillus* spp. and eight *Pseudomonas* spp. strains was selected for further

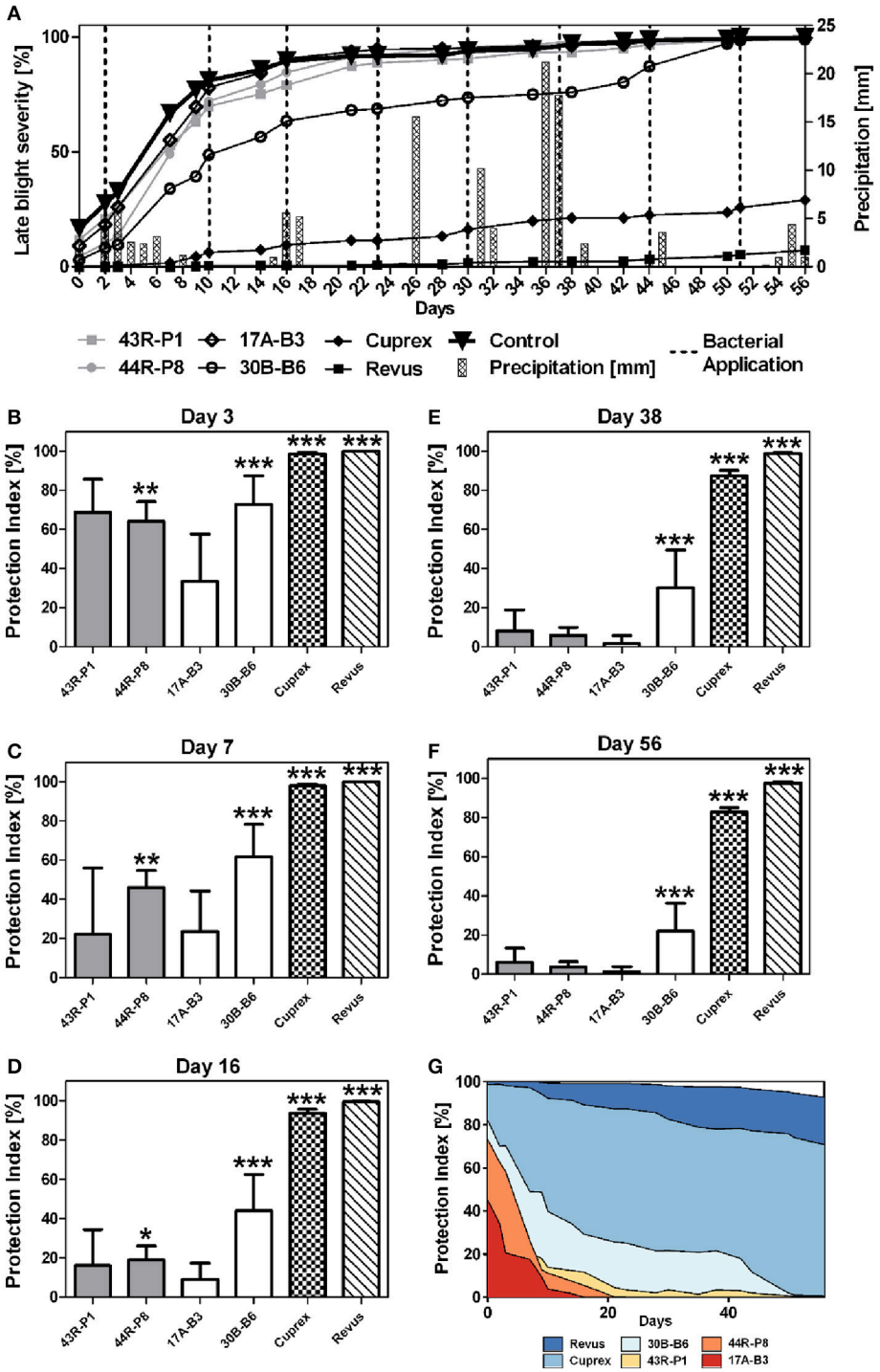


FIGURE 4 | (A) Mean of late blight severity (percentage of symptomatic leaf area) observed in field trial on "Challenger" variety potato plants: non-treated (▼) and treated with: *Pseudomonas* spp. strains 43R-P1 (■) and 44R-P8 (●), *Bacillus* spp. strains 17A-B3 (◊) and 30B-B6 (○), fungicides Cuprex® (♦) and Revus® (■). **(B–F)** Mean of normalized protection index (PI) against late blight in field assay on semi-resistant "Challenger" variety of potato plants observed after foliar spray with *Pseudomonas* spp. (gray), *Bacillus* spp. (white), fungicides Cuprex® and Revus® on days 3–56. **(G)** Mean of normalized PI evolution. Variance analysis (ANOVA) showed that at the end of pilot field trial, normalized PI is statistically significant for *Bacillus* 30B-B6 and fungicide treatments (*p-value < 0.1; **p-value < 0.01; ***p-value < 0.01).

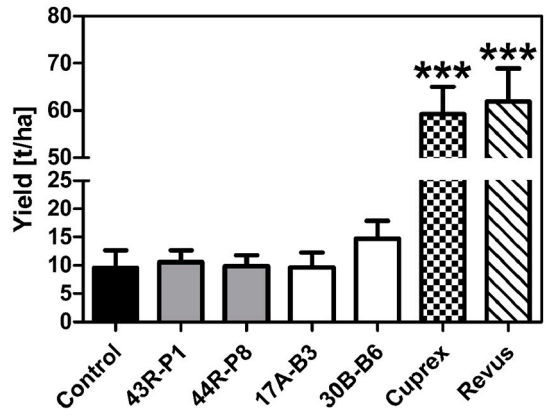


FIGURE 5 | Mean yield obtained at the end of the pilot field trial with the "Challenger" variety of potato plants non-treated (control) and treated with: *Pseudomonas* spp. strains 43R-P1 and 44R-P8, *Bacillus* spp. strains 17A-B3 and 30B-B6, fungicides Cuprex® and Revus®. Variance analysis (ANOVA) showed that yield enhancement is statistically significant for fungicide treatments (***p-value < 0.001).

analyses based on their significant *in vitro* antagonistic activities against important potato pathogens. Sequencing of 16S rRNA gene of selected bacteria revealed that the *Pseudomonas* strains belong to the *P. fluorescens* group (3), to the *P. putida* group (1), to the *P. chlororaphis* group (1) and to undefined groups, while antagonistic *Bacillus* strains belong mainly to the *B. subtilis* and *B. cereus* groups (Jensen et al., 2003; Wang et al., 2007). Antagonistic activities of *P. fluorescens* have already been described (Weller et al., 2002; de Souza et al., 2003; Haas and Defago, 2005) and were associated with specific metabolites such as DAPG. Nevertheless, our PCR screening for known antagonistic metabolites revealed only one strain (44R-P8) positive for the detection of genetic determinants involved in DAPG biosynthesis. This is an encouraging perspective for novel bio-active compound discovery because the other three *Pseudomonas* spp. strains (43R-P1, 42R-P4, 42R-P6) were highly effective against *P. infestans* and negative to all the other target genes.

Bacillus spp. also possess numerous interesting properties for industries and agriculture. As a matter of fact, *Bacillus* spp. based products are present on the market as bioinsecticides since early fifties with strains of *B. thuringiensis* that represent around 2% of the total insecticidal market (Bravo et al., 2011). Various products based on *Bacillus* spp. with other interesting properties have also been commercialized. These bacteria based products are claimed to be active through various mode of action due to production of a large set of bio-active metabolites. Nevertheless, the harmlessness of the use of biological control agents (BCA) is not always taken into account. Therefore, a screening for potential virulence factors was performed on our core collection. This screening revealed that no *Pseudomonas* spp. were positive to any of the potential virulence factor tested. With the exception of one *B. pumilus*, only strains belonging to *B. cereus s.l.* group (*B. cereus* and *B. mycoides*) were tested positive to some potential virulence factors (i.e., CerO, Nhe and HBL) but none for the lethal toxin cereulide or the cytotoxin K1 enterotoxin. Moreover, the well-known biopesticides *B. thuringiensis* serovar *kurstaki* HD-1 (Day et al., 2014) and *B. cereus* UW85 (Handelsman et al., 1990; Lozano et al., 2016) also harbor the genetic determinants coding for these potential virulence factors (data not shown) and they are used worldwide.

In this work, an extensive PCR screening performed on our core collection indicated numerous bio-active metabolites potentially produced by strains displaying remarkable antagonistic activities. It is worth noting that the mechanisms of action of many of these antagonistic molecules remain poorly understood and are associated with generic concepts such as plant growth and defense promotion, or antibiosis. The diversity in the metabolic arsenal of strains such as *B. amyloliquefaciens* (17A-B3), *B. subtilis* (15A-B91, 15A-B92, 15A-B93, 27A-B4 and 30BB6) and *P. protegens* (44R-P8) is particularly broad. Interestingly, despite the systematic search for known bio-active compounds via biomolecular and *in vitro* approaches, a large set of strongly effective strains remained negative to these screenings. Although those approaches have intrinsic limitations, these results are particularly encouraging in the prospection of discovering novel bio-active metabolites.

Among the 22 *Bacillus* spp. strains positive for the bacilysin-related genes, 14 were very effective against *P. infestans* (GIP $\geq$ 50%). These observations suggest that (part of) the strong *in vitro* inhibition against *P. infestans* might be related to the production of bacilysin by some *B. pumilus* and *B. subtilis* strains. Bacilysin is a 270 Daltons dipeptide composed of L-alanine and L-anticapsin known for its antifungal activity through inhibition of fungal mannoprotein and chitin biosynthesis (Milewski et al., 1986). Moreover, bacilysin also has antibacterial activity since it is an inhibitor of glucosamine synthetase (Chmara, 1985) and hence interferes with the bacterial peptidoglycan biosynthesis. Therefore, it might interfere with cellulose biosynthesis which is required by *P. infestans* to successfully infect potato (Grenville-Briggs et al., 2008). The potential involvement of bacilysin in antagonistic interactions with the oomycete *Phytophthora capsici* suggested by Chung et al. (2008) has not been clearly demonstrated since the observed activity could be related to a blend of known antifungal metabolites as iturin or mersacidin. Therefore, the potential mode of action of bacilysin against oomycete pathogens should be further investigated.

Besides bacilysin, other bio-active compounds might be involved in antagonistic activity. Our results revealed that the *B. amyloliquefaciens*, *B. subtilis* and *Pseudomonas* spp. strains effective *in vitro* and *in vivo* against *P. infestans* (GIP $\geq$ 70%) were also able to produce both bio-surfactants and siderophores. Bio-surfactant as lipopeptides from iturin and fengycin families produced by *B. amyloliquefaciens* and *B. subtilis* species are known to exhibit direct antifungal and anti-oomycetal activity (Yu et al., 2002; Ongena et al., 2005; Raaijmakers et al., 2010). Their fungi-toxicity is mediated by mechanisms involving pore formation in the plasmic membrane (Maget-Dana et al., 1985; Ongena and Jacques, 2008). Similarly, cyclic lipopeptides (cLPS) produced by *Pseudomonas* spp. have direct antifungal and anti-oomycetal activities (Yang et al., 2014). Cyclic lipopeptides

produced by *P. fluorescens* SBW25, for instance, have a specific activity on *P. infestans* zoospores (de Bruijn et al., 2007). Their exposure to cLPS results in their complete immobilization and subsequent lysis. The mechanism of action suggested, as for *Bacillus* lipopeptides, is a solubilization of the zoospore membranes. Among the five *Pseudomonas* strains selected in this work and highly active against *P. infestans*, three were shown to possess the genetic determinants involved in lipopeptide biosynthesis and all were positive to bio-surfactant production tests. Similar observations were done for *B. amyloliquefaciens* and *B. subtilis* strains regarding the presence of the genetic determinants coding for lipopeptides of fengycin, iturin, and surfactin families and they were positive to bio-surfactant production, too. Moreover, three of the active *Pseudomonas* spp. strains against *P. infestans* (i.e., 31A-P4, 42R-P6, and 48R-P9) were able to induce systemic resistance in *A. thaliana* (unpubl. results). Indeed, some lipopeptides produced by *Pseudomonas* spp., as well as lipopeptides from the surfactin and fengycin family produced by *Bacillus*, are known to have an indirect antagonistic activity triggering ISR in plant (Ongena et al., 2007; Tran et al., 2007; Jourdan et al., 2009; Nihorimbere et al., 2012). This indirect activity might be one explanation to the poor correlation observed between growth inhibition in *in vitro* assays and the protection index obtained in *in vivo* assays.

Lipopeptides are not the only metabolites able to trigger plant defenses. Iron chelating siderophores, produced either by *Bacillus* spp. or *Pseudomonas* spp., are also associated with indirect antagonistic mechanisms such as plant defenses and/or growth promotion (Leeman et al., 1996; de Boer et al., 2003; Meziane et al., 2005; Yu et al., 2011; Verbon et al., 2017). For example, pseudobactin siderophore produced by *P. putida* WCS358 is able to act as an elicitor and prime plant tissues in tomato for enhanced defenses against the pathogen *Botrytis cinerea* (Meziane et al., 2005). The ISR-triggering activity of cathecolic siderophore bacillibactin, produced by *B. subtilis* CAS15, has also been pointed out by Yu et al. (2011) on another *Solanaceae*, *Capscicum annuum* L., confronted to *Fusarium* wilt. However, siderophores with high and specific affinities are best known for their antagonistic activities through efficient competition for iron uptake, making it unavailable for pathogens (Kloepper et al., 1980; Thomashow and Bakker, 2015). Even though direct antifungal activity of the harzianic acid siderophore produced by *Trichoderma harzianum* has been highlighted (Vinale et al., 2013), to our knowledge, no direct activity of siderophores produced by *Pseudomonas* spp. or *Bacillus* spp. has been demonstrated. The difficulty to distinguish siderophores from common antibiotic definition has already been pointed out by Haas and Defago (2005). They suggested that siderophores contribute to disease suppression in some situations, but are not acting alone. Based on our observations, we suggest the complementary roles of lipopeptides and siderophores explaining the high *in vivo* antagonistic activity against the oomycete *P. infestans*. Indeed, among our core collection, all *Pseudomonas* spp., *B. amyloliquefaciens* (17A-B3), and *B. subtilis* (15A-B92, 27A-B4, 30B-B6) that were able to produce both siderophores and bio-surfactants, strongly inhibit *P. infestans,* whereas *B. licheniformis* strains, only producing siderophores, and *B. subtilis* that does not produce any of these metabolites, were not very effective against *P. infestans*. Therefore, considering the production of these two metabolites might be an interesting way of selecting effective bio-controlling agents (BCA) from *B. subtilis* and *P. fluorescens* groups. Moreover, a potential co-production, synergistic or any other complementary activities of those bio-active metabolites could also be a valuable explanation to effectiveness against late blight and should be further investigated.

This work also revealed the interest in other bio-control mechanisms dealing with competition for space, nutrient or oxygen between microorganisms and their ability to quickly colonize an ecological niche. This capacity is illustrated by the *in vitro* confrontation tests with strains of *B. mycoides* and their distinct rhizoid phenotype (Figure S2 and **Table 2**). Our results showed that *B. mycoides* strains have a strong *in vitro* antagonistic activity against *P. infestans,* which is thought to be mediated by competition for surface colonization. Additional assays with the lyophilized *B. mycoides* 18A-B9 strain, applied as tuber treatment, showed its ability to colonize "Bintje" potato plant from soil to upper foliar levels (unpubl. results). The same strain was also able to induce systemic resistance in *A. thaliana* (unpubl. results). This *Bacillus* species is already known in bio-control strategies as a plant defense and plant growth promoter (Bargabus et al., 2002; Bach et al., 2016). Similarly, *B. mycoides* 15A-B2 was very effective *in vivo* against *P. infestans* conferring a significant protection to "Bintje" potato plant. Under greenhouse conditions (foliar spraying), this strain also significantly decrease late blight severity (PI: 65%) which suggests that it is not only able to survive but could also colonize the foliar surface and allow a direct competition with *P. infestans* by invading its ecological niche and preventing infection of plant tissue. The production of anti-oomycete compounds at foliar level should not be excluded. Indeed, PCR screenings showed that the *B. mycoides* strains were almost exclusively positive to AHL-lactonase and exochitinase-related genes.

Pseudomonas spp. and *Bacillus* spp. bacteria are known to be producers of numerous bio-active compounds with versatile capacities but their *in vitro* activities are not easily transferable to *in vivo* assays, especially under field conditions (Ravensberg, 2011; Glare et al., 2012). This highlights the necessity to develop efficient way of selection. In the present study, *Bacillus* and *Pseudomonas* strains selected after extensive characterization for greenhouse trials showed promising, repeated and statistically relevant decrease in late blight severity going from 46% to almost 90%. Moreover, pilot field trial revealed the effectiveness of two out of four strains tested. *P. protegens* strain 44R-P8 conferred a significant protection (PI: 19%) against naturally occurring late blight until 16 days after first apparition of symptoms while *B. subtilis* 30B-B6 strain was still able to decrease from 44% the late blight severity and showed significant protection until the end of the pilot field trial (PI: 22%). These levels of protection are very promising considering the fact that no formulation was used and only crude bacterial suspensions were applied once a week, regardless of environmental conditions that were extremely severe, promoting *P. infestans* development and propagation.

Although treatments based on BCA are not sufficiently effective to be used alone, the application of *B. subtilis* 30B-B6 allowed a shift in time of 5 days to reach same level of late blight severity in first 2 weeks of infection. This shift in time could delay the first treatment with fungicides. Therefore, it would imply a decreasing in the number of required fungicide pulverizations that would be economically and environmentally attractive. However, developing a sustainable pest management approach must integrate various parameters such as appropriate crop rotation, appropriate fertilization, use of resistant cultivars, use of bio-control agents and pesticides, among others. In this regard, compatibility of active strains with specific cultivars and with a pest management scheme encompassing chemical pesticides are required and currently investigated. Taken together, this work highlights the benefit to combine molecular screening for bio-active metabolites with *in vitro* and greenhouse assays to successfully select field effective BCAs and have an insight in their potential mode of actions.

AUTHOR CONTRIBUTIONS

CB, JM, and AL conceived the study. PM and ND performed sampling and preliminary *in vitro* screenings. SC, AG, FL, and ML completed antagonistic *in vitro* screenings. AG performed strains identification and analyzed sequencing data. AG and SC performed genetic screening for bio-active metabolites and virulence factors. SC performed *in vitro* screening for bio-active metabolites production. ML conducted plant assays in greenhouses. GC carried out pilot field trial. SC analyzed the data of *in vitro* screenings, genetic screenings, plant assays and pilot field trial. SC and AG wrote the manuscript with help of AL, JM, and CB. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00143/full#supplementary-material

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Involvement of the Transcriptional Coactivator ThMBF1 in the Biocontrol Activity of *Trichoderma harzianum*

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Trichoderma harzianum is a filamentous fungus well adapted to different ecological niches. Owing to its ability to antagonize a wide range of plant pathogens, it is used as a biological control agent in agriculture. Selected strains of *T. harzianum* are also able to increase the tolerance of plants to biotic and abiotic stresses. However, little is known about the regulatory elements of the *T. harzianum* transcriptional machinery and their role in the biocontrol by this species. We had previously reported the involvement of the transcription factor THCTF1 in the *T. harzianum* production of the secondary metabolite 6-pentyl-pyrone, an important volatile compound related to interspecies cross-talk. Here, we performed a subtractive hybridization to explore the genes regulated by THCTF1, allowing us to identify a multiprotein bridging factor 1 (*mbf1*) homolog. The gene from *T. harzianum* T34 was isolated and characterized, and the generated *Thmbf1* overexpressing transformants were used to investigate the role of this gene in the biocontrol abilities of the fungus against two plant pathogens. The transformants showed a reduced antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* race 2 (FO) and *Botrytis cinerea* (BC) in confrontation assays on discontinuous medium, indicating that the *Thmbf1* gene could affect *T. harzianum* production of volatile organic compounds (VOC) with antifungal activity. Moreover, cellophane and dialysis membrane assays indicated that *Thmbf1* overexpression affected the production of low molecular weight secreted compounds with antifungal activity against FO. Intriguingly, no correlation in the expression profiles, either in rich or minimal medium, was observed between *Thmbf1* and the master regulator gene cross-pathway control (*cpc1*). Greenhouse assays allowed us to evaluate the biocontrol potential of *T. harzianum* strains against BC and FO on susceptible tomato plants. The wild type strain T34 significantly reduced the necrotic leaf lesions caused by BC while plants treated with the *Thmbf1*-overexpressing transformants exhibited an increased susceptibility to this pathogen. The percentages of Fusarium wilt disease incidence and values of aboveground dry weight showed that T34 did not have biocontrol activity against FO, at least in the 'Moneymaker' tomato variety, and that *Thmbf1* overexpression increased the incidence of this disease. Our results show that the *Thmbf1* overexpression in T34 negatively affects its biocontrol mechanisms.

Keywords: biological control, antifungal activity, multiprotein bridging factor, volatile organic compounds, *Fusarium oxysporum* f. sp. *lycopersici*

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INTRODUCTION

Trichoderma is a genus of filamentous fungi distributed worldwide, extremely well suited to live in different ecological niches (Druzhinina et al., 2011). This is due to its remarkably diverse metabolism, capable of catabolising a broad variety of substrates as well as of producing a huge diversity of secondary metabolites (Mukherjee et al., 2013). *Trichoderma* (where known, the teleomorphs belong to *Hypocrea*) includes species currently used as biological control agents due to their ability to antagonize a wide range of plant pathogens (Harman et al., 2004), *Trichoderma harzianum* being one of the species most widely used in biocontrol (Monte, 2001; Lorito et al., 2010). Selected *Trichoderma* rhizosphere-competent strains have been shown to exert beneficial effects on plants, increasing growth and stimulating defences against biotic and abiotic damage (Shoresh et al., 2010; Hermosa et al., 2012; Rubio et al., 2017).

Transcriptional coactivators play a crucial role in eukaryotic gene expression by connecting TATA-binding proteins (TBP) and the associated basal transcription machinery to transcription factors (TFs) (Suzuki et al., 2005). Some TFs have been functionally characterized in *Trichoderma* spp. (Aro et al., 2003; Casas-Flores et al., 2004; Stricker et al., 2006; Rubio et al., 2009; Fu et al., 2012; Gruber and Zeilinger, 2014, among others). However, little is known about other regulatory elements of the *Trichoderma* spp. transcriptional machinery and their role in biocontrol. Members of the highly conserved multiprotein bridging factor 1 (MBF1) protein family function as non-DNA-binding transcriptional coactivators. These mediator proteins are involved in regulating metabolic and developmental pathways in different organisms ranging from fungi to animals (Li et al., 1994; Takemaru et al., 1997). It has been demonstrated that MBF1 proteins interact with TFs or with different hormone receptors and link them with TBP, as observed in yeasts (Takemaru et al., 1998), fruit flies (Liu et al., 2003) or humans (Brendel et al., 2002; Kabe et al., 2005). MBF1 is also crucial for response to oxidative stress in human cells (Miotto and Struhl, 2006). In plants, MBF1 of *Arabidopsis thaliana* is encoded by three genes: *Mbf1a* and *MBf1b*, which are regulated developmentally (Tsuda et al., 2004), and *Mbf1c* that was related to expression changes of 36 transcripts during heat-stress (Suzuki et al., 2008). The potato MBF1 protein is induced in response to attack by a pathogen (Godoy et al., 2001) as well as to heat and oxidative stresses (Arce et al., 2006).

Most studies addressing fungal MBF1 have been carried out in yeasts. This coactivator mediates the general control non-derepressible (GCN4) protein-dependent transcriptional activation in *Saccharomyces cerevisiae* (Takemaru et al., 1998). GCN4 is a TF controlled at multiple levels by diverse signals of starvation and stress. This master regulator of gene expression acts modulating the transcription of amino acid biosynthesis genes, among others (Hinnebusch and Natarajan, 2002). In filamentous fungi, the cross-pathway control 1 gene, *cpc1*, encodes a protein similar to the yeast GCN4 (Paluh et al., 1988).

Little has been reported about MBF1 in filamentous fungi. In *Fusarium fujikuroi*, deletion of the *areA* gene, encoding a TF that mediates nitrogen metabolite repression, leads to an up-regulation of amino acid biosynthesis genes as well as *cpc1* and its putative co-regulator *mbf1*, both under nitrogen starvation and abundance (Schönig et al., 2008). However, Δ*mbf1* mutants of *F. fujikuroi* do not show differences in gene expression regulated by the factor CPC1 (Schönig et al., 2009). Thus, CPC1 mediates cross-pathway control independently of MBF1, at least in this fungus (Schönig et al., 2009). Furthermore, it has been identified and characterized an *mbf1* homolog in *Beauveria bassiana* and *Magnaporthe oryzae* as being involved in hyphal growth and stress responses (Ying et al., 2014; Fan et al., 2017). In addition, it has been demonstrated that the lack of *BbMBF1* in *B. bassiana* reduced its pathogenicity level against *Galleria mellonella* larvae (Ying et al., 2014), and that *MoMBF1* contributes to the virulence of *M. oryzae* in rice plants (Fan et al., 2017).

In a previous study working with *Thctf1*-null mutants from *T. harzianum* T34, we demonstrated that the TF THCTF1 was related to the biosynthesis of 6-pentyl-2*H*-pyran-2-one (6-PP) derivatives and biocontrol activity in this fungus (Rubio et al., 2009). Here, an *mbf1* homolog was identified in a suppression subtractive hybridization between the wild type strain T34 and a *Thctf1*-null mutant. The aim of this study was to functionally characterize *mbf1* in *T. harzianum*. We overexpressed the *Thmbf1* gene in strain T34 and studied its involvement in the antagonistic activity of T34 against *Fusarium oxysporum* f. sp. *lycopersici* and *Botrytis cinerea* in *in vitro* assays, and in the biocontrol potential against these two pathogens on tomato plants in greenhouse assays. Expression levels of *Thmbf1* and *cpc1* genes in *T. harzianum* strains grown on rich and minimal media were analyzed to investigate whether both genes might be functionally related.

MATERIALS AND METHODS

Bacterial and Fungal Strains and Tomato Seeds

Escherichia coli DH5α was used as a host for plasmid construction and propagation. This bacterial strain was grown in Luria-Bertani (LB) broth or on LB agar dishes, supplemented with ampicillin (100 μg/ml), X-gal (40 μg/ml) and IPTG (10 μg/ml), when required.

Trichoderma harzianum T34 (CECT 2413, Spanish Type Culture Collection, Valencia, Spain) was used as a source of DNA to clone the *Thmbf1* gene and also as a host in the transformation experiments to overexpress the *Thmbf1* gene. *Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (FO), determined as race 2 (Di Pietro and Roncero, 1998) and *Botrytis cinerea* B05.10 (BC), were used as plant pathogenic microorganisms in *in vitro* and *in vivo* assays. Fungal strains were routinely grown on PDA (Difco Becton Dickinson, Sparks, MD), and conidia were stored at −80°C in 30% glycerol.

Tomato (*Solanum lycopersicum*) seeds of 'Moneymaker' (Dobies & Paignton, Devon, United Kingdom) and 'Marmande' (Thompson & Morgan, Ipswich, United Kingdom) varieties were

used in greenhouse assays. Seeds were superficially disinfected in 70% ethanol for 10 min and in 2% sodium hypochlorite for 10 min. Later on, they were rinsed thoroughly three times in sterile distilled water before use, and air-dried on a sterile gauze sheet.

Selection and Isolation of *Thmbf1*

A suppression subtractive hybridization (SSH) between cDNAs from *T. harzianum* strains T34 and ΔD1-38, a Δ*Thctf1* mutant affected in the production of 6-PP derivatives (Rubio et al., 2009), was carried out. Mycelia from both strains were obtained after growth under identical conditions as follows: 100 ml of CM medium (0.5% malt extract, 0.5% yeast extract, 0.5% glucose) was inoculated with 10^8 conidia from PDA cultures, and incubated at 28°C in an orbital incubator at 250 rpm for 20 h. Then, 18 ml of the CM culture was inoculated into a Roux flask, containing 250 ml of MM containing 0.5% glucose (Penttilä et al., 1987). The culture was incubated statically at 28°C for 7 days in a growth chamber with a 12 h light/12 h dark photoperiod. *Trichoderma* mycelia were harvested by filtration through nytal (30-μm pore diameter), and RNA was extracted as previously described (Cardoza et al., 2006b). The mRNA was purified by oligo (dT) cellulose columns (Stratagene, La Jolla, CA, United States). Five microgram of mRNA were used for cDNA synthesis, using a cDNA synthesis system (Roche Diagnostics, Mannheim, Germany) and following the manufacturer's instructions.

The cDNAs from both strains were used for a subtractive hybridization using the PCR-Select cDNA subtraction kit (Clontech laboratories, Palo Alto, CA, United States) (Diatchenko et al., 1996), following the manufacturer protocol.

The complete sequence of the *Thmbf1* gene was obtained from a screening of a *T. harzianum* T34 lambda genomic library (Lora et al., 1995) as previously described (Rubio et al., 2009). DNA-binding elements were found by looking for consensus sequences described elsewhere or by using the MatInspector program[1] with the TRANSFAC database restricted to fungi.

Conventional PCR Amplification and Sequencing

PCR amplifications were accomplished using the *Taq* polymerase system (Biotools, Edmonton, AB, Canada), following the manufacturer's instructions. The *Thmbf1* cDNA was PCR-amplified with the primers MBF1-5 (5′- ATGTCTAACCAGGACTGGGATT-3′) and MBF1-3 (5′-TTATTTCTTCTTGGGGCCCAAG-3′) and T34 cDNA as template. Screening of *T. harzianum* T34 *Thmbf1* overexpressing transformants was performed by PCR with the primers Phleo-3 (5′-GGTGTTGGTCGGCGTCGG-3′) and GPD-3 (5′-GGTGTGTCGGCGGGGTTG-3′) to amplify a 645-bp fragment from the p43b1MBFa plasmid.

The PCR products were purified from agarose gels using the NucleoSpin Extract II Kit (Macherey-Nagel) according to the manufacturer's protocol. PCR fragments were sequenced and the sequences were analyzed using the DNASTAR package (Lasergene, Madison, WI, United States).

[1]http://www.genomatix.de/matinspector.html

Plasmid Constructions and *Trichoderma* Transformation Procedure

Plasmid p43b1MBFa was used for the transformation. To construct it, plasmid pAN52.1 (Punt et al., 1987), which contained the *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) gene promoter and the *trpC* gene terminator from *Aspergillus nidulans,* was digested with *Nco*I, treated with Klenow fragment and dephosphorylated with calf intestine alkaline phosphatase (CIAP). Then, it was ligated to the *Thmbf1* cDNA, which was amplified using the oligonucleotides MBF1-5 and MBF1-3. As result, the pAN52.1-MBF1a (6367 bp) plasmid was obtained. This plasmid was *Pst*I-digested, treated with Klenow fragment, and the resulting 3571 bp fragment, containing the *Thmbf1* expression cassette, was gel-purified. This fragment was ligated to the pJL43b1 plasmid (Gutiérrez et al., 1997), which contained the *ble* gene from *Streptoalloteichus hindustanus* under the control of the *gpdA* gene promoter, previously digested with *Kpn*I, treated with Klenow fragment, and CIAP-dephosphorylated. The resulting plasmid, p43b1MBFa (8067 bp), was used to transform protoplasts of the T34 strain (Cardoza et al., 2006a). In parallel, strain T34 was also transformed with pJL43b1 to obtain empty vector transformants; one of them was included in assays as a transformation control. Transformants were selected for phleomycin resistance.

Hybridization Experiments

For Southern blot analysis, total DNA was extracted as previously described (Cardoza et al., 2006a). Then, 10 μg of genomic DNA was *Xho*I- and *BamH*I-digested, electrophoresed on a 0.7% agarose gel, and transferred to a Hybond-N^+ membrane (Amersham, Piscaway, NJ, United States). The *Thmbf1* cDNA gene was labeled using the DIG High Prime kit (Roche, Penzberg, Germany), following the manufacturer's instructions, and used as a probe. Hybridization, washes and detection were carried out as previously described (Tijerino et al., 2011).

Phenotypic Assays

The growth of the wild type, transformation control and transformant strains was tested under different culture media. Two hundred conidia of each strain were used to inoculate dishes containing PDA or minimal medium (Penttilä et al., 1987) and incubated at 28°C for 3 days. These assays were performed in triplicate.

Mycelia from the transformation control Thmbf-CT and transformants were collected from both culture conditions and used to analyze the expression levels of *Thmbf1*and *cpc1* genes

In Vitro Antifungal Assays

Dual Confrontations

In vitro confrontation assays between *Trichoderma* strains and the pathogens FO and BC were carried out on PDA at 28°C as previously described (Rubio et al., 2009) and photographs were taken after 10 days. These assays were performed in triplicate, and single cultures of *Trichoderma* strains and pathogens were used as controls.

Confrontations on Discontinuous Medium

Strains of *T. harzianum* were also confronted with pathogens FO and BC on PDA using 90-mm Petri dishes separated in two halves. *Trichoderma* and pathogen were inoculated to their respective half. Cultures were incubated at 28°C in the dark, and colony diameter measures and photographs were taken 5 days after inoculation. Pathogen cultures grown alone were used as controls.

Growth Assays on Membranes

Five-mm-diameter PDA plugs of *T. harzianum* T34, transformation control or transformants were placed, at the center of Petri dishes containing PDA, on cellophane sheets or on 14 kDa-cut-off dialysis membranes. After 2 days of incubation at 28°C, the membranes were removed from the dishes and a single 5-mm diameter mycelial plug of FO or BC was placed at the center of each dish. In parallel, each pathogen was grown on PDA (control dishes). Each condition was tested in triplicate and the results were expressed as growth diameters of each pathogen after incubation for 2 days on PDA.

Real-Time Quantitative PCR

Gene expression was analyzed by real-time quantitative PCR (qPCR). cDNAs were synthesized from 1 μg of total RNA, using the Transcriptor First Strand cDNA Synthesis kit (Takara Inc., Tokyo, Japan) with an oligo(dT) primer. Reaction mixtures and amplification conditions were performed as previously described (Montero-Barrientos et al., 2010). PCRs were carried out in triplicate for three different biological replicates. Data are expressed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The following primer pairs were used and checked for dimer formation: 414 (5′-CTCAGCTTGACGTTG ACGAC-3′) and 415 (5′-CTACACCCGACCAGACCATT-3′), Cpc1-bf (5′-CGTCGATTTGGACGACTTCAC-3′) and Cpc-br (5′-GAGGAGACACGGTGCCAAGATT-3′), and Act-1 (5′-ATC GGTATGGGTCAGAAGGA-3′) and Act-2 (5′-ATGTCAACAC GAGCAATGG-3′), amplifying fragments of the *Thmbf1*, *cpc1* and *actin Trichoderma* genes, respectively. The primer pair Cpc-bf and Cpc-br was designed using a sequence alignment of the *cpc1* gene identified in the annotated genomes of *T. atroviride*, *T. reesei* and *T. virens*. Standard curves were measured for dilution series of pooled cDNA samples, and calculated using Applied Biosystems software.

Biocontrol Assays in Tomato Plants

The biocontrol ability of four *T. harzianum* strains (T34, Thmbf-CT, Thmbf-ov3 and Thmbf-ov4) against FO and BC on susceptible tomato plants was evaluated in *in vivo* assays.

FO Assays

Two independent assays were performed only differing in the method of inoculation with the *T. harzianum* strains: 'Moneymaker' tomato seed or substrate applications. In the first assay, surface-sterilized seeds as described above were coated with 1 ml of an aqueous suspension containing 1×10^8 conidia per ml or with 1 ml of sterile water (control) as previously described (Pérez et al., 2015). One ml was used to coat 30 seeds. Coated seeds were sowed in multi-cell growing trays containing a mixture of commercial substrate (Projar Professional-Comercial Projar, Valencia, Spain) and vermiculite (3:1), previously autoclaved for 1 h at 121°C on two successive days. In the second assay, surface-sterilized seeds were sown in 0.7–l pots (one seed per pot) containing 200 g of the above described autoclaved commercial substrate inoculated with *T. harzianum* (10^8 conidia per pot). In both assays, seedlings were maintained under greenhouse conditions at 22 ± 4°C and a photoperiod of 16 h light:8 h dark. Fourteen days after sowing, when the first true leaf was fully expanded, seedlings were uprooted, the excess of peat removed by shaking and roots cut to about 2.5 cm. The cut-root seedlings were dipped in a FO conidial suspension, adjusted to 2×10^7 conidia per ml, and planted in 0.7–l pots containing the above indicated mixture. FO conidia were obtained from 7 days-PDB cultures. Ten pots per treatment and one seedling per pot were used for each assay. Seedlings dipped with sterile water were included as a control. After FO inoculation, seedlings were maintained in the greenhouse under the conditions described above for 3 weeks, and watered as needed.

The six treatments tested were as follows: untreated (control), FO, T34 + FO, Thmbf-CT + FO, Thmbf-ov3 + FO, and Thmbf-ov4 + FO. In both assays, ten plants were used per treatment in a completely randomized design. The disease index was calculated using the following symptom severity scale (0–4): 0, healthy plant; 1, 2, and 3, slight, moderate and severe wilting plant, respectively; and 4, dead plant; and values used to determine the disease incidence (DI) percentage, as previously described (Song et al., 2004). Aboveground dry weights were also recorded for the assay *T. harzianum* treated-seeds.

Assay of BC

Surface-sterilized 'Marmande' tomato seeds were coated with an aqueous suspension of *T. harzianum* conidia or water (control) as described above. Seeds were sowed in 0.7–l pots (one seed per pot) containing the autoclaved mixture above indicated and seedlings were maintained under the indicated greenhouse conditions for 4 weeks. The sensitivity of plants to BC was evaluated as previously described (Pérez et al., 2015), except that two leaves from each plant were inoculated in a single point. Necrotic leaf area was evaluated after 3 days using ImageJ free software. Five plants were considered for each of the six treatments tested: untreated (control), BC, T34 + BC, Thmbf-CT + BC, Thmbf-ov3 + BC and Thmbf-ov4 + BC.

Statistical Analyses

Each data set was submitted to analysis of variance (ANOVA) and means compared by Tukey test ($P < 0.05$) using Statistica 7 software (Statsoft Inc., Palo Alto, CA, United States).

RESULTS

The *T. harzianum* T34 *Thmbf1* Gene

A SSH method was carried out with cDNAs from the wild type strain *T. harzianum* T34 and the ΔD1-38 knock-out mutant.

ΔD1-38 had been previously used to explore 6-PP biosynthesis-related genes regulated by the TF THCTF1 (Rubio et al., 2009). A total of 202 differentially expressed clones were isolated, sequenced and analyzed using BlastX software. As a result, 96 clones showed homology with known genes (Supplementary Table S1). Six of them (6.5% of the total identified clones) corresponded to an *mbf1* homolog, which was selected for further characterization. The 340-bp fragment isolated from the *T. harzianum* subtractive library was used as a probe to screen a lambda genomic library. A total of 657 bp containing the complete open reading frame (ORF) of *Thbmf1* and 119 bp of the promoter region were sequenced from a positive phage. *Thmbf1* has a length of 538 bp and contains one intron of 70 bp. The ORF, excluding the intron, contains 468 bp and encodes a protein of 156 amino acids with a theoretical molecular mass of 16.4 kDa and an isoelectric point of 10.2. The nucleotide sequence of *Thmbf1* was deposited in the GenBank database under Accession No. CCG26107. One single copy of *Thmbf1* homolog was detected in publicly available *Trichoderma* spp. genomes such as *T. reesei* (94% protein identity, ID 122457 protein), *T. virens* (94% protein identity, ID 73623 protein) and *T. atroviride* (90% protein identity, ID 151694 protein). Analysis of the 156 amino acids of the predicted *T. harzianum* T34 ThMBF1 protein revealed the presence of one DNA-binding helix-turn-helix (HTH) domain (amino acids 81-117), as described previously for eukaryotes (Aravind and Koonin, 1999), and the prevalence of the alpha-helix conformation. A high degree of similarity (70% amino acid sequence identity) was also found with the MBF1 proteins from fungi such as BbMBF1 of *B. bassiana* (XP008595149).

Overexpression of *Thmbf1* in *T. harzianum* T34

In order to functionally characterize the *Thmbf1* gene, the plasmid p43b1MBF1a was constructed and transformed in *T. harzianum* T34. Thirty transformants showing phleomycin resistance were checked by PCR. A 645-bp PCR product was amplified in nine of the thirty transformants analyzed, using the primer pair Phleo-3 and GPD-3. Four PCR-positive putative transformants were randomly chosen for further analysis by Southern blot (Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4) and determination of the additional *Thmbf1* copies due to the insertion of the transformation cassette in their genomes (**Supplementary Figure S1**). DNAs from strains T34 and Thmbf-CT, an empty vector transformant, were included as controls. One 0.8 kb signal, which corresponded to the endogenous gene, was observed in all lanes, indicating that *Thmbf1* is present as a single copy in *T. harzianum* T34 and that the transformation cassette is not present in strain Thmbf-ov1. Several blotted bands corresponding to the *Thmbf1* gene were observed in DNAs from three out of four transformant strains analyzed, indicating that the transformation cassette had been inserted into the Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 genomes at several *loci*.

Additional PCR reactions were carried out with DNA from the strain Thmbf-ov1 and the primer pairs MBF1-5 and MBF1-3 and Pleo-3 and GPD-3. A 558-bp PCR fragment was amplified with the pair MBF1-5 and MBF1-3, but no PCR product was observed when Pleo-3 and GPD-3 primers were used. Moreover, strain Thmbf-ov1 lost its ability to grow on PDA containing 100 μg/mL of phleomycin.

Thmbf1 and *cpc1* Expression Patterns under Different Culture Conditions

We analyzed the expression level of the *Thmbf1* gene by qPCR with the primer pairs 414&415 and Act-1&Act-2 in the Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 strains after growing on PDA and minimal media using the expression level in strain Thmbf-CT as a reference condition. The calibration slope, R^2 and efficiency of these primer pairs were: – 3.26, 0.95 and 114.55%, for 414 and 415, and – 3.38, 0.95 and 96.61%, for Act-1 and Act-2. Transformants Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 showed higher *Thmbf1* transcript levels than those observed for Thmbf-CT after growing on both media (**Figure 1A**), whereas no differences were detected between Thmbf-ov1 and Thmbf-CT strains.

In order to identify the influence of *Thmbf1* on the transcription of *cpc1*, the transcript levels of this latter were also examined in strains Thmbf-CT, Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 after growing under identical culture conditions. We used the primer pairs Cpc-Bf and Cpc-Br, which had values of – 3.13, 0.99, and 108.73% for calibration slope, R^2 and efficiency, respectively, and Act-1 and Act2. No significant expression differences were observed among the five tested strains (**Figure 1B**).

Antagonistic Activity

Dual confrontations assays between T34, Thmbf-CT, Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4, and the pathogens FO or BC were performed to investigate the effect of *Thmbf1* overexpression on the antagonistic activity of *T. harzianum* T34. All the assayed *T. harzianum* strains inhibited the growth of both pathogens on PDA, although they were not able to grow over the colonies of FO and BC (**Supplementary Figure S2**). No different behavior was observed among the wild type or the transformation control and the strains Thmbf-ov1, Thmbf-ov3 and Thmbf-ov4, whereas less ability to inhibit colony growth of FO and BC was observed for the Thmbf-ov2 strain. On PDA and minimal media, the Thmbf-ov2 strain displayed a smaller growth phenotype than the other strains, whereas no differences were observed between the rest of them (data not shown). At this stage, transformants Thmbf-ov3 and Thmbf-ov4 were selected for further analyses.

In order to analyze whether *Thmbf1* is involved in the production of volatile organic compounds (VOC) with antifungal activity, dual confrontations between *T. harzianum* strains T34, Thmbf-CT, Thmbf-ov3 and Thmbf-ov4, and the pathogens FO or BC were carried out on PDA discontinuous medium. The antifungal activity due to hydrolases and metabolites released to the culture medium was avoided since each fungus grew in different halves of the Petri dish. Strains Thmbf-ov3 and Thmbf-ov4 differed significantly in their ability to inhibit colony growth

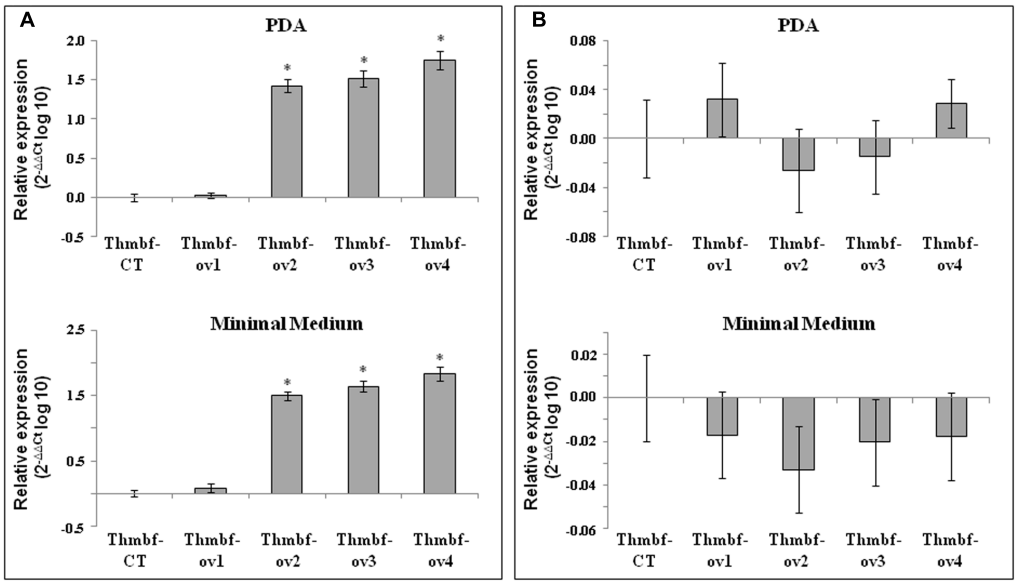


FIGURE 1 | Transcript levels of *Thmbf1* **(A)** and *cpc1* **(B)** in four putative *Thmbf1* overexpressed transformants -*T. harzianum* Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4- by qPCR. Values correspond to relative measurements against the *Thmbf1* or *cpc1* transcripts in the control transformant *T. harzianum* Thmbf-CT ($2^{-\Delta\Delta Ct} = 1$), and are expressed as $\log_{10}$. The experiment was carried out with mycelia grown at 28°C for 3 days on PDA and minimal media. *T. harzianum actin* was used as an internal reference gene. Bars represent the standard deviations of the mean of three replicates. Asterisk (*) represents statistically significant differences ($P < 0.05$).

TABLE 1 | Growth of *F. oxysporum* f. sp. *lycopersici* (FO) and *B. cinerea* (BC) confronted with *T. harzianum* strains on discontinuous medium.

	Colony diameter (cm)	
Thesis	**FO**	**BC**
Control	4.15 ± 0.15b	3.52 ± 0.03a
T34	3.15 ± 0.05c	1.10 ± 0.13c
Thmbf-CT	2.90 ± 0.10c	1.25 ± 0.05c
Thmbf-ov3	3.95 ± 0.25b	1.45 ± 0.05b
Thmbf-ov4	4.60 ± 0.20a	1.62 ± 0.03b

Trichoderma harzianum strains correspond to the wild-type (T34), the transformation control (Thmbf-CT) and the Thmbf1 overexpressing transformants (Thmbf-ov3 and Thmbf-ov4). Colony diameters (cm) were measured after 5 days growing on discontinuous PDA medium at 28°C. Values are means of three replicates with the corresponding standard deviations. For each column, values followed by different letter are significantly different ($P < 0.05$).

of FO and BC (**Table 1**), showing less antifungal activity than T34 or Thmbf-CT (**Figure 2**). Particularly, *Thmbf1* overexpressing transformants did not reduce the colony size of FO when they were tested in discontinuous medium.

To examine the role of the *Thmbf1* gene in the secretion of different molecular weight (MW) compounds with antifungal activity in *T. harzianum*, assays were performed with cellophane (allowing small and large compounds to pass through) and dialysis membranes with a MW cut-off of 14 kDa (allowing only metabolites < 14 kDa to pass through). **Table 2** summarizes the colony diameters of FO and BC after growing on PDA medium containing *Trichoderma* extracellular compounds. All *T. harzianum* strains assayed were able to inhibit the growth of both pathogens. No differences were detected between both types of membranes, indicating that small compounds secreted by *T. harzianum* are major contributors to the inhibitory activity observed against FO and BC. Moreover, significantly lower FO growth inhibition was recorded for strains Thmbf-ov3 and Thmbf-ov4 compared to that of T34 and Thmbf-CT on both cellophane and dialysis membranes. However, no significant BC growth inhibition differences were observed among the four *T. harzianum* strains on both types of membranes.

Effect of Overexpressing *Thmbf1* Gene on *T. harzianum* Biocontrol Capability Against *Botrytis* Leaf Lesions in Tomato Plants

Four-week-old 'Marmande' tomato plants previously seed-coated with an aqueous solution (control) or treated with conidia of T34, Thmbf-CT, Thmbf-ov3 or Thmbf-ov4 were leaf inoculated with BC. Necrotic spots were observed 3 days after inoculation of BC whereas no lesions were detected in BC-uninoculated plants, results are shown in **Figure 3**. The lowest necrotic leaf area was observed in plants of the T34 and Thmbf-CT treatments, and no significant statistically differences were detected between them. However, plants treated with *Thmbf1*-overexpressing transformants showed the highest lesion sizes, being similar to those observed in the control plants. These results indicate that T34 is able to control BC in 'Marmande' plants and the overexpression of *Thmbf1* gene in this strain reduces its biocontrol ability against the pathogen BC.

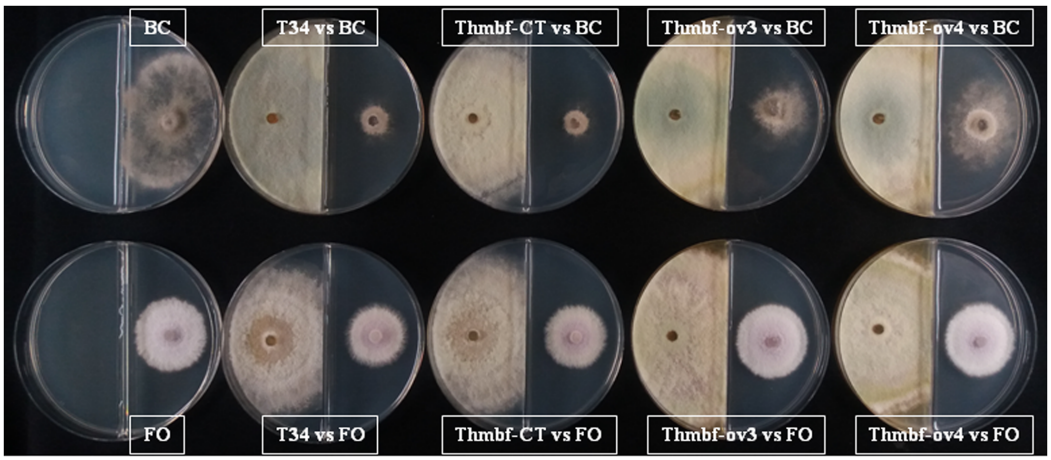


FIGURE 2 | Dual cultures of strains T34, Thmbf-CT, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 of *T. harzianum* and the pathogens *B. cinerea* (BC) and *F. oxyxporum* (FO) on discontinuous PDA medium. Plates only with the pathogen were used as controls. All plates were incubated at 28°C for 5 days.

TABLE 2 | Growth of *F. oxysporum* f. sp. *lycopersici* (FO) and *B. cinerea* (BC) on PDA medium, where *T. harzianum* wild-type (T34), transformation control (Thmbf-CT) or *Thmbf1* overexpressing transformants (Thmbf-ov3 and Thmbf-ov4) strains were previously grown on cellulose (cut-off 14 kDa) or cellophane membranes for 2 days at 28°C.

Thesis	Colony diameter (cm)			
	FO		BC	
	Cellophane	Cellulose	Cellophane	Cellulose
Control	2.33 ± 0.05a	2.33 ± 0.05a	2.75 ± 0.18a	2.75 ± 0.18a
T34	0.50 ± 0.00c	0.50 ± 0.00d	0.57 ± 0.10b	0.63 ± 0.10b
Thmbf-CT	0.50 ± 0.00c	0.50 ± 0.00d	0.58 ± 0.13b	0.65 ± 0.23b
Thmbf-ov3	0.67 ± 0.05b	0.63 ± 0.10c	0.50 ± 0.00b	0.50 ± 0.00b
Thmbf-ov4	0.70 ± 0.06b	0.78 ± 0.04b	0.50 ± 0.00b	0.55 ± 0.08b

Measurements were taken after 2 days on PDA medium. Controls are cultures without a previous T. harzianum growth. Values are means of three replicates with the corresponding standard deviations. For each column, values followed by different letter are significantly different ($P < 0.05$).

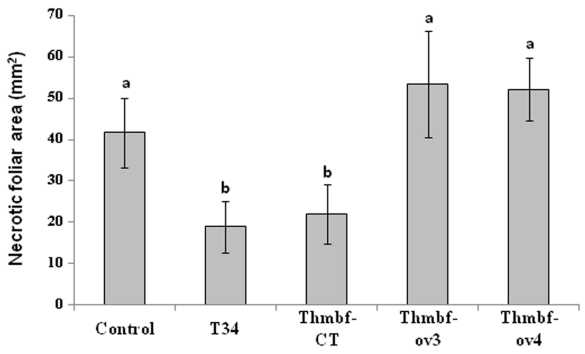


FIGURE 3 | Necrotic leaf area (mm^2)* caused by *B. cinerea* in 4-week-old 'Marmande' tomato plants from seeds treated with water (control) or *T. harzianum* T34, Thmbf-CT, Thmbf-ov3 or Thmbf-ov4 strains. Five plants were considered for each condition and foliar area and foliar necrotic area were evaluated using ImageJ software. *Two leaves from each plant were inoculated on one point using 10 μl containing 2500 *B. cinerea* conidia/point and the necrotic leaf area was evaluated after 3 days. In each bar, means with different letters are significantly different ($P < 0.05$).

Against Fusarium Wilt in Tomato Plants

To evaluate the effects of pretreatment with *T. harzianum* T34 and the *Thmbf1* overexpression on the development of Fusarium wilt disease caused by FO in 'Moneymaker' tomato plants, *in vivo* assays were performed using two *T. harzianum* application methods (**Table 3**). **Supplementary Figure S3** shows the phenotype of tomato plants derived from *T. harzianum*-treated seeds and inoculated with FO. Typical symptoms of wilt disease were first observed 10 days after inoculation of FO with both *T. harzianum* application methods; the uninoculated tomato seedlings showed no symptoms. DI recorded at 21 days in FO-inoculated plants ranged from 50 to 56.2% and 48.5 to 53.7% for *T. harzianum*-treated seeds and *T. harzianum*-inoculated substrate, respectively. The lowest DI values were observed in T34 + FO and Thmbf-CT + FO treatments in the substrate inoculation assay. No differences were detected between FO and Thmbf-CT + FO treatments. However, higher DI values were recorded for plants coming from *T. harzianum*-treated seeds, and later infected with FO, compared to those directly infected with FO. The highest DI values were observed in plants from Thmbf-ov3 + FO and Thmbf-ov4 + FO treatments, those with the *Thmbf1*-overexpressed transformants, in both assays. In addition, the lowest dry weight values were also observed in tomato plants previously seed-coated with a *Thmbf1*-overexpressing transformant and no significant differences were detected among plant dry weights from the treatments FO, T34 + FO and Thmbf-CT + FO. Although the disease of tomato plants appears to be influenced by the method of inoculation of *T. harzianum*, taken all together, these results indicate that strain T34 did not show a biocontrol activity against FO in 'Moneymaker' plants, and that *Thmbf1* overexpression in T34 reduced its antifungal activity against FO, leading to increased Fusarium wild disease.

TABLE 3 | Effect of *T. harzianum* wild-type (T34), transformation control (Thmbf-CT) or *Thmbf1* overexpressing transformants (Thmbf-ov3 and Thmbf-ov4) treatments on the development of disease caused by *F. oxysporum* f. sp. *lycopersici* (FO) in 'Moneymaker' tomato plants. *Trichoderma* strains were applied by inoculation of the substrate or by treatment of seeds.

	Inoculated substrate with *T. harzianum*	*T. harzianum*-treated seeds	
	DI (%)	DI (%)	Dry weight (g)*
Control	0.0	0.0	0.44 ± 0.07a
FO	52.5	50.0	0.19 ± 0.03b
T34 + FO	50.0	52.7	0.18 ± 0.06b
Thmbf-CT + FO	48.5	50.0	0.14 ± 0.06b
Thmbf-ov3 + FO	53.7	54.2	0.07 ± 0.04c
Thmbf-ov4+FO	53.7	56.2	0.10 ± 0.05c

*Disease incidence (DI), expressed in percentage, was determined after both application methods. Aboveground dry weights were also recorded for the assay T. harzianum treated-seeds. Controls are plants without a previous T. harzianum or FO treatment. *Values are means of ten replicates with the corresponding standard deviations, and those followed by different letter are significantly different ($P < 0.05$).*

DISCUSSION

MBFs are highly conserved transcriptional coactivators present in Archaea and Eukarya (Takemaru et al., 1997; Tsuda et al., 2004; Suzuki et al., 2005), although not found in bacteria. This fact evidences the emergence of MBFs mediator proteins after the separation of the last archaeal common ancestor from the bacterial lineage (Forterre, 2013). MBF1 proteins control different physiological and/or developmental processes and, although few studies have been reported in fungi, they have also been related to virulence in *B. bassiana* and *M. oryzae* (Ying et al., 2014; Fan et al., 2017). In the present work, we explored the role of *Thmbf1* gene in the antifungal activity of *T. harzianum*, since this species is one of the most cited as active ingredient in commercial biocontrol products (Lorito et al., 2010).

We identified the *Thmbf1* gene in a subtractive library prepared with cDNAs from *T. harzianum* T34 wild type and *Thctf1* null mutant affected in the production of 6-PP derivatives (Rubio et al., 2009). These VOC are released by *Trichoderma* spp. as a component of their antifungal machinery (Mukherjee et al., 2013), and it has been described that a decreased production of 6-PP is correlated with loss of antifungal activity against pathogens such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum* or *F. oxysporum* (Reithner et al., 2005; Rubio et al., 2009). The 6-PP is a major VOC biosynthesized by *T. harzianum* or *T. atroviride* species (Reino et al., 2008; Daoubi et al., 2009; Garnica-Vergara et al., 2016). It is able to induce growth promotion and reduce disease symptoms when applied at low concentrations to plant growth media or directly onto the leaves (Vinale et al., 2008). It has been demonstrated that Arabidopsis root responses to 6-PP involves components of auxin transport as well as a master regulator of the ethylene-depending response pathway (Garnica-Vergara et al., 2016).

We have isolated the *Thmbf1* gene using a T34 genomic library (Lora et al., 1995). Since the frequency of homologous recombination in *Trichoderma* is very low and therefore null mutants are not easy to obtain in *T. harzianum* (Rosado et al., 2007; Rubio et al., 2009), the function of *Thmbf1* was studied following an overexpression strategy. This approach limits comparison of the results with those from the three studies performed in filamentous fungi, in which a disruption strategy was followed (Schönig et al., 2009; Ying et al., 2014; Fan et al., 2017).

Southern blot data, showing one hybridization signal in both T34 and Thmbf-CT strains, as well as a single *Thmbf1* homolog identified in the publicly available *Trichoderma* genomes, indicate the existence of a single copy of this gene in the genus. These results are in agreement with those observed in other filamentous fungi, where a single *MBF* ortholog has been described (Ying et al., 2014), whereas other organisms such as plants contain several *MBF* genes (Tsuda et al., 2004). Multiple additional copies of the gene were observed in three out of the four putative *Thmbf1*-transformants analyzed by Southern blotting (**Supplementary Figure S1**). However, there was no correlation between the expression levels and the additional gene copies. This lack of correlation has been also reported in *Trichoderma* transformant strains for genes such as *chit33*, *hsp23* or *hsp70* (Limón et al., 1999; Montero-Barrientos et al., 2007, 2008). The fact that Thmbf-ov1 did not show higher *Thmbf1* transcript levels than the transformation control Thmbf-CT, after growing on rich or minimal medium, together with the absence of additional copies of the gene in its genome demonstrate that this is not an overexpressing transformant. When Thmbf-ov1 was further checked by PCR and grew in the presence of antibiotic, we could assess that this strain had not maintained the transformation cassette in its genome and the transforming DNA had been lost.

Since the expression of *cpc* gene was not significantly modified in none of the five strains tested after growing in two different media, ThMBF1 does not appear to be linked to the master regulator CPC1 in *T. harzianum*. Our results are in contrast with the findings reported in yeast (Takemaru et al., 1998), but they are in agreement with those obtained by yeast two-hybrid assays carried out in *F. fujikuroi* (Schönig et al., 2009) and *M. oryzae* (Fan et al., 2017), where no interaction between MBF1 and CPC1 proteins was observed.

We used different *in vitro* assays to study the involvement of *Thmbf1* in the antifungal activity of *T. harzianum* using two target phytopathogenic fungi. Thmbf-ov2 was the only transformant that showed a reduced antagonistic activity against FO and BC on dual culture assays (**Supplementary Figure S2**). However, it could be due to the low growth rate observed in this transformant. For this reason, we selected the strains Thmbf-ov3 and Thmbf-ov4 for further analysis. The fact that these *Thmbf1* overexpressing transformants showed lower antifungal activity against both pathogens when using a discontinuous medium, is indicative that VOC production is affected by *Thmbf1*. It is clear that a *Thmbf1* overexpression in *T. harzianum* T34 modifies the communication mediated by VOC between the two physically separated fungi; it reduces the antifungal activity of the T34 strain. However, our results are not enough to conclude whether the observed differences are due to VOC produced by *T. harzianum* or there are also involved other compounds from the pathogen in that scenario. VOC are

considered ideal info-chemicals that play important roles in the short- and long-distance interactions between physically separated microorganisms (Effmert et al., 2012; Schmidt et al., 2017). There is evidence that VOC play a role in *T. harzianum*-*F. oxyxporum* confrontations, and that this pathogen induces the production of these type of compounds in the antagonist fungus (Zhang et al., 2014). The relationship between *Thmbf1* and VOC in *T. harzianum,* deduced from assays performed in a discontinuous medium, should not be surprising since this gene has been identified in a SSH approach performed with a null mutant unable to produce several 6-PP derivatives (Rubio et al., 2009).

In addition to VOC, *Thmbf1* could be involved in the production of low MW metabolites and/or enzymes with antifungal activity in *T. harzianum* since a significantly lower FO inhibition was detected for the two *Thmbf1* overexpressing transformants in cellophane and dialysis membrane assays compared to those of wild type and the transformation control strains.

Trichoderma harzianum has demonstrated biocontrol potential in a wide range of crop plants against different pathogens (Monte, 2001; Harman et al., 2004; Lorito et al., 2010). In our study, according to the results obtained in the *in vivo* assays, T34 was able to reduce the lesions produced by BC in tomato plants but this ability was not observed in *Thmbf1*-overexpressing transformants. Considering that in this assay *T. harzianum* and BC were not in physical contact, the systemic defense mechanisms activated by strain T34 in the plant were not elicited enough when the *Thmbf1* gene was overexpressed in the fungus. The beneficial effects of *Trichoderma* spp. regarding not only a direct antagonistic activity, but also the activation of systemic defense responses in the plant, depend on the strain, the genotype and age of the plant, the type of pathogen and the interaction conditions (Hermosa et al., 2012; Martínez-Medina et al., 2014). It is recognized that *Trichoderma* spp. are able to induce systemic resistance against necrotrophs like BC by signaling jasmonic acid (JA) and ethylene (ET)-dependent defense pathways (Shoresh et al., 2010). Moreover, they can activate the salicylic acid (SA)-dependent defense responses (Rubio et al., 2014, 2017), which are crucial against biotrophic pathogens like FO (Glazebrook, 2005).

Despite of the wide host range shown by *F. oxysporum*, individual isolates are able to infect only one or a few plant species (Michielse and Rep, 2009). For *in vivo* assays we selected 'Moneymaker' tomato plants and used FO strain 4287 as the target pathogen because of its known virulence for this variety (Di Pietro and Roncero, 1998; Niño-Sánchez et al., 2016). Although T34 showed antifungal activity against FO in *in vitro* assays, no biocontrol efficacy was observed against this pathogen in two *in vivo* assays using different *T. harzianum* inoculation methods. Furthermore, tomato plants from the treatments with the *Thmbf1*-overexpressing transformants displayed the highest levels of Fusarium wilt disease. It is well known that the antifungal activity observed in *in vitro* assays for *Trichoderma* strains against different pathogens should not be extrapolated to other situations such as field or greenhouse conditions (Hermosa et al., 2000). However, it has also been reported that *Trichoderma* spp. induce the above indicated JA/ET- and SA-dependent defense responses in tomato plants (Salas-Marina et al., 2011; Rubio et al., 2014), and that these fungi have demonstrated potential for suppressing Fusarium wilt development in tomato plants (Cotxarrera et al., 2002; Taghdi et al., 2015). These last works also demonstrated that the potential to suppress Fusarium wilt depends on the *Trichoderma* strain. Previous studies have shown that T34 is able to colonize the tomato rhizosphere (Morán-Diez et al., 2009; Samolski et al., 2012), and successful root colonization is considered a major prerequisite for the beneficial effects exerted by *Trichoderma* on plants (Lorito et al., 2010; Shoresh et al., 2010; Hermosa et al., 2012). Our greenhouse results indicate that *T. harzianum* T34 has the ability to reduce the lesion caused by BC in 'Marmande' tomato plants but it is not able to suppress the Fusarium wilt caused by FO in 'Moneymaker', at least under the assayed conditions. We have also found that the *Thmbf1* overexpression in *T. harzianum* T34 negatively affected the biocontrol activity of this strain. Considering that reduced *B. bassiana* and *M. oryzae* virulence was observed in absence of MBF1 (Ying et al., 2014; Fan et al., 2017), and that *Thmbf1* overexpressing transformants showed lower biocontrol potential than the wild type, it could be thought that adequate levels of MBF1 are needed to mediate the transcriptional pathways involved in the interactions of filamentous fungi with pathogens and plants.

In summary, we can conclude that the transcription coactivator MBF1 plays an important role in the biocontrol ability of *T. harzianum*, affecting the production and secretion of different antifungal compounds, and that the success of this fungus as a biocontrol agent depends on a suitable expression level of this fine adjustment regulator.

AUTHOR CONTRIBUTIONS

MBR and AP carried out *in vitro* assays. RC constructed the overexpressing plasmid and obtained transformants. SG made the subtractive cDNA library and the Southern blot. MBR and RH carried out greenhouse assays, prepared tables, figures and additional material. EM, RH, and MBR wrote the manuscript. RH designed and led the study. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02273/full#supplementary-material

FIGURE S1 | Southern blot analysis of wild-type (T34) and transformant strains. Genomic DNAs were *Xho*I- and *BamH*I-digested and the *Thmbf1* cDNA was used as a probe. Lanes correspond to *T. harzianum* T34 (lane 1), Thmbf-CT (control transformant, lane 2), Thmbf-ov1 (lane 3), Thmbf-ov2 (lane 4), Thmbf-ov3 (lane 5) and Thmbf-ov4 (lane 6). *EcoR*I-*Hind*III-digested λ DNA was used as a marker and molecular sizes are indicated in kbp (lane 7).

FIGURE S2 | Dual cultures of strains T34, Thmbf-CT, Thmbf-ov1, Thmbf-ov2, Thmbf-ov3 and Thmbf-ov4 of *T. harzianum* and the pathogens *F. oxyxporum* (FO) **(A)** and *B. cinerea* (BC) **(B)** on continuous PDA medium. Plates only with the pathogen were used as controls. All plates were incubated at 28°C for 10 days.

FIGURE S3 | Phenotype of 'Moneymaker' tomato plants derived from *T. harzianum*-treated seeds and inoculated with FO. The wild-type T34, the transformation control (Thmbf-CT), and the *Thmbf1* overexpressing transformants (Thmbf-ov3 and Thmbf-ov4) were applied as *T. harzianum* strains. Plants without *T. harzianum* or FO treatment were used as controls. Photographs were taken 3 weeks after FO inoculations.

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Modulation of Tomato Response to *Rhizoctonia solani* by *Trichoderma harzianum* and Its Secondary Metabolite Harzianic Acid

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The present study investigated the transcriptomic and metabolomic changes elicited in tomato plants (*Solanum lycopersicum* cv. Micro-Tom) following treatments with the biocontrol agent *Trichoderma harzianum* strain M10 or its purified secondary metabolite harzianic acid (HA), in the presence or the absence of the soil-borne pathogen *Rhizoctonia solani*. Transcriptomic analysis allowed the identification of differentially expressed genes (DEGs) that play a pivotal role in resistance to biotic stress. Overall, the results support the ability of *T. harzianum* M10 to activate defense responses in infected tomato plants. An induction of hormone-mediated signaling was observed, as shown by the up-regulation of genes involved in the ethylene and jasmonate (ET/JA) and salicylic acid (SA)-mediated signaling pathways. Further, the protective action of *T. harzianum* on the host was revealed by the over-expression of genes able to detoxify cells from reactive oxygen species (ROS). On the other hand, HA treatment also stimulated tomato response to the pathogen by inducing the expression of several genes involved in defense response (including protease inhibitors, resistance proteins like CC-NBS-LRR) and hormone interplay. The accumulation of steroidal glycoalkaloids in the plant after treatments with either *T. harzianum* or HA, as determined by metabolomic analysis, confirmed the complexity of the plant response to beneficial microbes, demonstrating that these microorganisms are also capable of activating the chemical defenses.

Keywords: *Trichoderma harzianum*, secondary metabolites, tomato, biological control, beneficial microbes, resistance response, DEGs, harzianic acid

INTRODUCTION

The complex network of interactions established by plants with the rhizosphere microbiota greatly affect crop health and fitness (Raaijmakers et al., 2009; Berendsen et al., 2012). The demonstrated benefits include stimulation of plant growth, enhanced pathogen control, improved abiotic stress resistance, increased nutrient availability and uptake, higher yield and better product quality

(Berg, 2009; Berg et al., 2014; Trivedi et al., 2016; Martínez-Medina et al., 2017; Pascale et al., 2017). Plant-microbe interactions also include cases that may be detrimental, compromising plant growth and health, although the establishment of a disease status is often counteracted by the presence of a beneficial soil microbial community that can hinder infection by disease pathogens (Matos et al., 2005; Walters et al., 2008). Mycoparasitic fungi such as *Trichoderma* and *Gliocladium* have antagonistic activity on many soilborne pathogens (including *Verticillium*, *Sclerotinia*, *Rhizoctonia, Fusarium, Pythium*) due to a variety of mechanisms, including: parasitism, competition for nutrients and space, antibiosis, and production of lytic enzymes, as well as the ability to trigger the induction of systemic resistance (ISR) (Harman et al., 2004b; Lorito et al., 2010; Doornbos et al., 2012; Hermosa et al., 2014; Amira et al., 2017; Nawrocka et al., 2018).

To date, disease management in agriculture has relied mainly on the application of chemical pesticides. Even though this protection strategy may be efficient and in many cases cost-effective, it poses serious risks to human health and the environment (Leach and Mumford, 2008). With the implementation of Directive 2009/128/EC, the European Community promotes specific actions to support the establishment of sustainable agriculture through the reduced use of chemical pesticides in favor of alternative non-chemical products, and the promotion of integrated pest management (IPM).

Therefore, the development of bio-based strategies to enhance crop production and food safety has become a cutting-edge research topic in biological and agricultural sciences. Numerous beneficial microorganisms are already in use as active ingredients in bio-pesticides, bio-fertilizers, bio-stimulants, plant growth enhancers and soil integrator formulations (Verma et al., 2007; Harman et al., 2010; Lorito et al., 2010; Woo et al., 2014). Many of them are based on endophytic or plant-root associated fungi belonging to the genus *Trichoderma* due to the ability of these microbes to control different phytopathogens and exert a number of positive effects on crops. Some *Trichoderma* spp. are characterized by competency for the rhizosphere environment, being able to extensively colonize the root system starting from the germinating seed (Yedidia et al., 1999; Harman, 2000; Harman et al., 2004a). Production of auxins or auxin–like compounds by the fungus stimulates root formation and development, thus expanding the area available for colonization (Vinale et al., 2008, 2012; Contreras-Cornejo et al., 2009; Hermosa et al., 2012).

The plant-*Trichoderma* interaction may be classified as a facultative symbiosis characterized by the establishment of reciprocal advantages. The fungus occupies a strategic ecological niche, obtains nutrients from the host, and in turn provides beneficial effects to the plant that include direct protection to pathogen attack, solubilization of nutrients, improvement of growth and vigor, plus the "alerting" (priming) of defense response (Harman et al., 2004b; Vargas et al., 2009, 2011; Shoresh et al., 2010). Furthermore, tomato plants subjected to biotic and/or abiotic stresses release specific root exudates that act as chemo-attractants for *Trichoderma*, which are able to recognize "help" signals and grow toward the stressed plants (Lombardi et al., 2018). Root colonization by *Trichoderma* induces root-hair development and defense responses, generates substantial changes in diverse metabolic pathways and triggers the expression of genes involved in plant defense mechanisms mainly associated to jasmonic acid- (JA) and ethylene- (ET) dependent signaling pathways (Vinale et al., 2008; Contreras-Cornejo et al., 2015; Martínez-Medina et al., 2017). In the case of *Arabidopsis,* colonization by *Trichoderma,* prior to infection by biotrophic or necrotrophic phytopathogens, activated a priming status that was able to systemically enhance resistance (Salas-Marina et al., 2011; Hermosa et al., 2013). Moreover, some *Trichoderma* strains can produce microbe-associated molecular patterns (MAMPs) that induce plant defense responses including the production of elicitors such as a xylanase (Xyn2/Eix) in tomato and tobacco, an endopolygalacturonase in *Arabidopsis thaliana*, and a swollenin (TasSwo) in cucumber (Rotblat et al., 2002; Brotman et al., 2008; Morán-Diez et al., 2009).

Trichoderma spp. are important producers of secondary metabolites (SM) that provide selective advantages in processes like competition, symbiosis, metal transport, growth differentiation, signaling, mycoparasitic activity etc. (Sivasithamparam and Ghisalberti, 1998; Woo et al., 1999; Harman et al., 2004b; Reino et al., 2008; Lorito et al., 2010). Harzianic acid (HA), a tetramic acid produced by *Trichoderma harzianum* M10 strain, demonstrated remarkable biological properties, including plant growth promotion, antimicrobial activity against different pathogens such as *Pythium irregulare, Sclerotinia sclerotiorum,* and *Rhizoctonia solani*, plus an ability to chelate soil iron ($Fe3^{+}$) thus facilitating its uptake by the plant (Vinale et al., 2013).

In many instances, the application of a purified compound, such as a SM, was found to produce effects comparable to those obtained by treating the crop with the living microbe (Vinale et al., 2008; Pascale et al., 2017). The use of these naturally-derived compounds in alternative to, or in combination with the living microbe may contribute to developing novel plant protection products and biofertilizers that may be more effective and reliable when applied to a variety of crops and environment conditions.

The effects of living *Trichoderma* on the plant transcriptome, metabolome, and proteome have been extensively studied (Marra et al., 2006; Chacón et al., 2007; Lorito et al., 2010; Morán-Diez et al., 2012, 2015; Mendoza-Mendoza et al., 2018). However, the role of purified secondary metabolites produced by the same fungus in the interaction with the plant has not yet been fully clarified.

In order to dissect the molecular basis of defense responses and induction of resistance mechanisms activated during *Trichoderma*-plant interactions, we tested and compared the effect of *T. harzianum* strain M10 and its secondary metabolite HA. The patterns of differentially expressed genes (DEGs) and differentially abundant plant secondary metabolites were determined by analyzing transcriptomic and metabolomic changes occurring in tomato plants colonized by *T. harzianum* M10 or treated with HA, in the presence or the absence of the soilborne pathogen *R. solani.*

MATERIALS AND METHODS

Fungal Strains and Plant Material

Trichoderma harzianum strain M10, isolated from composted hardwood bark in Western Australia, was maintained and routinely propagated to complete sporulation on Potato Dextrose Agar (PDA; SIGMA, St Louis, MO) slants at room temperature and sub-cultured monthly. Ten 7-mm diameter plugs of actively growing M10 were obtained from the margins of PDA cultures and inoculated to 5-L conical flasks containing 2 L of sterile potato dextrose broth (PDB; SIGMA). Cultures were incubated for 30 days at 25°C without agitation, then the fungal biomass was removed and the liquid culture was filtered through Whatman paper (No. 4, Brentford, UK) under vacuum. For the production of HA, the fungal culture filtrate was extracted exhaustively with ethyl acetate (EtOAc). The purification and biochemical characterization of HA was carried out as previously reported (Vinale et al., 2009, 2013, 2014).

Fresh conidia were collected in sterile water from sporulating fungal cultures of M10 grown for 7 days on PDA at 25°C. Tomato (*Solanum lycopersicum* cv. Micro-Tom) seeds were surface sterilized using 70% (v/v) ethanol for 2 min, followed by 2% (v/v) sodium hypochlorite solution for 2 min, rinsed well with sterile distilled water, and left to air-dry.

The soilborne fungal pathogen *R. solani* was isolated from naturally infected tomato seedlings. Cultures were maintained on PDA for 1 week at 25°C, then ten 7-mm diameter mycelia plugs were inoculated to 5-L conical flasks containing 2 L of sterile PBD. The culture was incubated for 15 days at 25°C, with orbital agitation at 150 rpm, then paper filtered under vacuum to harvest the biomass.

Plant Treatments

The experiment was divided into two blocks based on the absence (Block 1) or the presence (Block 2) of the pathogen. Thirty tomato seeds were employed for each treatment and sown in 500-mL pots containing sterile soil, with three independent biological replicates used for each treatment.

Treatments without the *Rhizoctonia* pathogen were comprised of:

- Plant-H_2O [P]. Seeds were treated with water and plants only received water for the duration of the experiment.
- Plant-M10 [P+T]. Seeds were coated with *T. harzianum* M10 spores (1×10^7 spores/mL) in water, stirring to uniformly coat the entire surface, left to air-dry, and stored at 4°C until planting in sterile soil.
- Plant-HA [P+HA]. Seeds were treated with water, and sown in sterile soil, then 30 days after emergence, all plants were treated with a 50 mL volume of the HA solution [10^{-6} M] by foliar spray.

Treatments with the *Rhizoctonia* pathogen were comprised of:

- Plant-*R. solani* [P+R]. Seeds were treated with water and sown in sterile soil. One month after the emergence the soil was inoculated with 50 mL of *R. solani* biomass homogenized in water (2% w/v).
- Plant-M10-*R. solani* [P+T+R]. Seeds were coated with *T. harzianum* M10 spores (1×10^7 sp/mL) and sown in sterile soil; then, 1 month after emergence, the soil was infected with *R. solani* biomass (2% w/v).
- Plant-HA-*R. solani* [P+HA+R]. Seeds were treated with water, and sown in sterile soil, then 30 days after emergence, were treated with a 50 mL volume of the HA solution [10^{-6} M] by foliar spray. Then 24 h following the treatment, the soil was infected with the *R. solani* biomass (2% w/v).

All plants were placed in a growth chamber under controlled conditions, temperature (25°C), RH 70% and photoperiod (16 h of light/8 dark). Whole plants were harvested at the same time, equivalent to 48 h after pathogen inoculum (if present), frozen in liquid nitrogen, ground to a fine powder and stored at −80°C until further processing.

Biocontrol Assay of *Rhizoctonia solani*

Biocontrol assays were conducted as described in the above section for Block 2, with 1-month old plants infected with *R. solani*, to test the effect of the *Trichoderma* M10 (seed coating) and the HA (foliar spray) on disease development. Each treatment consisted of 15 plants, in 3 replicates (total of 45 plants,) with the experiment arranged in random block design. The disease development was evaluated every 24 h for a 6 day period after pathogen inoculation, and the disease incidence was determined as the percentage of plants demonstrating symptoms of *R. solani* crown rot infection.

RNA Extraction and Microarray Analysis

Each treatment consisted of 20 plants. Total RNA was extracted and purified using PureLink® RNA Mini Kit (Ambion Inc., Austin, TX) from a pool of equal amounts of the powdered vegetative material obtained from the 20 tomato plants of each treatment. Removal of genomic DNA was performed by digestion with DNase I, Amplification Grade (Invitrogen, USA). Total RNA quantity and quality was assessed using NanoDrop 1000 (Thermo Scientific, Waltham, MA). The quality of RNA is a critical factor in hybridization performance, therefore only RNA samples with 230/260 and 260/280 ratios >2 were used.

Total RNA (2 μg) was amplified and labeled using the RNA ampULSe kit (Kreatech Biotechnology, Amsterdam, Netherlands). Microarray hybridization was performed with 4 μg of Cy5 labeled aRNA inoculated at 45°C for 16 h in the Pre-hybridization Solution. After incubation, arrays were washed with different Washing Solutions at 45°C (6X SSPET Wash, 5 min with gentle rotation; 3X SSPET Wash, 1 min; 0.5X SSPET Wash, 1 min; PBST Wash, 1 min) and two times with PBS Wash at room temperature for 1 min. Labeled aRNA was hybridized to the TomatoArray 2.0 (CombiMatrix microarray platform, Italy), then analyzed using a Perkin Elmer ScanArray 4000 XL scanner. Images were acquired with ScanArray Express Microarray Analysis System (Version 4.0) software. The samples analyzed are reported in **Table 1A**. Microarrays were stripped for reuse

TABLE 1 | Plant material used in the transcriptomic and comparative analysis obtained by treating tomato (P) with Trichoderma M10 (T) or harzianic acid (HA), and the pathogen Rhizoctonia (R) pathogen.

No Pathogen	With Pathogen
(A) HYBRIDIZATION	
Plant-H_2O [P]	Plant + *R. solani* [P+R]
Plant + *T. harzianum* M10 [P+T]	Plant + *T. harzianum* M10 + *R. solani* [P+T+R]
Plant + Harzianic Acid [P+HA]	Plant + HA + *R. solani* [P+HA+R]
(B) COMPARISONS	
	Plant + M10 + *R. solani* [P+T+R] vs. Pathogen [P+R]
Plant-M10 [P+T] vs. Plant-H_2O [P]	Plant + HA + *R. solani* [P+HA+R] vs. Pathogen [P+R]
	Plant + HA + *R. solani* [P+HA+R] vs. Plant + M10 + *R. solani* [P+T+R]

A, Sample conditions used in tomato CombiMatrix microarray hybridizations; B, Combinations of treatments used in the comparison analyses.

(each chip was used up to three times) using the CombiMatrix CustomArray Stripping Kit according to the manufacturer's instructions. Two biological replicates were employed to evaluate the differential gene expression.

Chip Annotation and Bioinformatic Analysis

Transcriptome analysis was performed using the 90 K TomatoArray 2.0 chip with probes synthesized at the Functional Genomics Centre of the University of Verona using the CombiMatrix platform. The TomatoArray 2.0 chip has 25,789 oligonucleotide probes of 35-40-mer, randomly distributed in triplicate across the array. Each probe was constructed to be complementary to a Tentative Consensus sequence (TC), resulting from DFCI Tomato Gene Index Release 12.0. Eight bacterial oligonucleotides provided by CombiMatrix, as well as 40 probes of *Bacillus anthracis, Haemophilus ducreyi,* and *Alteromonas,* were included in the chip design as negative controls.

The TCs reported in Tomato Gene Index Release 12.0 (ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Solanum_lycopersicum/) were mapped to tomato chromosomes, blasting the probes used to design the chip against the Database WGS Chromosomes (SL 2:50) of Sol-Genomics Network (https://solgenomics.net/). Only the data with an e-value between 0 and 1.0^{-6} were considered for further analysis. Each Solyc ID obtained from TCs was removed from the tomato annotation files ITAG 2.40, thus allowing the annotation of the complete chip. The DEGs were identified using the software R (R Core Team, 2013 http://www.R-project.org/). In particular, the acquired CombiMatrix arrays were analyzed through Bioconductor package (Gentleman et al., 2004). The software MapMan ORC 3.1 (Thimm et al., 2004) was used to map the DEGs for all the experimental conditions, and KEGG database (http://www.genome.jp/kegg/) was used for further reconstruction of the biosynthetic pathways.

Expression Profiling by qPCR

One μg of purified total RNA was used as a template for first-strand cDNA synthesis using SuperScript® III Reverse Transcriptase (Invitrogen). Seven genes for each treatment were amplified to validate the microarray results. These genes were selected from DEGs lists obtained for each condition. *S. lycopersicum* primer sequences were designed using the Primer3 online tool (http://primer3.ut.ee/) and are listed in (**Table S1**). All samples were normalized to *apha-tubulin* as reference housekeeping gene. Gene transcript levels were measured using Power SYBR® Green PCR Master Mix (Applied Biosystems®) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems®). Data were analyzed with 7900 V 2.0.3 evaluation software (Applied Biosystems®). The relative quantitative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Plant Metabolome Analysis

Ten mg of ground tomato from the infected plants treated with *Trichoderma* and HA were used for metabolite extraction in 0.8 mL of 20% methanol in water. Samples were centrifuged (10 min at 16,100 g, 4°C), and the supernatant was filtered through a 0.2 μm polyvinylidine fluoride (PVDF) filter (Chromacol, Welwyn Garden City, UK). Sample extracts (7 μl) were loaded onto a Poroshell 120EC-C18 1.8 Pm, 2.1 × 5 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA) for metabolite profiling performed in a 6540 UHD Accurate Mass QTOF LC-MS/MS mass spectrometer (Agilent Technologies, Palo Alto, USA), in MS mode coupled to a 1200 series Rapid Resolution + HPLC. Mobile phases consisted in water (Cromasolv® Plus, LC-MS-Sigma) with 0.1% LC-MS grade formic acid (A) and acetonitrile (Cromasolv® Plus, LC-MS-Sigma) with 0.1% LC-MS grade formic acid (B). The separation was carried out by the following gradient: 0 min−5% B; 12 min−100% B; 15 min−100% B; 17 min−95% B; 20 min−95% B, 2 min post-time. The flow rate was 0.6 ml min^{-1} and the column temperature was held at 35°C. The source conditions for electrospray ionization were the following: nitrogen gas temperature was 350°C with a drying gas flow rate of 11 L min^{-1} and a nebulizer pressure of 45 psig. The fragmentor voltage was 180 V and skimmer voltage 45 V. The range acquisition of TOF spectra was from 50 to 1,600 *m/z* with an acquisition rate value of three spectra sec^{-1}. The data were collected in positive ion mode. The mass spectra were submitted to the Agilent MassHunter Profinder software and then to MassProfile Professional Software to compute the annotation and statistical analysis.

Statistical Analysis

Data from biocontrol experiments were subjected to statistical analysis using "Agricolae" package of R software, from the R project (www.r-project.org) of a repository CRAN, de Mendiburu and de Mendiburu (2017). Statistical analysis was performed by ANOVA followed by Least Significant Difference (LSD) *post-hoc* test using Bonferroni correction ($p < 0.05$).

The DEGs were identified using the software R (R Core Team, 2013 http://www.R-project.org/). In particular, the acquired CombiMatrix arrays were analyzed through Bioconductor package (Gentleman et al., 2004). The software MapMan ORC 3.1 (Thimm et al., 2004) was used to map the DEGs for all the experimental conditions. The fluorescence data were normalized applying the quantile normalization and the expression value was estimated using the empirical Bayesian approach (Wu et al., 2004). Subsequently, data were filtered for an adjusted $p \leq 0.05$ and a Fold Change (FC) $\geq \pm 1$, in order to obtain the complete list of DEGs. The comparison analysis used to identify DEGs in each type of interaction is reported in **Table 1B**. The software MapMan ORC 3.1 (Thimm et al., 2004) was used to map the DEGs for all the experimental conditions.

Enrichment analysis of the complete set of DEGs obtained was carried out using the binomial statistics tool to determine over- or under- representation of PANTHER or GO ontology classification categories (http://www.geneontology.org/). Each DEGs list was compared to a reference list using the binomial test (Cho and Campbell, TIGs 2000) for each Gene Ontology category. The settings for this analysis were as below:

- Annotation data set: PANTHER GO-slim.
- Reference gene list: All genes in PANTHER database for the selected organism (*S. lycopersicum*).
- Correction: Bonferroni.

The data from mass-spectrometry were analyzed considering a minimum absolute abundance of 5,000 counts, minimum number of ions 2. FC was calculated using the infected plant [P+R] as a control and only entities with $-2 < FC > 2$ were selected. Subsequently, the entities filtered were identified using ID browser identification associated to a Metlin Library (Agilent). A total of 25 compounds were found. The entities list was loaded in the clustering analysis tool using a hierarchical algorithm. The map was built on the three analyzed conditions [P+R, P+HA+R, P+T+R] using normalized log FC values, the Euclidean metric distance and the Ward's Linkage Rule.

RESULTS

Biocontrol Assay of *R. solani*

The biocontrol activity of *T. harzianum* M10 and HA against *R. solani* on tomato was evaluated as the percentage of infected plants observed at 7 different time points (**Figure 1**). The first *R. solani* disease symptoms, indicated with the appearance of brown to reddish lesions on the stem/crown zone, appeared 24 h post-inoculum (HPI) in more than 30% of the *R. solani* infected plants, and a significant increase (77%) was noted after 48 h. Plants treated with M10 (P+T+R) or HA (P+HA+R) showed a much slower progression of disease development, with a reduction in infection at all measured time points significantly different in comparison to control (P+R). At the end of the experiment, only 35% of M10 and 38% of HA-treated plants were infected, in comparison to 100% infection rate observed in the control group. Interestingly, M10 and HA treatments demonstrated comparable efficacy in controlling disease (**Figure 1**). This experiment was useful to determine the optimal time point for the collection of samples to use in the following transcriptomic analysis. The 48 HPI time point was selected because it allowed the collection of a sufficient number of infected but still healthy plants for each experimental group, which is critical for obtaining a good quality RNA suitable for transcriptomic analysis.

Plant-*Trichoderma harzianum* M10 Interaction

To monitor the global gene expression changes in tomato after colonization with *Trichoderma* M10 [P+T vs. P], a microarray analysis was performed 30 days post-inoculum (DPI). The complete DEG lists of each analyzed condition are reported in Supplementary Material **Data Sheets 1–3**. A

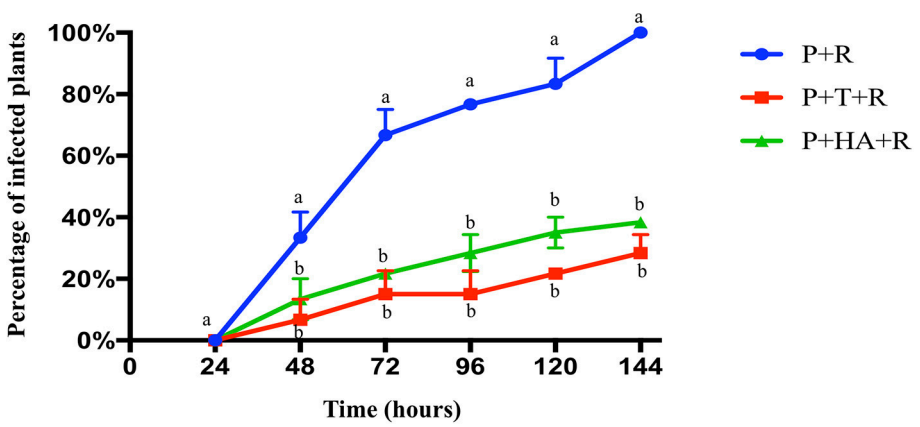


FIGURE 1 | Biocontrol activity of *T. harzianum* M10 and harzianic acid on the development of *R. solani* disease symptoms on tomato. Pathogen attack was noted by the appearance of lesions on plant stem. Plant treatments included: P+R = *R. solani* - infected plants (blue); P+T+R = *Trichoderma* + *R. solani* (red); and P+HA+R = harzianic acid + *R. solani* (green). Data are presented as the mean of three independent experiments ± SEM. Different letters indicate significantly different values after two-way ANOVA followed by Bonferroni correction ($p < 0.05$) for each time point measurement.

TABLE 2 | Number of differentially expressed genes (DEGs) up-regulated (Up) and down-regulated (Down) in tomato plants treated with *Trichoderma, HA* and/or *Rhizoctonia*.

Comparisons	DEGs	Up	Down
[P+T vs. P]	1,227	1,142	85
[P+HA vs. P]	–	–	–
[P+T+R vs. P+R]	1,142	630	512
[P+HA+R vs. P+R]	2,317	1,456	861

[T+P vs. P], plants treated with Trichoderma compared to control plants (water); [P+T+R vs. P+T], pathogen-infected plants treated with Trichoderma compared to untreated infected plants; [P+HA+R vs. P+R], infected plants treated with HA compared to untreated infected plants.

total of 1,227 DEGs were observed, of which 1,142 were up-regulated and 85 down-regulated (**Table 2**). To identify the processes in which these genes are involved, DEGs obtained from each comparison were mapped to the main metabolic pathways using the MapMan software. The DEGs involved in the "Cellular Function" category, which represents an overview of all the pathways identified, were obtained for each of the following interaction comparisons [P+T vs. P], [P+T+R vs. P+R], and [P+HA+R vs. P+R] (**Table 3**). Among the categories noted in the [P+T vs. P] comparison, the most prevalent genes were involved in "protein synthesis/degradation" (231 genes), "RNA metabolism" (129 genes), "redox reactions" (24 genes), "signaling" (79 genes), "photosynthesis" (32 genes), "cell wall synthesis/degradation" (17 genes), "secondary metabolism" (28 genes), and "stress (biotic/abiotic)" (50 genes) (**Table 3A**). Based on MapMan prediction, several genes mapped to these categories were found to be putatively involved in biotic stress-related processes (**Figure 2A**). Over-expression of genes associated to ethylene production was predominant among hormone signaling pathways, and a general over-expression of genes coding for transcription factors such as ERF, bZIP, WRKY, and MYB was also observed. Several genes directly involved in pathogen recognition and plant defense were up-regulated; among them 2 pathogenesis related (PR) proteins coding transcripts and 1 gene involved in signaling (Enhanced disease susceptibility 1-*EDS1*). Furthermore, numerous genes involved in isoprenoids, phenylpropanoids, flavonoids, alkaloids and aromatic amino acids biosynthesis were over-expressed. **Figure 3** presents enriched Gene Ontology (GO) terms obtained by Singular Enrichment Analysis (SEA) of the 1227 DEGs. The "Biological process" GO category contained 12 GO terms, while "Molecular function" and "Cellular component" categories contained 10 GO terms each. The majority of the tomato DEGs were associated to "cellular process," "metabolic process," "primary metabolic process," "binding," "catalytic activity"; these terms were found dominant (**Figure 3**). The transcriptomic analysis of HA-treated plants compared to untreated plants ([P+HA]) did not reveal any DEGs.

Plant–*Trichoderma harzianum* M10-*Rhizoctonia solani* Interaction

Results from expression analysis of tomato responses in the plant–*T. harzianum* M10–*R. solani* interaction were compared to those from the plant-pathogen interaction [P+T+R vs. P+R]. A total of 1,142 DEGs were noted, of which 630 genes were over-expressed and 512 were down-regulated and found distributed in the list of pathways identified by Mapman in the "Cellular Function" category (**Tables 2**, **3B**). Among these, the most represented DEGs were involved in "protein synthesis/degradation" (241 genes), "RNA metabolism" (142 genes) and "signaling" (49 genes). Concerning response to biotic stress, 298 genes belonging to several MapMan categories were found to have their expression level modified, with 32 of them also involved in responses to abiotic stress (**Figure 2B**). Additionally, the occurrence of DEGs associated to JA and SA biosynthesis, cell wall synthesis (27 genes), proteolysis (88 genes), signaling (49 genes), and ethylene (14 genes) was observed (**Figure 2B**). Remarkably, one of the cellular processes most affected by the tripartite interaction appeared to be the metabolism of oxygenic compounds with 23 genes up-regulated (**Figure 2B**, Redox state, Peroxidases, Gluthatione-S-transferase). Many genes related to different transcription factors (TFs) were found down-regulated (WRKY, MYB) while bZIP and *DOF* were up-regulated (**Figure 2B**). Infected plants treated with M10 showed the over-expression of nine genes involved in plant secondary metabolism. Specifically, four genes were associated to phenylpropanoid metabolism, three were involved in isoprenoid biosynthesis, while two genes were associated to flavonoid production (**Figure 2B**). The GO analysis revealed 37 enriched GO terms, 17 associated to Biological process, 12 to Molecular function and eight to Cellular component, respectively (**Figure 4**). Most of the enriched GO terms found in the bipartite interaction were in common with those found in the tripartite interaction GO enrichment. However, the number of DEGs identified in the latter analysis was much higher than that obtained for the bipartite interaction (**Table 2**).

Plant–HA-*Rhizoctonia solani* Interaction

The expression analysis of plant response in the Plant–HA–*R. solani* interaction [P+HA+R vs. P+R] revealed a total of 2,317 DEGs, of which the majority (1,456) were over-expressed (**Table 2**). In all cases, there were more DEGs activated by the treatment with HA and the pathogen than by the treatments of *Trichoderma* alone or by *Trichoderma* plus *Rhizoctonia*, suggesting a strong plant response to the fungal metabolite. As reported in **Table 3C**, the most represented MapMan categories were "protein synthesis/degradation" (505 genes), "RNA processing-regulation of transcription" (284 genes), "signaling" (122 genes) and "hormone metabolism" (63 genes).

The response to biotic stress affected the expression of 648 genes, six of which were directly involved in pathogen recognition and plant defense (**Figure 2C**). Up-regulation of genes involved in ethylene (29 genes), JA (7 genes), and oxygenic compounds (42 genes) pathways was also observed. All transcription factor families putatively involved in defense responses were also represented (ERF, bZIP, WRKY, MYB, DOF) among the up-regulated genes. Furthermore, infected plants treated with HA showed a remarkable activation of secondary metabolism with the over-expression of 44 genes involved in the

TABLE 3 | List of pathways obtained from Cellular Function overview (MapMan category) with the related number of DEGs involved.

A		B		C	
Pathway name	**No. of genes**	**Pathway name**	**No. of genes**	**Pathway name**	**No. of genes**
Photosynthesis	32	Photosynthesis	29	Photosynthesis	44
Major carbohydrate metabolism	12	Major carbohydrate metabolism	5	Major carbohydrate metabolism	12
Minor carbohydrate metabolism	8	Minor carbohydrate metabolism	4	Minor carbohydrate metabolism	10
Glycolisys	10	Glycolisys	5	Glycolisys	11
Fermentation	2	Fermentation	4	Fermentation	7
Gluconeonese/glyoxylate cycle	1	Gluconeonese/glyoxylate cycle	2	Gluconeonese/glyoxylate cycle	3
OPP.oxidative PP.6-phosphogluconolactonase	4	OPP.oxidative PP.6-phosphogluconolactonase	2	OPP.oxidative PP.6-phosphogluconolactonase	9
Tricarboxylic acid (TCA) cycle	6	Tricarboxylic acid (TCA) cycle	9	Tricarboxylic acid (TCA) cycle	23
Mitochondrial electron transport/ATP synthesis	9	Mitochondrial electron transport/ATP synthesis	12	Mitochondrial electron transport/ATP synthesis	23
		Synthesis	12	Synthesis	18
Cell wall synthesis/degradation	17	Cell wall synthesis/degradation	27	Cell wall synthesis/degradation	54
Lipid metabolism	23	Lipid metabolism	24	Lipid metabolism	49
N-metabolism	5	N-metabolism	3	N-metabolism	4
Amino acid metabolism	27	Amino acid metabolism	21	Amino acid metabolism	44
				S-assimilation	2
Metal handling	2	Metal handling	4	Metal handling	9
Secondary metabolism	28	Secondary metabolism	15	Secondary metabolism	33
Hormone metabolism	28	Hormone metabolism	30	Hormone metabolism	63
Co-factor and vitamin e metabolism	3	Co-factor and vitamin e metabolism	5	Co-factor and vitamin e metabolism	6
Tetrapyrrole synthesis	9	Tetrapyrrole synthesis	7	Tetrapyrrole synthesis	12
Stress biotic/abiotic	50	Stress biotic/abiotic	42	Stress	93
Redox reactions	24	Redox reactions	16	Redox reactions	33
Polyamine metabolism	2	Polyamine metabolism	1	Polyamine metabolism	5
Nucleotide metabolism	5	Nucleotide metabolism	11	Nucleotide metabolism	21
Biodegradation of Xenobiotics	4	Biodegradation of Xenobiotics	3	Biodegradation of Xenobiotics	4
		C1-metabolism	5	C1-metabolism	6
Miscellaneous	67	Miscellaneous	55	Miscellaneous	121
RNA metabolism	129	RNA metabolism	142	RNA metabolism	284
DNA synthesis	21	DNA synthesis	25	DNA synthesis	43
Protein synthesis/degradation	231	Protein synthesis/degradation	241	Protein synthesis/degradation	505
Signaling	79	Signaling	49	Signaling	122
Cell cycle	41	Cell cycle	54	Cell cycle	83
		MicroRNA, natural antisense	1	MicroRNA, natural antisense	1
Development	29	Development	35	Development	59
Transport	52	Transport	50	Transport	109
Not assigned	287	Not assigned	223	Not assigned	454

A, [P+T vs. P]; B, [P+T+R vs. P+R]; C, [P+HA+R vs. P+R]. [T+P vs. P]: Plants treated with Trichoderma compared to control plants (water); [P+T+R vs. P+R]: Infected plants treated with Trichoderma compared to untreated infected plants; [P+HA+R vs. P+R]: infected plants treated with HA compared to untreated Infected plants.

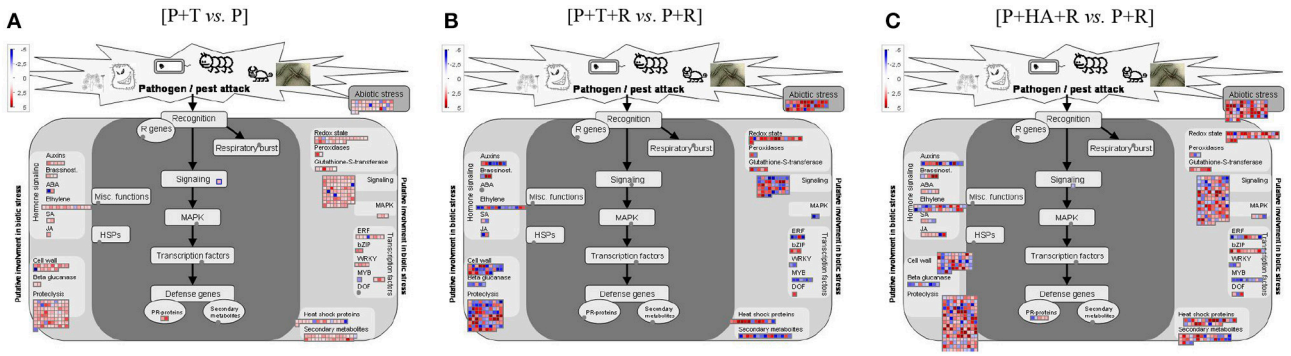


FIGURE 2 | Diagrammatic representation of DEGs involved in "Biotic stress" category as visualized by MapMan. **(A)** [P+T vs. P]: plant treated with *Trichoderma* compared to Control plants (water); **(B)** [P+T+R vs. P+R]: infected plants treated with *Trichoderma* compared to infected plants (*R. solani*); **(C)** [P+HA+R vs. P+R]: infected plants treated with HA compared to infected plants (*R. solani*). Each gene is symbolized by a box, the gene expression level is color-encoded (red = up-, blue = down-regulation). Genes with experimental indication of involvement in the biotic stress are grouped in the main central panel (colored in dark gray), while genes and pathways that are putatively involved in biotic stress pathway are shown on the left and right sides (colored in light gray).

biosynthesis of flavonoids, isoprenoids, phenylpropanoids and aromatic amino acids.

GO analysis highlighted the presence of 32 enriched terms belonging to Biological process category, 16 to Molecular function and 8 to Cellular component (**Figure 5**). The most represented GO terms were "cellular process," "metabolic process," and "primary metabolic process" and many genes involved in protein metabolism, transport, localization and response to stress were identified.

Plant Responses Comparisons—With and Without Pathogen

DEGs obtained comparing the expression analysis of the tripartite and bipartite interactions were studied in order to identify common genes mainly associated to *Trichoderma* colonization in plants infected and non-infected by the pathogen [P+T+R vs. P+T]. Interestingly, 215 DEGs were present in both conditions and a significant portion of them (39%) showed opposite expression values (**Figures 6A**; **Table S2**). In the comparison analysis of the DEGs from [P+T+R vs. P+R] and [P+HA+R vs. P+R], used to identify the HA-dependent effect in *Rhizoctonia* infected plants, a total of 1,053 genes were found to be shared, while 1,264 and 89 genes resulted specifically associated to [P+HA+R vs. P+R] and [P+T+R vs. P+R], respectively (**Figure 6B**). The comparison between all interactions ([P+HA+R vs. P+R] vs. [P+T+R vs. P+R] vs. [P+T vs. P]) allowed to identify 208 shared DEGs (**Figure 7A**). Interestingly, 119 of them were over-expressed in all the conditions, 12 were up-regulated only in infected plants [P+T+R vs. P+R] and [P+HA+R vs. P+R] and down regulated in non-infected plants [P+T vs. P], while 6 genes resulted up-regulated in [P+T vs. P] and [P+HA+R vs. P+R] and down-regulated in [P+T+R vs. P+R] (**Figure 7B**). Among the up-regulated genes in pathogen-infected plants treated with the beneficial fungus *Trichoderma* or its metabolite HA, many were found to be involved in processes like photosynthesis, cell wall modifications, cell organization and development (**Table S3**). On the other hand, DEGs common to [P+T vs. P] and [P+HA+R vs. P+R] conditions were mostly associated to photosynthesis, cellular redox state, protein synthesis/degradation and ABC transporters. Finally, 68 genes were up-regulated only in [P+T vs. P] and down-regulated in the multiple interaction ([P+HA+R vs. P+R] and [P+T+R vs. P+R]). Several of these DEGs resulted involved in transcriptional regulation, protein synthesis/degradation, signaling, and hormone metabolism.

Microarray Validation by RT-qPCR

Quantitative real time PCR (qPCR) was used as a validation tool to confirm expression data obtained with microarray analysis. In particular, 21 genes (7 for each interaction) involved in plant defense responses were selected among the DEG lists. As shown in **Figure 8**, qPCR confirmed the results obtained for DEGs with microarray analysis in each interactions. In particular, in plant-*Trichoderma* interaction, genes involved in ethylene pathway (*ERT* and *EDS1*) and in reactive oxygen species (ROS) detoxification (superoxide dismutase-*SOD*) were found to be significantly up-regulated. In infected plants treated with M10 [P+T+R], *HSP90* as well as 1-aminocyclopropane-1-carboxylate synthase (*ACCs*) were up-regulated, while the expression of both chitinase and strictosidine synthase (*STR*) genes were both down-regulated. In the P+HA+R interaction, the *KTI* (Kunitz trypsin inhibitor) and the *PR1* genes were over-expressed. The results confirmed the reliability of the microarray approach.

Metabolomic Analysis

A comparison of the plant metabolites obtained from the treatments of [P+HA], [P+M10], [P+R] with the water control indicated 10 compounds differentially produced. However, the subsequent bioinformatics analysis did not reveal any molecule annotation predictions, and since it was not possible to determine the molecular formula for many of the compounds, these treatments were not furtherly considered in the comparison analyses (data not shown).The untargeted metabolomic analysis of the methanol extracts from *Rhizoctonia*-infected and M10-

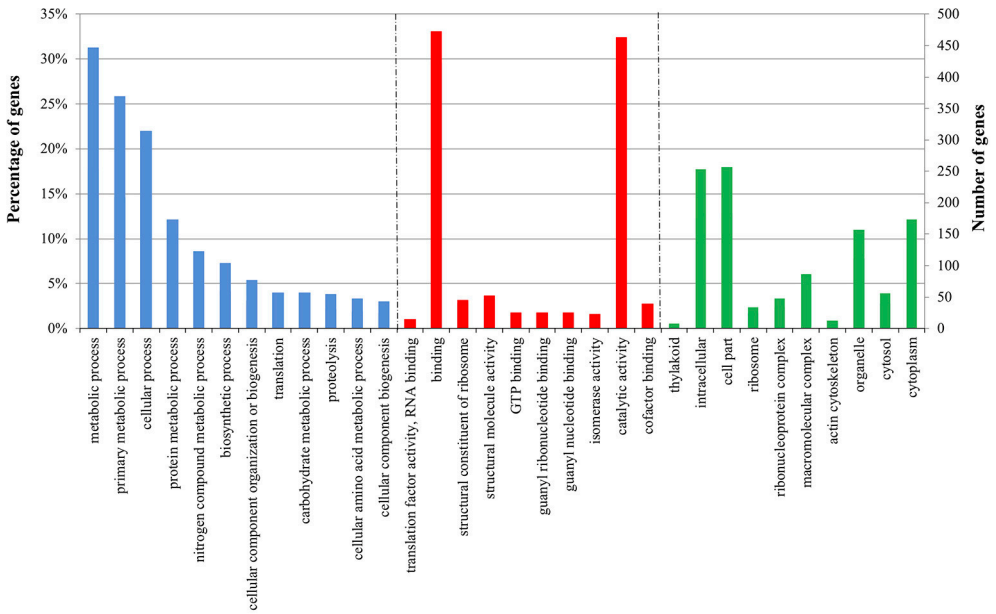


FIGURE 3 | Enriched Gene Ontology (GO) terms distribution of tomato DEGs in plant-*Trichoderma* [P+T vs. P] interaction 30 days post-inoculum. The GO terms displayed along the X-axis are grouped in three GO categories: Biological Process (blue); Molecular function (red); and Cellular component (green). The values on the Y-axis indicate tomato genes in each GO term represented as: on the left, the percentage of total genes; and on the right, the number of genes detected in each GO term.

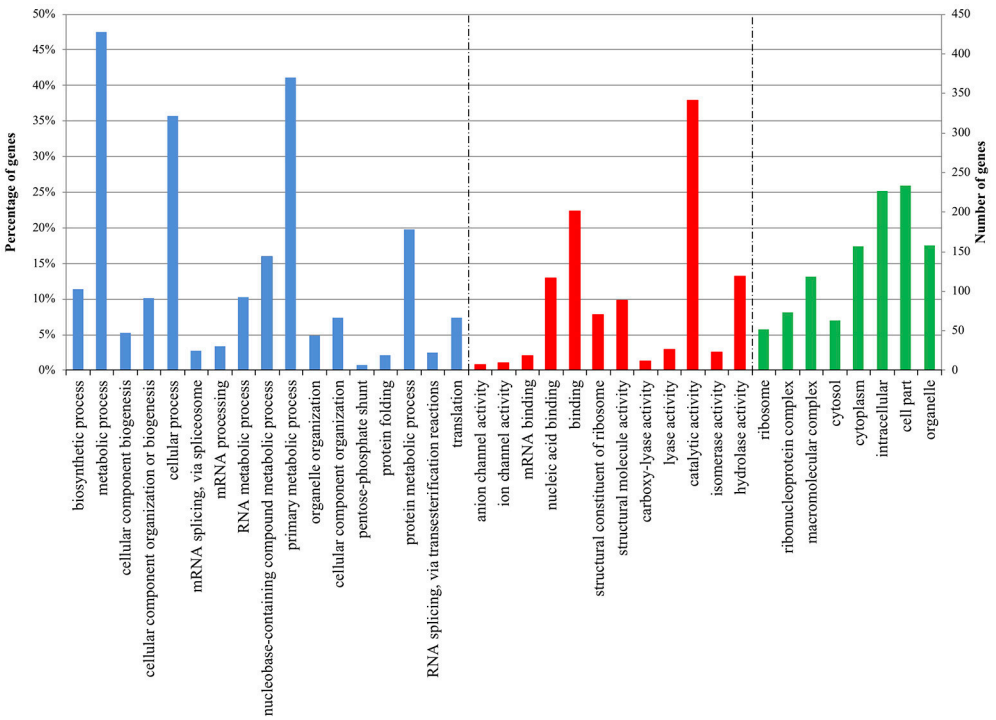


FIGURE 4 | Enriched Gene Ontology (GO) terms distribution of tomato DEGs in plant-*Trichoderma-Rhizoctonia* [P+T+R vs. P+R] interaction 30 days post-inoculum. The GO terms displayed along the X-axis are grouped in three GO categories: Biological Process (blue); Molecular Function (red); and Cellular Component (green). The values on the Y-axis indicate tomato genes in each GO term represented as: on the left, the percentage of total genes; and on the right, the number of genes detected in each GO term.

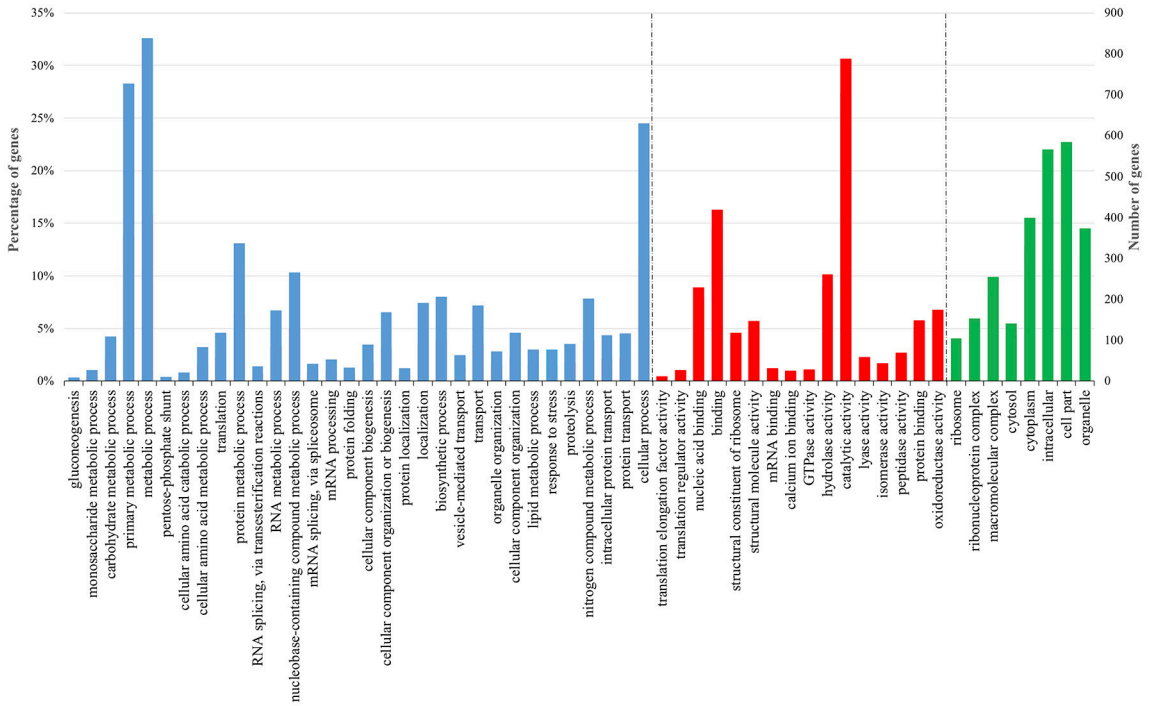


FIGURE 5 | Enriched Gene Ontology (GO) terms distribution of tomato DEGs in plant—harzianic acid—*Rhizoctonia* [P+HA+R vs. P+R] interaction 30 days post-inoculum. The GO terms displayed along the X-axis are grouped in three GO categories: Biological Process (blue); Molecular Function (red); and Cellular Component (green). The values on the Y-axis indicate tomato genes in each GO term represented as: on the left, the percentage of total genes; and on the right, the number of genes detected in each GO term.

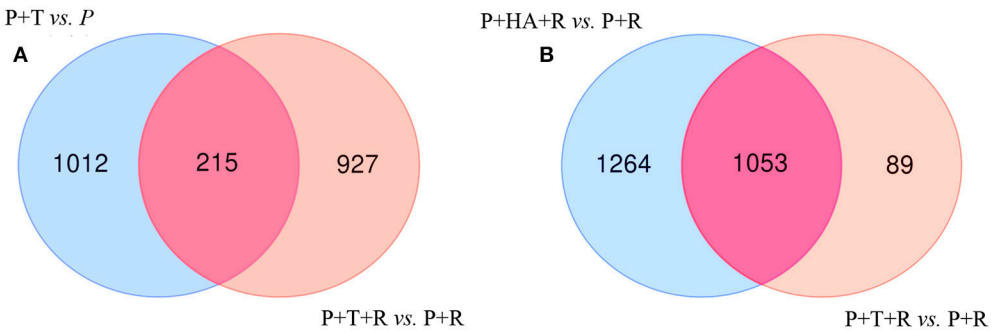


FIGURE 6 | Venn Diagrams showing the number of unique and overlapping DEGs in bipartite and tripartite interactions. **(A)** [P+T vs. P] (non- infected plants treated with M10) vs. [P+T+R vs. P+R] (infected plant treated with M10); **(B)** [P+HA+R vs. P+R] (infected plant treated with HA secondary metabolite) vs. [P+T+R vs. P+R] (infected plant treated with M10).

or HA-treated plants allowed to identify 25 compounds of which 15 were automatically annotated, while the remaining 10 resulted "unknown" (**Table 4**). Tomato treated with HA or M10 increased the abundance of more than 90% of the metabolites in comparison with plants only infected by the pathogen, as depicted by the hierarchical clustering shown in **Figure 9**. Intriguingly, only compound 22 was found to be more abundant in infected plants in comparison with treated groups. Compound 23 was the only substance to be up-regulated in HA-treated plants in comparison to M10-treated plants, whereas in the latter case it was decreased (**Figure 9**; **Table 4**). On the other hand, no significant differences in metabolite abundance were found comparing treated groups (i.e., [P+HA+R] vs. [P+T+R]). In fact, out of 25 compounds, 18 showed an FC value close to zero. Interestingly, several putatively identified compounds belong to the class of steroidal glycoalkaloids (SGAs). These molecules are synthetized from isopenthyl-phosphate via mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways. In [P+T+R] only genes involved in MVA pathway resulted up-regulated, whereas in [P+HA+R], besides those of the MVA

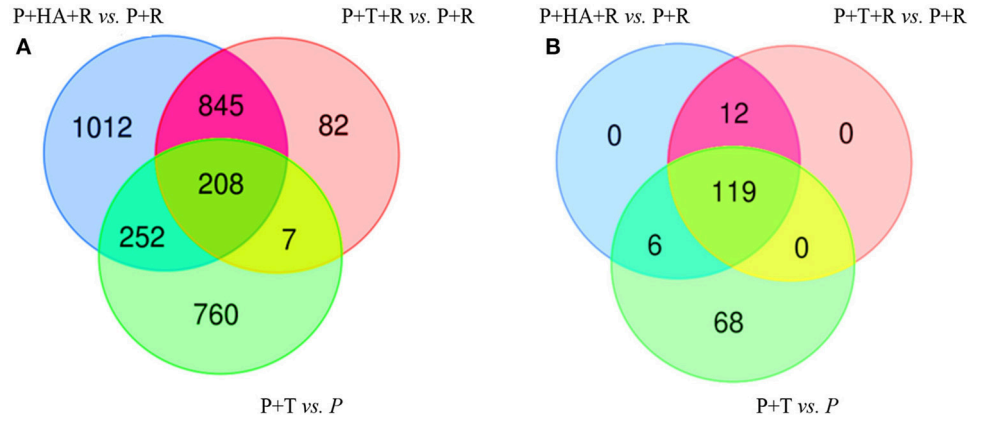


FIGURE 7 | Venn Diagrams showing the number of unique and overlapping DEGs in bipartite and tripartite interactions. **(A)** Comparative analysis of DEGs obtained from [P+T vs. P] vs. [P+T+R vs. P+R] vs. [P+HA+R vs. P+R]; **(B)** Fold changes value of the 208 DEGs found in common in **(A)**; 119 genes resulted over-expressed in all the conditions, 12 were up-regulated in [P+T+R vs. P+R] and [P+HA+R vs. P+R] and down in [P+T vs. P], while 6 genes resulted up-regulated in [P+T vs. P] and [P+HA+R vs. P+R] and down-regulated in [P+T+R vs. P+R]. [P+T vs. P]: plant treated with *Trichoderma* compared to control plants (water); [P+T+R vs. P+R]: infected plants treated with *Trichoderma* compared to infected plants (*R. solani*); [P+HA+R vs. P+R]: infected plants treated with HA compared to infected plants (*R. solani*).

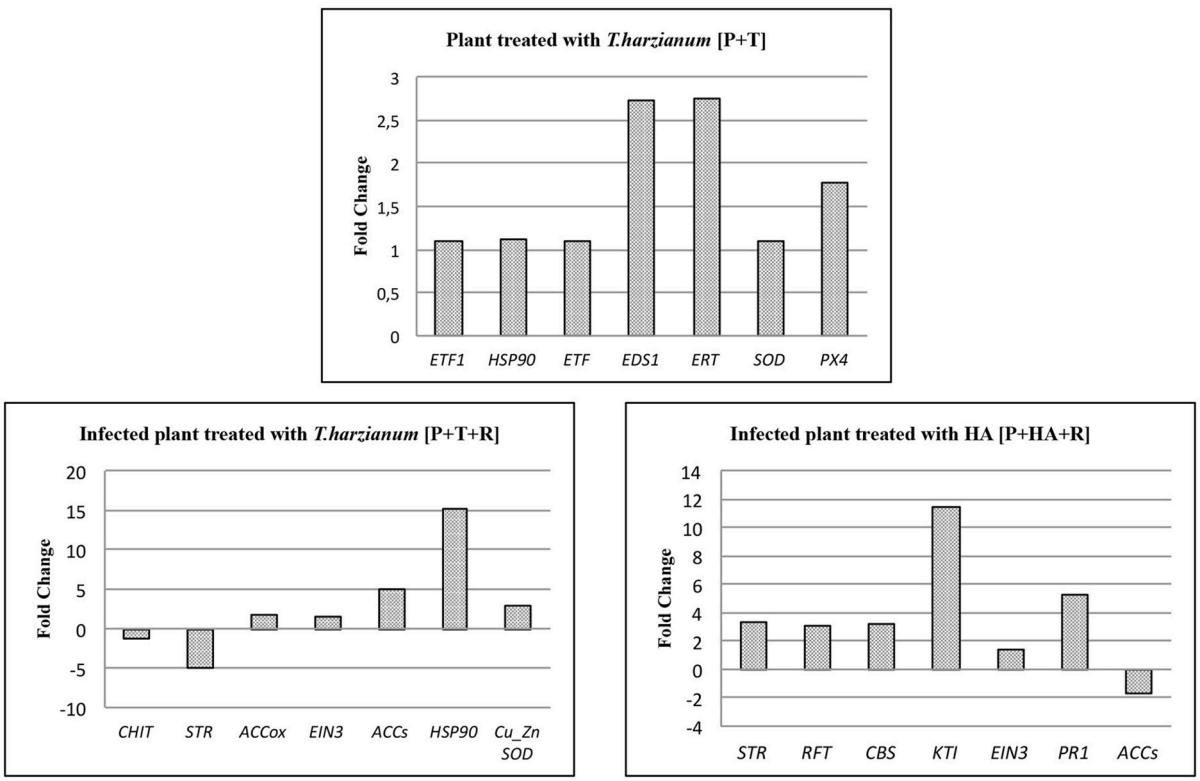


FIGURE 8 | qPCR assay of 7 target genes in each treatment identified by microarray analysis. Bars indicate relative expression measurements (Fold change) of target genes in treated plants respect to the calibrator (control plants). *ETF1*, Ethylene responsive transcription factor 1*; HSP90*, Heat shock protein 90; *ETF,* Ethylene responsive transcription factor*; EDS1*, Enhanced disease susceptibility 1; *ERT*, Ethylene responsive transcript; *SOD*, Superoxide dismutase; *PX4*, Peroxidase 4; *Chi*t, Chitinase; *STR*, Strictosidine synthase family protein; *ACCox*, 1-aminocyclopropane-1-carboxylate oxidase; *EIN3*, Ethylene insensitive 3 class transcription factor; *ACCs*, 1-aminocyclopropane-1-carboxylate synthase; *CuZnSOD*, Cu/Zn-superoxide dismutase copper chaperone; *RFP*, Reticulon family protein; *CBS*, CBS domain-containing protein-like*; KTI*, Kunitz trypsin inhibitor; *PR1*, Pathogenesis related protein 1. The significance of the $2^{-\Delta\Delta Ct}$ values was calculated as $p < 0.05$; Student's *t*-test.

TABLE 4 | List of identified compounds in metabolomic analysis.

Compound	[P+T+R] vs. [P+HA+R]	[P+HA+R]	[P+T+R]	Mass	RT	Molecular formula	Putative identification/formula	Id. score	Composite spectrum
1	0,444101	0,939036	1,383138	1033,5474	6,9830	C50H83NO21	Tomatine	96.94	1034.5547- 1035,5583-1036.5615
2	0,368933	1,582161	1,951094	577,3991	6,9840	C34H51N5O3	Unknown	97.86	578.4063-579.4097-580.41205
3	0,277088	1,130523	1,407611	1031,5320	6,9000	C50H81NO21	Dehydrotomatine	89.22	1032.5393-1033.5428-1034.5494
4	0,187634	0,995527	1,183162	415,3459	6,9830	C27H45NO2	Tomatidine	98.62	416.35303-417.3561-418.35898
5	0,953433	0,394625	1,348058	575,3835	6,9030	C34H49N5O3	Unknown	NI	576.3907-577.39386
6	0,367650	0,944839	1,312489	103,1002	1,6770	C5H13NO	2-Amino-3-methyl-1-butanol	86.8	104.1074-105.11036
7	0,487186	0,896811	1,383997	137,0480	1,7850	C7H7NO2	Methyl nicotinate-(Trigonelline)	86.76	138.05537-139.05818
8	1,07620330	0,94239616	2,01859950	1048,5581	6,9010	C37H72N22O14	Unknown	NI	525.2868-525.78828-526.2919
9	1,45554260	17,51499600	18,97054000	413,3296	6,9040	C27H43NO2	5alpha-Tomatidan-3-one	81.25	414.33713-415.34024
10	−16,15150800	17,28217900	1,13067050	739,4512	6,9800	C39H65NO12	Lycoperoside D-γ tomatine	67.78	740.4583-741.4614
11	−0,47575950	1,95826770	1,48250820	921,0026	1,4960	NA	Unknown	NI	922.0098-923.0124
12	1,05304240	0,47741318	1,53045560	1063,5562	6,9370	C49H73N15O12	Unknown	NI	1064.5641-1065.5673-1066.5693
13	−13,95523300	1,50859030	1,13067050	380,2901	17,0060	C21H42O4	MG(0:0/18:0/0:0)	76.76	381.29742-382.30084
14	0,55803300	0,76031590	1,31834890	109,0013	1,5240	C4HN2O2	Unknown	NI	110.00861-111.0102
15	0,36856365	0,97294044	1,34150410	162,0528	6,9830	C6H10O5	D-Fructofuranose 1,2′:2,3′-dianhydride	47.48	325.11313-163.0601)
16	0,32262707	1,15023610	1,47286320	386,1730	10,5240	C15H26N6O4S	CysHysLys	62.48	387.18027-388.18338-425.136-409.16208
17	−0,34325027	1,89385750	1,55060720	577,3975	6,5100	C17H45N20O3	Unknown	NI	578.40515-579.4085
18	0,49116898	0,79987430	1,29104330	456,1478	6,9810	C17H28O14	Unknown	NI	457.15494-458.15833
19	17,57123200	0,33272552	17,90395700	731,4610	9,6850	C41H65NO10	Unknown	68.73	732.4684-733.4719
20	0,44027900	0,86345770	1,30373670	1033,5444	6,5100	C50H83NO21	Tomatine	87.43	1034.5525-1035.5558-1036.5592
21	0,45742893	0,86361220	1,32104110	1031,5314	6,9000	C50H81NO21	Dehydrotomatine	58.68	1032.5393-1033.5428
22	0,79794500	−17,17997700	−16,38203000	1050,5719	6,5100	C27H44NO7P	unknown	NI	526.2937-526.7954
23	−15,39738200	1,82587770	−13,57150500	1031,5294	6,2150	C50H81NO21	Dehydrotomatine	60.32	1032.5375-1033.54
24	0,29756737	2,93896630	3,23653360	426,2018	13,3600	C20H30N2O8	Dolychil phosphate D mannose	83.47	427.20892-428.2124
25	0,30474758	1,14425370	1,44900130	763,5235	17,7890	NA	Unknown		764.5308-765.5342

Compounds are reported with relative normalized logFC values, masses, retention times (RT), molecular formulas, putative identifications, mass scores, and composite spectra. See ***Figure 9****.*

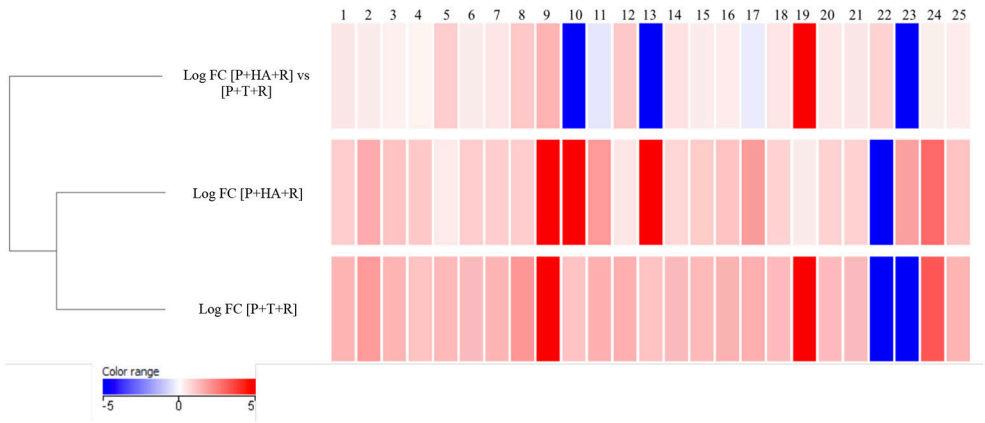


FIGURE 9 | Hierarchical clustering of compounds identified in methanol extract of treated plants using LC-MS in positive mode. Values are expressed in Log FC (normalized by quantile). Each compound was associated to an ID number. The identification of compounds is reported in **Table 4**.

pathway, also three genes involved in the MEP pathway were found over-expressed (**Figure 10**).

DISCUSSION

In the case of *Trichoderma*, the establishment of a plant-fungus interaction in most cases produced multiple positive effects (Segarra et al., 2007; Fontenelle et al., 2011; Perazzolli et al., 2011; Brotman et al., 2012; Yoshioka et al., 2012; Martínez-Medina et al., 2017; Nawrocka et al., 2018). The objective of this work was to use transcriptomic and metabolomic analysis to obtain new insights into the molecular and biochemical processes underlying tomato resistance induced by the plant beneficial fungus *T. harzianum* M10 or its metabolite HA, both in healthy and pathogen-infected plants.

Plant Responses Induced by *Trichoderma* Colonization

In the bipartite interaction *Trichoderma*-plant [P+T vs. P], many genes involved in defense responses as well as in growth and development were found differentially expressed. Germin-like proteins and oxalate oxidase germin-like enzymes were over-represented indicating the activation of hydrogen peroxide production and defense responses against fungal pathogens (Wang et al., 2013). The accumulation of oxalate oxidase germin-like enzymes in the absence of the pathogen ([P+T vs. P]) may be caused by *Trichoderma* root colonization (Martínez-Medina et al., 2017). The presence of *Trichoderma* activated several hormone-signaling pathways (Contreras-Cornejo et al., 2015). For example, many genes directly involved in ethylene signaling, like ethylene receptors (*ER*), ethylene-responsive transcription factors (*ERF*s) and 1-aminocyclopropane-1-carboxylic acid synthase (*ACC*s) were found up-regulated. Microbial hydrolysis of ACC released in root exudates helps to maintain low ethylene levels and, at the same time, increases nitrogen bioavailability in soil, thus favoring plant growth (Glick et al., 2007; Viterbo et al., 2010).

Trichoderma Primes Plant Defense Responses

The genes encoding cathepsin B and epoxide hydrolase were over-expressed, suggesting that the induction of programmed cell death (PCD) by *Trichoderma* could be related to endoplasmic reticulum (ER) stress (Cai et al., 2018) in [P+T+R vs. P+R]. Epoxide hydrolase is essential to detoxify ROS, and it also seems to be involved in cutin biosynthesis, the main cuticle component that acts as a physical barrier against pathogen penetration (Pinot et al., 1992), and is involved in the process of biosynthesis as a precursor of JA (Hamberg and Gardner, 1992; Ziegler et al., 1997). Furthermore, the over-expression of two ERFs and ethylene insensitive 3 transcription factor (EIN3), which act as regulators of the plant defense response (Alonso et al., 2003; Yamazaki and Hirose, 2003; Zhong et al., 2009; An et al., 2010), confirmed the involvement of JA and ethylene signaling pathways in the establishment of ISR by *Trichoderma*. This is in accordance with the production of ROS, the accumulation of phenylalanine ammonia-lyase (PAL) and cinnamyl-alcohol dehydrogenase (CAD) transcripts and the synthesis of phenylpropanoids, metabolites that are produced during response to pathogen attack (Korkina, 2007). In general, these results demonstrate that pre-colonization by *Trichoderma* induces a stronger reaction of the plant upon challenge by a pathogen as compared to untreated plants (Lorito et al., 2010; Mauch-Mani et al., 2017).

Tradeoffs Between Growth and Defense

The comparison between the interactions [P+T+R vs. P+R] and [P+T vs. P] identified 215 shared genes, 83 of which showed opposite expression values (**Figure 6A**; **Table S2**). Many of the latter group, such as those encoding for NAC domain protein and transcription factors, cytochrome P450, subtilisin-like protease etc., were over-expressed in the absence but down-regulated in the presence of the pathogen. The lower expression of such genes may be due to the activation of defense responses to the pathogen with a consequent inhibition of other processes (development

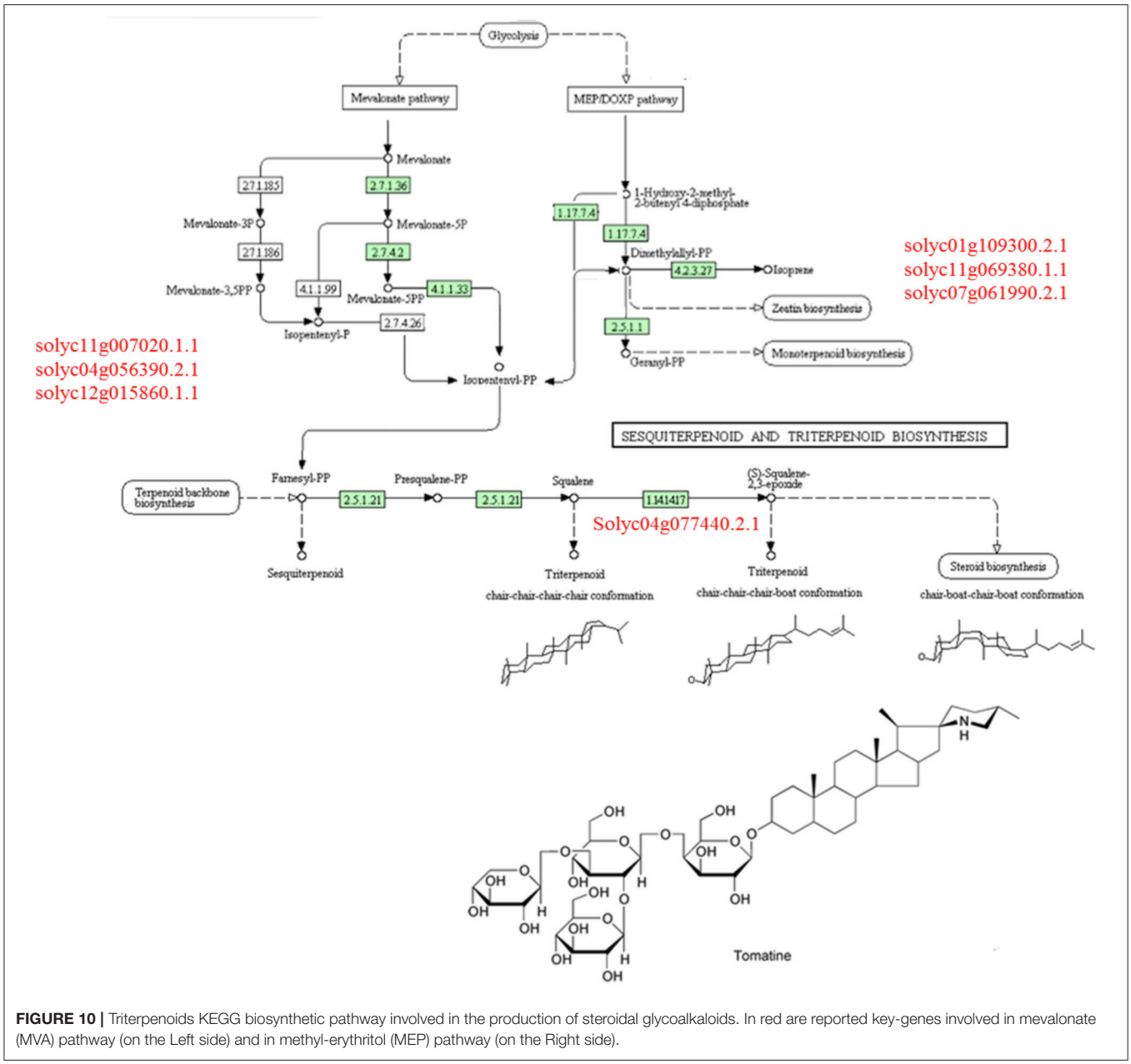


FIGURE 10 | Triterpenoids KEGG biosynthetic pathway involved in the production of steroidal glycoalkaloids. In red are reported key-genes involved in mevalonate (MVA) pathway (on the Left side) and in methyl-erythritol (MEP) pathway (on the Right side).

processes), in order to balance the energy framework. In any case, our findings only refer to an early phase (48 HPI) of the studied interaction, thus we cannot exclude that later on *Trichoderma* may promote growth also in the presence of the pathogen. Significantly changed pathway analysis indicated a strong involvement of the energy metabolism to support a simultaneous activation of growth and defense related processes (Harman et al., 2004b; Segarra et al., 2007; Shoresh and Harman, 2008). In fact, *Trichoderma* up-regulated plant genes involved in TCA, glycolysis, photosynthesis, and gluconeogenesis, as well as ethylene biosynthesis. Interestingly, we found several defense related genes whose transcription was enhanced by *Trichoderma* compared to the *Rhizoctonia*-treatment only.

HA Boosts the Plant Immune Response

The transcriptomic analysis of [P+HA] samples did not reveal any DEGs. However, the absence of a plant response to this treatment is probably not due to a lack of effect caused by HA, since other studies using the same *Trichoderma* compound demonstrated a clear effect also on tomato (Vinale et al., 2013, 2014). Most likely, the timing of collection of the plant material for the RNA extraction used in this work was not appropriate. A recent study by Stringlis et al. (2018) demonstrated that the *Arabidopsis* transcriptional response to treatments with a beneficial strain of *Pseudomonas* or two different flagellins plus one chitin preparation had different kinetics when purified substances were compared to the living microbes. In particular,

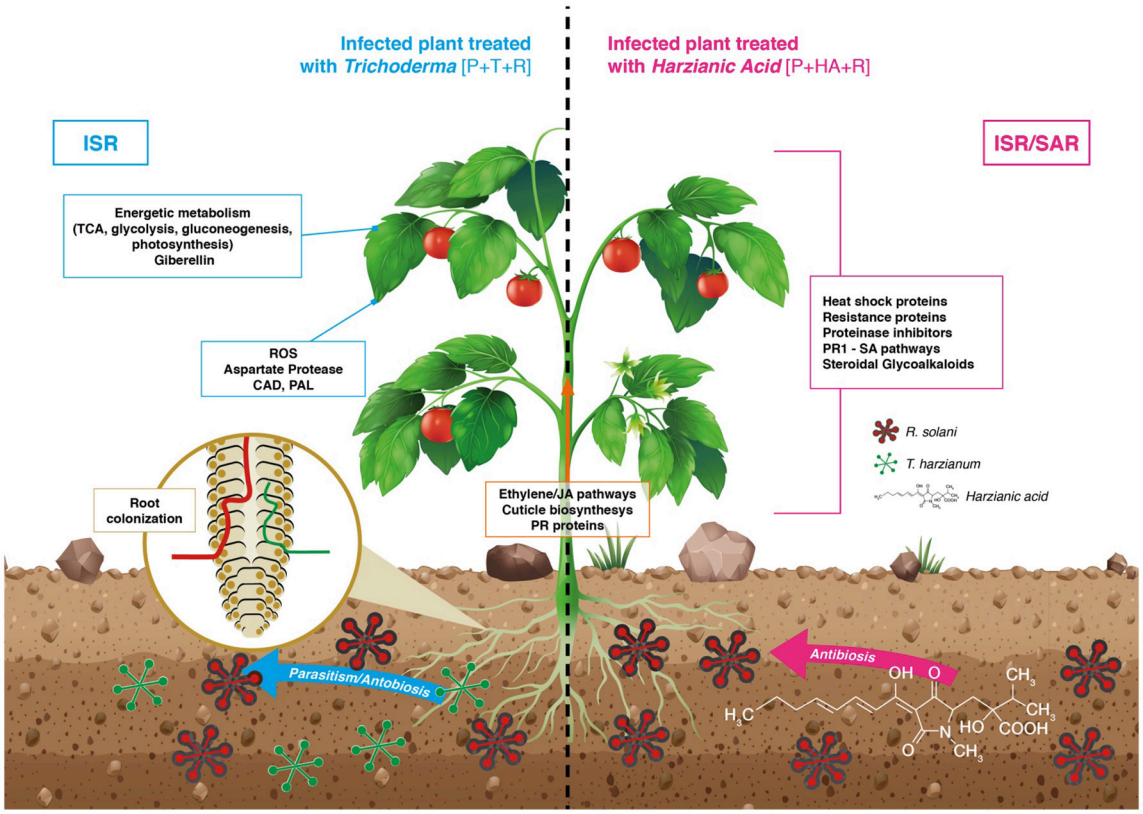


FIGURE 11 | Model of response of tomato plant infected with the soil pathogen *Rhizoctonia solani* (see figure legend) when pre-treated with *Trichoderma harzianum* or its bioactive metabolite harzianic acid (HA). In the soil *T. harzianum* limits pathogen proliferation through its mycoparasitic action and the release of antibiotic compounds like Harzianic Acid (HA). In the presence of pathogens, *T. harzianum* and its metabolite HA elicit defense responses mainly by the production of toxic secondary metabolites, strengthening of physical barriers and triggering ethylene/jasmonic acid biosynthetic pathways (induced systemic resistance-ISR). Additionally, HA carries out its beneficial effect activating salicylic acid (SA) pathway (systemic acquired resistance-SAR). Besides pathogen control, *T. harzianum* also enhances plant energetic metabolism thus promoting its growth and development.

chitin triggers the strongest activation of plant responses and the highest number of DEGs within 0.5 h post-inoculation (HPI), after which the transcriptional response gradually diminished over time. Furthermore, the effect of HA, also known to be a siderophore (Vinale et al., 2013), could be dependent upon the nutritional status of the plants. Trapet et al. (2016) demonstrated that microbial siderophores affect plant gene expression mainly on iron-deficiency conditions, and this was not the case in our study. On the other hand, it can be speculated that HA has a priming effect on gene expression since the number of DEGs identified in the [P+HA+R vs. P+R] was greater than those observed in the interactions with the *Trichodema* fungus [P+T+R vs. P+R], and 60% of those DEGS were over-expressed. Furthermore, the establishment of a priming status may have allowed HA to perform as well as the living fungus in the biocontrol experiment.

In the comparison of [P+T+R vs. P+R] with [P+HA+R vs. P+R] datasets, the plant response to HA-treatment resulted more intense in terms of activation of stress-induced and resistance genes. Transcripts of heat shock (*HSP*), early-response to dehydration and universal stress proteins, as well as of molecular chaperones (DNAK, DNAJ, DNAJ2), were also increased. In addition, overexpression of osmotin-like genes, involved in biotic and abiotic stress response was also observed (Hanin et al., 2011; Hakim et al., 2017).

The simultaneous over-expression of HSP90 and co-molecular chaperones is reported to be linked to the accumulation of disease resistance (R) genes encoding pathogen receptors (Holt et al., 2005). This is in agreement with the overexpression of genes encoding resistance proteins with an N-terminal coiled-coil (CC) domain, a central nucleotide-binding site (NBS) domain and a C-terminal leucine-rich repeat (LRR) domain (CC-NBS-LRR) found in the presence of HA and with the activation of SA, JA and ethylene biosynthetic pathway. The whole activation of defense responses was indicated by the over-expression of genes coding for pathogenesis-related proteins (PR), such as PR1, oxalate oxidase-like germin 171 and kunitz-type proteinase inhibitor. The up-regulation of *PR1* could be related to the activation of systemic acquired resistance (SAR) as well as to an ISR status. Interestingly, we found a simultaneously expression of genes involved in SA and JA biosynthesis only when plants were treated with HA

in the presence of *Rhizoctonia* and this may have resulted in an enhanced protection against the pathogen (Verhagen et al., 2006).

Trichoderma and HA Increase Plant Chemical Weapons

The untargeted metabolomic analysis revealed the presence of 25 compounds mainly annotated in the SGAs class. SGAs are specialized anti-nutritional metabolites constitutively produced in plants and frequently reported as determinants of resistance to fungal attack (Friedman, 2002; Bednarek and Osbourn, 2009).

In tomato, α-tomatine is important in the defense against fungi because of its specific effect on sterols that leads to membrane leakage (Sandrock and VanEtten, 1998). Lycoperoside belongs to the same chemical class and, similarly to other SGAs, its biosynthesis is strictly dependent on cholesterol. The level of lycoperoside was significantly increased in [P+HA+R], which is in agreement with the activation, as found in the microarray analysis, of cholesterol biosynthesis observed in pathogen-infected plants treated with HA. On the other hand, compounds such as tomatidanol and tomatine were predominant in plants infected by *R. solani* and treated with M10. These results indicate an activation of terpenoid and polyketide pathways with the biosynthesis of alkaloids related to chemical defense. Furthermore, N-methyl-nicotinic acid (Trigonelline), another important SM involved in defense response (Kraska and Schönbeck, 1993) was found particularly abundant in infected plants colonized by *Trichoderma*, which suggest a positive correlation between the presence of the biocontrol fungus and the accumulation of this compound.

CONCLUSIONS

A microarray analysis was used to study tomato gene expression in the interaction with the fungal biocontrol agent *T. harzianum* M10 or its secondary metabolite HA, in the presence of the soil-borne root pathogen *R. solani*. The over-expression of genes involved in glycolysis, TCA and photosynthesis, as well as those implicated in cell wall remodeling, is related to the growth promotion effect of *T. harzianum* M10 on healthy plants. In addition, despite the initial activation of defense responses, the promoted expression of genes involved in cellular homeostasis is in agreement with the beneficial nature of the *Trichoderma*-plant interaction (Hermosa et al., 2013).

The presence of the pathogen resulted in a strong overexpression of genes involved in different resistance mechanisms. When infected plants were colonized by *T. harzianum* or treated with HA, physical and chemical defenses involving cuticle biosynthesis and production of toxic secondary metabolites, were activated. Both treatments stimulated ethylene/JA pathways related to ISR activation. In particular, HA also up-regulated the SA pathway and the SAR marker PR1, thus causing the co-induction of ISR and SAR response (Van Wees et al., 2000; **Figure 11**).

This work provides a first overall view of the molecular response triggered in plant by the bioactive and commonly produced fungal metabolite HA. *T. harzianum* and HA treatments demonstrated generally comparable changes in defense-related gene expression and efficacy in *R. solani* containment in controlled conditions. Direct use of new, biocontrol-related fungal metabolites may help to overcome problems of efficacy, reliability and persistency of the effect related to the use a living beneficial microorganisms (Vinale et al., 2008).

AUTHOR CONTRIBUTIONS

GM executed microarray and molecular experiments, processed samples, conducted molecular studies, performed data elaboration and statistical analyses, interpreted the results, and was significantly involved in writing the manuscript. AS processed the microarray experiments, collected and analyzed data, interpreted the results. ME assisted in the experimental design, the microarray analysis, data interpretation. FV defined experimental protocols for the metabolomics analyses, performed data elaboration and analyses. SL, AP, MN, and NL executed the experiments and developed protocols, assisted in sample processing and data collection, and were involved in writing sections of the manuscript. ML assisted in experimental designs, interpretation of results, and writing of the manuscript. SW designed the study experiments and protocols, carried out sampling, coordinated molecular studies and data analysis, interpreted the results, and was significantly involved in writing of the manuscript.

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SUPPLEMENTARY MATERIAL

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Rhizosphere Competence and Biocontrol Effect of *Pseudomonas* sp. RU47 Independent from Plant Species and Soil Type at the Field Scale

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Biocontrol inoculants often show inconsistency in their efficacy at field scale and the reason for this remains often unclear. A high rhizosphere competence of inoculant strains is assumed to be a key factor for successful biocontrol effects as the biocontrol strain has to compete with the indigenous microbial community in the rhizosphere. It is known that many factors, among them plant species and soil type shape the rhizosphere microbial community composition. However, microbial community composition in the rhizosphere can also be influenced by the presence of a pathogen. We hypothesized that plant species, soil type, and a pathogen affect the rhizosphere competence of a biocontrol strain and its biocontrol effect against a soil-borne pathogen. To test the hypothesis, we used an experimental plot system with three soil types (diluvial sand, alluvial loam, loess loam) kept under similar agricultural management at the same field site for 12 years. We investigate the rhizosphere competence of *Pseudomonas* sp. RU47 in two plant species (potato and lettuce) and its biocontrol effect against Rhizoctonia diseases. The colonization density of a rifampicin resistant mutant of RU47 in the rhizosphere of both crops was evaluated by plate counts. Bacterial community compositions were analyzed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from total community DNA. The inoculant RU47 was able to colonize the rhizosphere of both model crops in a sufficient density and to reduce disease severity of black scurf on potato and bottom rot on lettuce in all three soils. DGGE indicated that RU47 affected the bacterial community composition stronger in the rhizosphere of lettuce than in the potato rhizosphere. In contrast, the effect of the pathogen *Rhizoctonia solani* on the bacterial community was much stronger in the rhizosphere of potato than in the lettuce rhizosphere. A significant effect of RU47 on the *Pseudomonas*-specific *gacA* fingerprints of the rhizosphere was only observed in lettuce in alluvial soil. The soil type and plant species independent biocontrol effects of RU47 and its minor influence on the indigenous bacterial community composition might be important criteria for the registration and use of RU47 as biocontrol strain.

Keywords: *Rhizoctonia solani*, plant health, biocontrol, plant disease, bacterial community, DGGE

INTRODUCTION

Diseases caused by soil-borne pathogens such as *Rhizoctonia solani* (Kühn) are difficult to control, and the use of bacterial inoculants for disease suppression represents an environmentally friendly control method (Alabouvette et al., 2009). Still biocontrol inoculants often showed a lack of consistency in their biocontrol activity at the field scale (Barret et al., 2011).

Over decades, efforts have been made to unravel factors affecting biocontrol efficacy of bacterial inoculants (Bais et al., 2006; Barret et al., 2011; Ghirardi et al., 2012). Variation in the ability of bacterial inoculants to colonize the rhizosphere and survive at sufficiently high cell densities was thought to be a reason for inconsistency in disease suppression (Ghirardi et al., 2012). Therefore, a high rhizosphere competence was identified as a prerequisite for the expression of beneficial effects on plants (De Bellis and Ercolani, 2001; Barret et al., 2011; Ghirardi et al., 2012).

Research presently is just starting to unravel the complex interplay between plants and their microbiome including bacterial inoculants applied (Berendsen et al., 2012; van der Heijden and Hartmann, 2016; Berg et al., 2017). Studies done by 16S rRNA gene-based fingerprints provided a growing body of evidence that biotic factors (e.g., plant species/genotype, developmental stage, and plant pathogens) and abiotic factors (e.g., weather conditions, agricultural management) affect the bacterial community composition in the rhizosphere (Berg and Smalla, 2009; Weinert et al., 2009; Schreiter et al., 2014a).

Several studies have clearly shown that both plant and soil type shape the rhizosphere microbiome (Bulgarelli et al., 2012; Peiffer et al., 2013; Schreiter et al., 2014b). Different soil types varying in physicochemical properties were shown to contain distinct bacterial communities from which a subset of populations was enriched in the rhizosphere of the plant due to root exudates (Costa et al., 2006; Schreiter et al., 2014b) resulting in an increased relative abundance in the rhizosphere compared to the corresponding bulk soil.

Until recently, limited information was available on the effect of different soil types and the plant species on the rhizosphere competence and biocontrol activity of bacterial inoculants at field scale. Results of a previous study showed that although the soil types harbor a specific bacterial community no impact on the ability of the inoculant strains *Serratia plymuthica* 3Re4-18 and *Pseudomonas* sp. RU47 to colonize the rhizosphere of lettuce in a sufficient density was given at the field scale (Schreiter et al., 2014c).

The strain RU47, initially identified as *Pseudomonas jessenii* based on the 16S rRNA gene sequence, has been recently reclassified as its genome sequence is phylogenetically related to strains from *P. koreensis* group (Eltlbany et al., under revision). Based on the results of this study, we assume that the often-reported inconsistency of biocontrol effects in the field is more likely due to plant characteristics. The plant modulates via root exudates the rhizosphere microbiome by stimulating microorganisms with traits beneficial for plant growth and health (Dennis et al., 2010; Doornbos et al., 2012). About 30–50% of photosynthetically fixed carbon are released through the root system in the rhizosphere (Kumar et al., 2006). Hence, root exudates play an important role in provoking a substrate-driven competition between rhizosphere microorganisms including an applied biocontrol inoculant in colonization of roots (Bais et al., 2006). The root exudation pattern and the amounts of various exudate compounds synthesized and exuded by the roots are under the plant's genetic control (Micallef et al., 2009). Therefore, the plant species and its unique root exudation pattern attract a specific rhizomicrobial community (Chapparo et al., 2014). A biocontrol inoculant introduced into the rhizosphere has to compete and interact with this plant specific microbial community. Moreover, the rhizosphere is a highly dynamic habitat due to the changing input by the plant. As a result, the rhizosphere microbial community composition and activity differs temporally and spatially (DeAngelis et al., 2009; Chowdhury et al., 2013).

The main objective of our study was to assess the rhizosphere competence and biocontrol activity of the bacterial inoculant RU47 against a soil-borne pathogen depending on the plant species in three soil types.

In 2011 the ability of RU47 to colonize the rhizosphere of lettuce and its biocontrol of *R. solani* was shown to be soil type independent but strikingly dependent on plant growth (Schreiter et al., 2014c). In the present study we compare the rhizosphere competence and biocontrol effects of the inoculant RU47 on lettuce (*Lactuca sativa* L.) with its rhizosphere competence and biocontrol effects on potato (*Solanum tuberosum* L.). There are great differences in systematic botany, phenotype and cultivation period between the model plants. The used model plant species potato and lettuce are members of different plant families (*Solanaceae* and *Asteraceae*, respectively) and the growing period for lettuce is around 5–6 weeks in the field, compared to potato with a cultivation period of around 4–5 months. We expect a pronounced different bacterial community structure in the rhizosphere of the used model plants. Both potato and lettuce are host plants of the soil-borne pathogen *R. solani*.

It is important to note that the fungal pathogen *R. solani* is a species complex of various genetic groups called anastomosis groups (AGs) with a distinct degree of host specificity. While *R. solani* AG1-IB is responsible for bottom rot on lettuce, *R. solani* AG3 causes black scurf disease on potato with striking differences in the genome (Wibberg et al., 2013) and in the interaction with the plant. Whereas *R. solani* AG3 colonizes the belowground parts of potato including the roots (Genzel et al., 2018), *R. solani* AG1-IB infects lettuce via the lower leaves in contact to the soil (Grosch et al., 2011). Hence, we expect a stronger impact of *R. solani* AG3 on the microbial community in the rhizosphere of potato than of *R. solani* AG1-IB in the rhizosphere of lettuce.

In view of the differences in plant-pathogen interaction of the two model plants we aimed to answer the question of whether RU47 is able to compete successfully with the bacterial community in the rhizosphere in both plant species independent from the soil type and to express biocontrol effects in the field on both crops. We used an experimental plot system with three soil types (diluvial sand, alluvial loam, loess loam) kept under similar agricultural management at the

same field site. The effects of the inoculant strain RU47 as well as the pathogens on bacterial community composition in the rhizosphere were assessed by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments amplified from total community (TC)-DNA. The synthesis of secondary metabolites involved in antagonistic interactions and disease suppression is positively controlled by the global response regulator gene *gacA* (Haas and Defago, 2005). Being highly conserved among *Pseudomonas* species, *gacA* serves as a reliable phylogenetic marker for community fingerprinting (De Souza et al., 2003). Hence, we used *gacA* in addition to the 16S rRNA gene to analyze the effect of RU47 and the pathogens *R. solani* AG3 and AG1-IB on the bacterial and the *Pseudomonas* community in the rhizosphere of both model plants.

MATERIALS AND METHODS

Field Experiments

A unique experimental plot system with independent experimental units for lettuce (unit 6) and potato (unit 7) was used for the experiments at the Leibniz-Institute of Vegetable and Ornamental Crops in Großbeeren (52° 33′ N, 13° 22′ E). Each unit consisted of three soil types characterized as Arenic-Luvisol (diluvial sand), Gleyic-Fluvisol (alluvial loam), and Luvic-Phaeozem (loess loam) that shared the comparable agricultural management at the same field site for more than 10 years (Rühlmann and Ruppel, 2005; Schreiter et al., 2014a). Each soil type was arranged in a separate block with 24 plots of 2 m × 2 m and a depth of 75 cm. From 2000 to 2011, following crops were cultivated in unit 6: pumpkin, nasturtium, pumpkin, amaranth, wheat, wheat, pumpkin, nasturtium, wheat, wheat, lettuce and lettuce; and in unit 7: broccoli, phacelia, broccoli, nasturtium, nasturtium, tomato, soybean, phacelia, wheat, phacelia, nasturtium, and phacelia.

On 5 May 2012, seed potato tubers (cv. Arkula, Norika GmbH, Groß Lüsewitz, Germany) were planted in unit 7 at a distance of 30 cm within a row, and with an intra-row distance of 65 cm. The number of tubers (diameter 35–60 mm) and the marketable tuber yield (MTY) were assessed at harvest 18 weeks after planting (on 5 September 2012). Additionally, the percentage of infestation of 20 randomly selected tubers per replicate with *Rhizoctonia* sclerotia was evaluated on a scale from 1 to 5 (1 – without sclerotia, 2 – <1% infestation, 3 – 5% infestation, 4 – 10% infestation, 5 – ≥15% infestation).

Seedling trays filled with the respective soil type were used to cultivate the lettuce seedlings (cv. Tizian, Syngenta, Bad Salzuflen, Germany) as described by Schreiter et al. (2014c). Lettuce seedlings were planted on 3 July 2012 at the three- to four-leaf stage 4 weeks after sowing with a within-row and intra-row distance of 30 cm to the experimental plot unit 6. The lettuce shoot dry mass (SDM) of each plant and the disease severity of bottom rot were assessed according to the following scale: (1) healthy plants without bottom rot symptoms; (2) symptoms on first lower leaves and small brown spots on the underside of leaf midribs; (3) brown spots on leaf midribs on lower and next upper leaf layers; and (4) severe disease symptoms on upper leaf layers and beginning of head rot to total head rot (Supplementary Figure S1) at harvest five weeks after planting (on August 8, 2012; typical size, form and firmness of head reached).

In both experimental units, plots of each soil type were adjusted to the same nitrogen amount of 157 kg ha^{-1} (Kalkamon, 27% N) before planting, based on nutrient analysis of the soil which was done according to the certified protocols of the Association of German Agricultural Analytic and Research Institutes (VDLUFA). The soils in unit 7 (potato) were also adjusted to the same amount of potassium of 210 kg ha^{-1} (Patentkali, K+S Kali GmbH, Kassel, Germany). Both crops were overhead irrigated based on the computer program 'BEREST.'

The following treatments of potato and lettuce were studied: treatment without *Pseudomonas* sp. RU47 and without pathogen application (control), treatment with *R. solani* inoculation (+*Rs*), treatment with RU47 but without pathogen inoculation (RU47), and treatment with RU47 and *R. solani* inoculation (RU47+*Rs*). Each treatment included four replicates (plots) with 21 (potato) or 36 (lettuce) plants per replicate, randomly arranged per soil type.

Inoculation of the Pathogens

The *R. solani* isolate Ben3 was obtained from sclerotia of mature potato tuber and characterized by molecular tools as AG3 (Kuninaga et al., 2000). The isolate *R. solani* AG1-IB 7/3/14 (AJ868459) was originally isolated from lettuce plants with bottom rot symptoms and characterized by conventional and molecular tools (Grosch et al., 2007). The inocula of both pathogens were prepared on barley kernels as described by Schneider et al. (1997).

For pathogen inoculation of potato at planting, seed tubers were covered with a 3 cm thick soil layer upon which five barley kernels infected with the black scurf pathogen *R. solani* AG3 were placed in plots with pathogen inoculation. Non-infected kernels were placed on tubers in plots without pathogen inoculation.

To ensure a homogeneous pathogen pressure for bottom rot in unit 6, a total of 36 lettuce plants (nine or more true leaves unfolded) were shredded and evenly incorporated in the top soil (10 cm) together with 40 g of barley kernels infected with *R. solani* AG1-IB or of non-infected barley kernels (plots without pathogen inoculation) with a rotary hoe 2 weeks before initiating the experiment.

Preparation of RU47 Cell Suspension and Application Mode

A rifampicin resistant mutant of *Pseudomonas* sp.RU47 (strain collection of the Julius Kühn-Institut) was used to assess the colonization density in the field (Adesina et al., 2009). The inoculum of RU47 was prepared on King's B agar plates (Merck KGaA, Darmstadt, Germany) for seed treatment, and in nutrient broth (NB II, SIFIN GmbH, Berlin, Germany) for treatment of young lettuce plants and seed potato tubers as described by Schreiter et al. (2014c). Both media were supplemented with rifampicin (75 μg mL^{-1}). After a cultivation time of 16 h the culture was centrifuged at 13,000 *g* for 5 min, the supernatant

discarded and the pellet was re-suspended in sterile 0.3% NaCl solution.

The potato seed tubers were inoculated with RU47 immediately before planting, spraying 20 mL suspension (10^8 colony forming units [CFU] mL^{-1}) on 10 tubers, and the emerged potato plants were inoculated four weeks after planting (WAP) by watering each plant with 100 mL suspension (10^8 CFU mL^{-1}).

A total of 100 lettuce seeds were coated on a Vortex Mixer (MSI, Minishaker, Staufen, Germany) with 250 µl of the bacterial cell suspension dropped on the seeds [10^8 CFU mL^{-1}]. Each lettuce seedling at its three-leaf stage was treated with 20 mL cell suspension of RU47 (10^7 CFU mL^{-1}) 1 week before transplanting them to the field plots and with 30 mL (10^8 CFU mL^{-1}) 2 days after planting. Regarding respective control treatments for lettuce and potato, watering was carried out with 0.3% NaCl.

Sampling, Sample Processing and Determination of RU47 Counts

The rhizosphere competence of *Pseudomonas* sp. RU47 was analyzed on potato and on lettuce at two time points (7 and 12 WAP for potato, 2 and 5 WAP for lettuce) and the bacterial and *Pseudomonas* community composition (based on 16S rRNA or *gacA* gene, respectively) at one time point (7 WAP for potato, 2 WAP for lettuce) during the growth period of both plants.

Potato plants were sampled 7 WAP of seed potato tuber at growth stage 2 (formation of basal side shoots below and above soil surface) and 12 WAP at growth stage 7 (50% of berries in the first fructification have reached full size). The roots of two potato plants per replicate were combined at the first sampling time (7 WAP), and the roots of one plant per replicate were sampled at the second sampling time (12 WAP).

Lettuce plants were sampled 2 WAP when plants had nine or more true leaves unfolded, and 5 WAP when lettuce head reached typical size, form and firmness. For each treatment the roots of three lettuce plants per replicate were combined as a composite sample and considered as one replicate. In both crops the rhizosphere competence of RU47 and the rhizosphere bacteria of four replicates per treatment were analyzed.

Adhering soil was removed by a quick root wash step, and the root system was cut into 1 cm long pieces with sterile scissors and mixed before microorganisms were extracted as follows: 5 g of roots were placed in sterile Stomacher bags and treated by a Stomacher 400 Circulator (Seward Ltd., Worthing, United Kingdom) for 30 s at high speed after adding 15 ml of sterile 0.3% NaCl. The Stomacher blending step was repeated three times and one ml was taken from the combined 45 ml for plate counts while the microbial rhizosphere/rhizoplane fraction was harvested by centrifugation steps as described by Schreiter et al. (2014a).

Stomacher blending steps were immediately processed to determine the CFU of RU47 by plating serial dilutions on King's B agar supplemented with rifampicin (75 µg mL^{-1}) and cycloheximide (100 µg mL^{-1}) after an incubation time of 48 h at 28°C. The CFU were calculated per gram root dry mass. For both plant species and all soil types, Stomacher supernatants obtained from the control plots were plated as well to determine the background of the indigenous rifampicin resistant bacteria.

Total DNA Extraction and Analysis of 16S rRNA and *gacA* Gene Fragments by DGGE Fingerprints

Total community DNA was extracted after a harsh lysis step by means of the FastDNA SPIN Kit for Soil® (MP Biomedicals, Heidelberg, Germany) from the same samples used for RU47 CFU determination (Schreiter et al., 2014a). PCR reactions were performed for amplification of 16S rRNA gene fragments with the bacterial primers F984-GC and R1378 using GoTaq® Flexi (Promega, Mannheim, Germany) (Nübel et al., 1996; Heuer et al., 1997). The PCR products were analyzed by DGGE as described by Weinert et al. (2009). For characterization of the effect of RU47 with (RU47+*Rs*) and without (RU47) *R. solani* inoculation on *Pseudomonas* community in the potato and lettuce rhizosphere, DGGE fingerprints of *gacA* gene amplicons were carried out according to Costa et al. (2007).

Data Analysis

All data obtained from the field were analyzed with the STATISTICA program (StatSoft Inc., Tulsa, OK, United States). The impact of the soil type, of the inoculant RU47 and of the pathogen *R. solani* on MTY of potato and SDM of lettuce was analyzed using three-way ANOVA combined with Tukey *post hoc* test ($P \leq 0.05$). Effects of RU47 and the pathogen on MTY and SDM were analyzed for each soil type by two-way ANOVA. The data of disease severity was analyzed using the non-parametric Kruskal–Wallis test followed by Mann–Whitney *U*-test ($P \leq 0.05$). The CFU per gram root dry mass (RDM) were calculated and logarithmically (Log_{10}) converted. The effects of the soil type, the growth period/plant age (sampling time), and the pathogen on the plate counts of RU47 were evaluated using three-way ANOVA ($P \leq 0.05$) combined with Tukey *post hoc* test ($P \leq 0.05$). DGGE fingerprints were evaluated with GELCOMPAR II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) as described by Schreiter et al. (2014a). The Pearson correlation coefficient as a curve-based method was chosen to obtain pairwise similarities between fingerprints and similarities were clustered using unweighted pair group method using average linkages (UPGMA). These were used for statistical analysis by Permutation tests ($P \leq 0.05$) where the *d*-value was calculated as average overall correlation coefficients within the groups minus the average overall correlation coefficients between samples from different groups as suggested by Kropf et al. (2004).

RESULTS

Plant Species Effect on Rhizosphere Competence of RU47 in Three Soils

Our results demonstrated that *Pseudomonas* sp. RU47 was able to colonize the rhizosphere of potato and lettuce at a density of more than 5 Log_{10} CFU per gram root dry

mass in all three soils at field scale at both sampling times. Three-way ANOVA revealed that neither the soil type (potato: $P \leq 0.19$; lettuce: $P \leq 0.08$) nor the presence of *R. solani* (AG3: $P \leq 0.10$; AG1-IB: $P \leq 0.44$) significantly affected the number of RU47 CFU counts in the rhizosphere of both crops. Two-way ANOVA showed for both crops significantly declined CFU counts of RU47 in the rhizosphere with increasing plant age in the treatments without (RU47) and with *R. solani* inoculation (RU47+*Rs*) in both loamy soils (alluvial loam: $P \leq 0.02$; loess loam: $P \leq 0.002$) but not in the diluvial soil ($P \geq 0.15$). Interestingly, no plant age-dependent decline in the density of RU47 was observed in the rhizosphere of both crops in the treatments with *R. solani* inoculation (RU47+*Rs*) in all soils, except for lettuce grown in alluvial soil (**Figure 1**).

Soil Type, *R. solani,* and of RU47 Effects on Yield of Potato and Lettuce

A significant effect of the soil type ($P \leq 0.0001$) and the pathogen *R. solani* AG3 ($P \leq 0.0001$) on MTY was found but no effect of RU47 ($P \geq 0.15$). Comparing the MTY of non-inoculated potato plants (control) in all soils, the lowest yield was recorded in diluvial sand (543.8 g plant^{-1}) which differed significantly from MTY in both loamy soils (alluvial loam, 841.3 g plant^{-1}; loess loam, 764.0 g plant^{-1}; $P \leq 0.008$) (**Table 1**). Two-way ANOVA indicated that the pathogen *R. solani* AG3 significantly reduced MTY of potato in both loamy soils ($P \leq 0.02$) but not in diluvial sand ($P \leq 0.18$) compared with control plants, respectively. In all soils, no effect by the inoculant RU47 on MTY of potato was observed in comparison to the non-inoculated control plants in absence (control vs. RU47) and in presence of *R. solani* (+*Rs* vs. RU47+*Rs*).

Three-way ANOVA indicated a significant effect of the soil type ($P \leq 0.0001$) and the pathogen ($P \leq 0.0001$) but also not of RU47 ($P \geq 0.07$) on lettuce SDM. In contrast to potato, the highest SDM of lettuce was recorded in diluvial sand (42.2 g plant^{-1}) which also differed significantly from SDM in both loamy soils (alluvial loam, 32.4 g plant^{-1}; loess loam, 30.6 g plant^{-1}; $P \leq 0.0003$). The SDM was significantly reduced by *R. solani* AG1-IB in all soils compared to the control plants (**Table 1**). The inoculant RU47 did not improve the SDM of lettuce in any of the soils in the treatments without *R. solani* inoculation (control vs. RU47; $P \geq 0.45$), but it significantly enhanced the lettuce growth in diluvial sand ($P \leq 0.01$) and alluvial loam ($P \leq 0.01$) in treatments with *R. solani* inoculation (+*Rs* vs. RU47+*Rs*).

Soil Type Effect on the RU47 Mediated Biocontrol Activity against *R. solani*

In all control treatments no, or only slight black scurf symptoms on potato tuber appeared at harvest. In contrast the inoculation of *R. solani* AG3 (+*Rs*) resulted in a significantly increased disease severity of black scurf on potato ($P \leq 0.05$) in all soils (**Table 1**). Comparing disease severity of black scurf symptoms on potato in the pathogen controls (+*Rs*) of all three soils the highest and significant ($P \leq 0.03$) disease severity was recorded on tubers harvested from diluvial sand. Comparable severity of black scurf ($P \leq 0.89$) was observed on tubers yielded from plants grown in alluvial loam and loess loam (**Table 1**). RU47 was able to significantly suppress ($P \leq 0.03$) the severity of black scurf on potato in all soils (+*Rs* vs. RU47+*Rs*).

Bottom rot symptoms on lettuce were revealed in the controls in each soil (**Table 1**). A significantly higher disease severity of bottom rot was revealed on plants in all soils after *R. solani* AG1-IB (+*Rs*) inoculation ($P \leq 0.05$), while no significant differences ($P \geq 0.06$) in disease severity were observed among the three soil types without (control) and with pathogen inoculation (+*Rs*; **Table 1**). The inoculant RU47 suppressed significantly ($P \leq 0.03$) the severity of bottom rot on lettuce in diluvial sand and loess loam (+*Rs* vs. RU47+*Rs*). The disease severity of bottom root

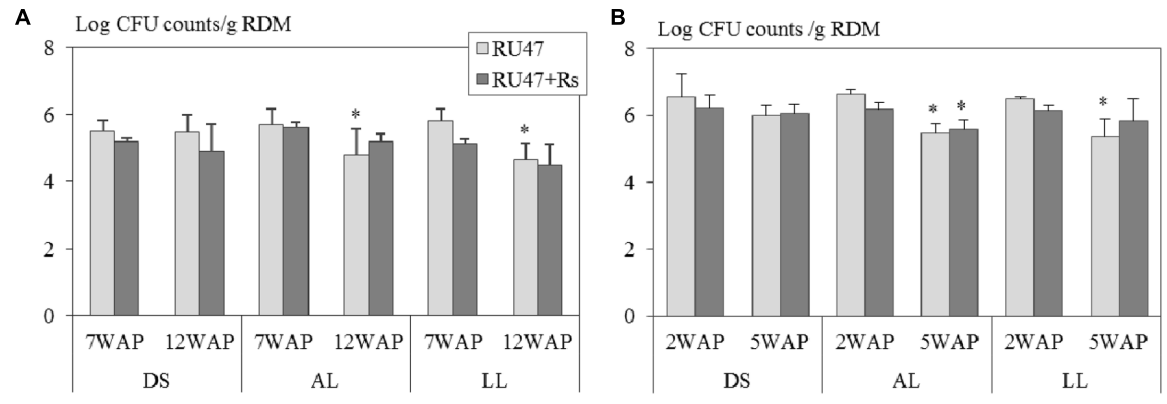


FIGURE 1 | Number of colony forming units (CFU) per gram root dry mass (RDM) of *Pseudomonas* sp. RU47 estimated in the rhizosphere of **(A)** potato (cv. Arkula) and **(B)** lettuce (cv. Tizian) with RU47 and with RU47 + *Rhizoctonia solani* inoculation (RU47+*Rs*) 7 and 12 weeks after planting (7 WAP, 12 WAP) of potato and 2 and 5 weeks after planting (2 WAP, 5 WAP) of lettuce in the 2012 season. Plants were grown in three soils [diluvial sand (DS), alluvial loam (AL), loess loam (LL)] at the same field site. No significant effect of the soil type and the presence of *R. solani* on the number of CFU of RU47 was revealed (ANOVA, $P \geq 0.05$). Asterisks indicate significant differences in CFU counts between the two sampling times within each soil type (Tukey *post hoc* test, $P \leq 0.05$). The bars represent the standard deviation.

TABLE 1 | Marketable tuber yield (MTY) of potato (Po, cv. Arkula) and shoot dry mass (SDM) of lettuce (Le, cv. Tizian) and disease severity of black scurf on potato and bottom rot on lettuce without (control) and with *Pseudomonas* sp. RU47 (RU47), and without and with *Rhizoctonia solani* inoculation (+*Rs*; RU47+*Rs*) in the 2012 season.

		SDM or MTY [g/plant]				Disease severity			
	Soil	**Control**	**RU47**	**+*Rs***	**RU47+*Rs***	**Control**	**RU47**	**+*Rs***	**RU47+*Rs***
Po	DS	543.8 aA	582.3 a	499.9 aA	516.8 a	1.1 aA	1.2 a	3.4 aA*	2.2 b
	AL	841.3 aB	737.4 a	705.6 aB*	643.2 a	1.1 aA	1.0 a	2.8 aB*	1.7 b
	LL	764.0 aB	711.3 a	606.1 aAB*	566.6 a	1.2 aA	1.1 a	2.8 aB*	1.8 b
Le	DS	42.2 aA	39.8 a	31.9 aA*	38.1 b	2.3 aA	2.4 a	2.8 aA*	2.5 b
	AL	32.4 aB	32.0 a	25.5 aB*	31.4 b	2.4 aA	2.2 a	3.0 aA*	2.5 a
	LL	30.6 aB	30.9 a	25.3 aB*	28.7 a	2.1 aA	2.1 a	2.6 aA*	2.3 b

Both crops were grown in three soils [diluvial sand (DS), alluvial loam (AL), loess loam (LL)] at the same field site. Asterisks denote significant differences in disease severity, SDM or MTY between control and pathogen control (+Rs), and different capital letters indicate significant differences comparing controls (control, +Rs; per column) of each soil type. Different small letters denote significant differences between treatments with RU47 and corresponding controls (control or +Rs) in each soil type (per row).

on plants grown in alluvial soil was reduced through RU47 application but not significantly ($P \leq 0.1$).

R. solani and RU47 Effects on the Rhizosphere Bacterial Community Composition

Effects of the plant species in three soil types, the pathogens *R. solani* AG3 and AG1-1B, and inoculant RU47 on the bacterial communities were detected based on 16S rRNA gene DGGE fingerprints for samples taken 7 or 2 WAP of potato or lettuce plants, respectively.

DGGE fingerprints revealed distinct rhizosphere bacterial communities depending on the plant species (Supplementary Figures S2–S4). Significant effects of *R. solani* AG3 on rhizosphere bacterial communities of potato plants were observed with high *d*-values (**Table 2**), while the effects of AG1-1B on the bacterial community composition of the lettuce rhizosphere were negligible, indicated by low and not significant *d*-values (**Table 2**). The highest impact of both pathogens (AG3 and AG1-IB) on the rhizosphere bacterial community of potato and lettuce plants was revealed in alluvial loam (**Table 2**), indicating a soil type effect on the interaction of pathogen and indigenous bacterial community in the rhizosphere.

The inoculant RU47 showed plant species dependent effects on the rhizosphere bacterial community composition as well. Based on the DGGE fingerprints and the *d*-values obtained by the comparison of the control and the treatment with RU47 fingerprints, the effects of RU47 were on average lower and not significant in the rhizosphere of potato in contrast to the rhizosphere of lettuce (**Table 2**).

An effect on the lettuce rhizosphere bacterial community composition was observed in the treatment with RU47 in presence of the pathogen *R. solani* for both crops. The highest effect was found consistently in alluvial loam albeit not significant for potato (**Table 2**).

RU47 and *R. solani* Effects on *Pseudomonas* Community in the Rhizosphere

The *Pseudomonas* community of the rhizosphere of both plant species was analyzed by DGGE of *gacA* genes for samples taken 7 or 2 WAP, respectively. A significant effect of the soil type was observed for lettuce in contrast to the potato rhizosphere which exhibited higher heterogeneity between replicates (control treatments in Supplementary Figures S5, S6). Inoculation with RU47 resulted only in small shifts of *Pseudomonas* community that were significant only for lettuce in alluvial loam soil

TABLE 2 | *D*-values obtained by bacterial DGGE analysis of rhizosphere samples from potato (Po, cv. Arkula) and lettuce (Le, cv. Tizian) grown in three soils [diluvial sand (DS), alluvial loam (AL), loess loam (LL)] at the same field site.

		Effect of *Rs*			Effect of RU47			Effect of RU47+*Rs*		
	Soil	***d*-value**	***p*-value**	**Figure**	***d*-value**	***p*-value**	**Figure**	***d*-value**	***p*-value**	**Figure**
Po	DS	16.91*	0.03	S2	6.23	0.09	S2	6.45*	0.03	S2
	AL	22.08*	0.03	S3	5.25	0.22	S3	16.7	0.06	S3
	LL	17.72*	0.03	S4	0.75	0.28	S4	3.69	0.17	S4
Le	DS	0.73	0.23	S2	5.88*	0.03	S2	7.83*	0.03	S2
	AL	3.75	0.08	S3	19.7*	0.03	S3	10.41*	0.03	S3
	LL	3.02	0.06	S4	7.68*	0.03	S4	4.38*	0.03	S4

D-values (in %) show the differences in the bacterial community composition between the control plots and plots treated with pathogen Rhizoctonia solani (Rs), biocontrol strain RU47, or RU47 and R. solani (RU47 + Rs). An asterisk indicates significant differences acquired by Permutation test as suggested by Kropf et al. (2004). A higher d-value indicates a more pronounced influence of the pathogen Rs, the inoculant strain RU47 or both RU47 + R. solani (RU47 + Rs).

(**Table 3**). The additional presence of *R. solani* AG3 (potato) or AG1-IB (lettuce) significantly changed the *Pseudomonas* community of potato grown in DS and LL and for lettuce grown in DS soil.

DISCUSSION

In this study, we compared the ability of RU47 to colonize the rhizosphere and to display biocontrol effects against Rhizoctonia diseases for two plant species grown in three different soil types. Hereby we confirm results of previous years obtained with lettuce in the same long term field trial and broaden our knowledge with a systematic, morphological different model plant (Schreiter et al., 2014a,b,c). Both, potato and lettuce are host plants of *R. solani* but the interaction of the model plants vary with the different anastomosis groups (AGs) of *R. solani*. The model plants were grown in the same three soil types at the same site. However, they were planted in two units of the experimental plot system which exhibited only a slight variation in cropping history. In contrast to other field studies the experimental set-up excluded site effects caused by different weather conditions and management practice on bacterial communities in the bulk soil. Potato plants have a much longer growth period than lettuce, and thus the analysis of bacterial community was performed 2 and 7 WAP (3 and 8 weeks after last application of RU47). We assumed that the bacterial community structure in the rhizosphere differed depending on the plant species in each soil and pyrosequencing (Schreiter et al., unpublished) and DGGE analysis underlined this assumption (Supplementary Figures S2–S4).

Contrasting effects of the soil types on MTY of potato and the SDM of lettuce in the untreated controls were assessed at harvest. The soil characteristics play an important role in the ability of a plant species to extract water and nutrients. Although the soil types were adjusted to the same amount of nitrogen it cannot be excluded that soil nitrogen, i.e., nitrate, leaches by irrigation water to a deeper, non-rooted soil level, especially in the diluvial sand. Availability of nitrogen was possibly limited in potato crop in diluvial sand within the growth period of 12 weeks compared to the loamy soils.

A negative effect of the pathogen *R. solani* AG1-IB on lettuce growth and of *R. solani* AG3 on the MTY of potato was observed in all soils. These results confirmed the observation for lettuce made in previous field experiments (Grosch et al., 2005; Schreiter et al., 2014c). The disease impact of the bottom rot pathogen was not revealed in the present study conducted in 2012 when plants were treated with RU47 confirming the biocontrol effects of RU47 observed in 2011 (Schreiter et al., 2014c). Our results are in accordance with findings for the inoculant strains *Bacillus amyloliquefaciens* FZB42 and *Serratia plymuthica* 3Re4-18 for lettuce (Grosch et al., 2005; Chowdhury et al., 2013). However, we found that the inoculant RU47 was not able to compensate the negative effect of the pathogen on MTY of potato. Compared to *R. solani* AG1-IB on lettuce which does not colonize the roots, *R. solani* AG3 was previously reported to affect all belowground parts of potato including the roots during the growth period (Atkinson et al., 2011; Genzel et al., 2018). Thus, it was not surprising that *R. solani* AG3 had a more pronounced effect on the composition of the bacterial community in the rhizosphere of potato as indicated by the higher *d*-values than *R. solani* AG1-IB on the rhizosphere bacterial community in lettuce. Windisch et al. (2017) showed that both the pathogen and the biocontrol strain induced changes in the root exudation pattern of lettuce, including higher amounts of antimicrobial compounds. An impact of pathogens such as *Verticillium* sp. and *Pythium* sp. on root exudation patterns of cotton and basil plants was reported by Bais et al. (2006) and Zhang et al. (2011). It is assumed that *R. solani* AG3 may also cause changes in root exudation patterns of potato and thus affect the interaction of RU47 with the plant. Although RU47 was found in a sufficient density in the rhizosphere of potato and was able to reduce severity of black scurf symptoms in all soils the potato yield losses were not compensated.

Our results revealed that despite the plant species dependent rhizosphere bacterial community composition observed (Supplementary Figures S2–S4), the inoculant RU47 was able to successfully colonize the rhizosphere of potato and lettuce. The rhizosphere competence of RU47 in potato and lettuce was not influenced by the soil type, as already revealed in a previous experiment (Schreiter et al., 2014c). Higher CFU counts of RU47 were found in the rhizosphere of lettuce in comparison to the potato rhizosphere. The finding might be due to the differences in sampling time after RU47 application (3 and 8 weeks after the last application for potato; 2 and 5 weeks after the last application for lettuce). Furthermore, the CFU counts were calculated per gram of root dry mass and not per cm^2 root surface. In contrast to potato, the fine root system of lettuce, which is likely to provide a higher root surface than the potato root system, might be a reason for the observed higher CFU counts in lettuce. A decline in the population density of RU47 within the growth period was

TABLE 3 | *D*-values obtained by *Pseudomonas*-specific DGGE analysis of rhizosphere samples from potato (Po, cv. Arkula) and lettuce (Le, cv. Tizian) grown in three soils [diluvial sand (DS), alluvial loam (AL), loess loam (LL)] at the same field site.

		Effect of RU47			Effect of RU47+*Rs*		
	Soil	***d*-value**	***p*-value**	**Figure**	***d*-value**	***p*-value**	**Figure**
Po	DS	6.6	0.11	S6	7.5*	0.04	S6
	AL	4.3	0.16	S6	5.3	0.16	S6
	LL	3.4	0.22	S6	8.3*	0.03	S6
Le	DS	5.2	0.12	S5	11*	0.03	S5
	AL	4.6*	0.03	S5	6.4	0.06	S5
	LL	2.1	0.09	S5	0	0.62	S5

D-values (in %) show the differences in the Pseudomonas community composition between the control plots and plots treated with biocontrol strain RU47 with or without inoculation of Rhizoctonia solani (RU47; RU47+Rs). An asterisk indicates significant differences acquired by Permutation test as suggested by Kropf et al. (2004). A higher d-value indicates a more pronounced influence of the pathogen R. solani or the inoculant strain RU47.

found for both crops and confirmed previous observations of RU47 and the inoculant strains 3Re4-18, and FZB42 (Chowdhury et al., 2013; Schreiter et al., 2014c). It is assumed that the decrease in CFU counts is likely linked to changes in root morphology as the number of fine roots decreased with the growing plant. In both crops no impact of *R. solani* AG3 or AG1-IB on the rhizosphere competence of RU47 was revealed. These results were consistent with the study of Chowdhury et al. (2013) where the rhizosphere competence of FZB42 was also not affected by the bottom rot pathogen *R. solani* AG1-IB in lettuce. Our results underline that the ability of the inoculant RU47 to colonize the rhizosphere of potato and lettuce plants in a sufficient density correlated with effective biocontrol activity in both crops and in all three soils.

Black scurf symptoms were observed only on potato tubers originating from treatments inoculated with *R. solani* AG3. In contrast, the bottom rot symptoms on lettuce were also detected in all control treatments and confirmed previous reports on the occurrence of *R. solani* AG1-IB in arable soils (Grosch et al., 2011). In accordance with the results of the 2011 conducted field experiment (Schreiter et al., 2014c), the lowest disease severity of bottom rot on lettuce (control, +*Rs*) was assessed in loess loam (+*Rs*). But the differences in disease severity between the soil types were not significant in the treatment with *R. solani* AG1-IB as determined previously. An impact of the soil type on bean hypocotyl rot severity caused by *R. solani* AG4 was also revealed by Nerey et al. (2010). In addition, Tamm et al. (2010) showed that the soil type is a major determinant for suppressiveness against soil-borne diseases. Several studies suggested that physicochemical properties of different soils have a strong effect on microbial community structure and function (reviewed in Berg and Smalla, 2009). In accordance, we found distinct rhizosphere bacterial communities in the different soil types with the best soil disease suppression in loess loam (Schreiter et al., 2014c). The disease severity of bottom rot in the growing period 2011 was much higher in all soils compared to the growing period 2012. Pathogen inoculum density affects the disease severity and additional inoculation increased the density in all soils. Moreover, results of previous experiments over different growing periods showed that also weather conditions have a pronounced impact on disease development (Grosch et al., 2011). In the growing period 2011 the conditions were more favorable for disease development of bottom rot compared to 2012.

Although DGGE fingerprints do not provide taxonomic information on community composition and responders to the treatments, they provide rapid insights into dynamic changes and the differences between treatments. Recently, we could show that the pyrosequencing data for lettuce rhizosphere and bulk soil from the experimental plot units confirmed the DGGE findings (Schreiter et al., 2014a,b).

The effect of RU47 on rhizosphere bacterial communities differed depending on plant species and soil type as revealed by bacterial and *Pseudomonas*-specific DGGE fingerprints. A significant effect of RU47 was found in all three soils in lettuce in the treatments without and with additional pathogen inoculation. This is in accordance to previous findings on lettuce where the impact of RU47 was assessed over three consecutive years. In the previous study an increasing effect of RU47 on rhizosphere bacterial communities could be observed over the years 2010 until 2012. Nevertheless, the influence of RU47 was negligible compared to community changes caused by the soil type (Schreiter et al., 2014b). The comparison of this and the previous study conducted in 2011 confirmed the results obtained for lettuce which therefore shows the importance of repeating field experiments for validation and reliable assessment of biocontrol agents. Furthermore, also a comparison of the impact of a biocontrol agent between economically important model plants is needed. Therefore, we used TC-DNA extracted from the same lettuce root samples for 2012 as used in Schreiter et al. (2014b), performed a separated amplification, DGGE run and analysis to compare to potato. In contrast to lettuce, on average a minor influence of RU47 on the total bacterial as well as *Pseudomonas* communities was revealed in the potato rhizosphere and might be explained by differences in the response of the plant-specific microbial community to RU47 inoculation. Recent results of Schreiter et al. (2014b) on lettuce in the 2011 field experiment indicated that different taxonomic groups responded to RU47 application depending on the soil type. An increased relative abundance of bacteria belonging to the genera *Bacillus* and *Paenibacillus* was observed in the rhizosphere of lettuce grown in alluvial loam. Thus, mutualistic effects of the inoculant with distinct enriched populations or indirect effects involving other bacterial rhizosphere populations cannot be excluded. Numerous strains of these genera were previously reported to display antagonistic activity and may support the observed biocontrol effect of RU47 (Kröber et al., 2014). The observed effect on rhizosphere bacterial communities in potato of the treatment RU47 in presence of the pathogen (RU47+*Rs*) seems to be caused by *R. solani* AG3. The strong effect of the pathogen on bacterial and *Pseudomonas* communities could not be compensated by the inoculant RU47.

CONCLUSION

The results underline that the bacterial community structure in the rhizosphere differed depending on the plant species in all soils analyzed. Despite the influence of the plant, the soil type, and the pathogen, the inoculant RU47 was capable of colonizing the rhizosphere of both crops in a density sufficient manner to reduce black scurf disease severity on potato and bottom rot on lettuce in all three soils. In both crops the rhizosphere competence of RU47 was neither affected by the soil type nor by the presence of the respective pathogen *R. solani*. In contrast to lettuce, the colonization of the belowground part of potato including the roots by the pathogen *R. solani* AG3 has had a pronounced effect on the bacterial community composition in the potato rhizosphere. Interestingly, the pathogen *R. solani*

affected the *Pseudomonas* community in both crops whereas the inoculant RU47 influenced especially the bacterial as well as the *Pseudomonas* community in the rhizosphere of lettuce. The plant species and soil type independent rhizosphere competence of RU47 and biocontrol of Rhizoctonia diseases observed under field conditions underlines the biocontrol potential of RU47.

AUTHOR CONTRIBUTIONS

KS and RG preparing the project proposal for third-party funds. KS, RG, and SS project planning, design and performance of experiments. SS and RG determination of rhizosphere competence and plant characteristics, disease assessment, analysis of these data. SS and DB molecular work and analysis of microbial community composition in the rhizosphere. SS, RG, KS, and DB preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00097/full#supplementary-material

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Relationships between Root Pathogen Resistance, Abundance and Expression of *Pseudomonas* Antimicrobial Genes, and Soil Properties in Representative Swiss Agricultural Soils

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Strains of *Pseudomonas* that produce antimicrobial metabolites and control soilborne plant diseases have often been isolated from soils defined as disease-suppressive, i.e., soils, in which specific plant pathogens are present, but plants show no or reduced disease symptoms. Moreover, it is assumed that pseudomonads producing antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG) or phenazines (PHZ) contribute to the specific disease resistance of suppressive soils. However, pseudomonads producing antimicrobial metabolites are also present in soils that are conducive to disease. Currently, it is still unknown whether and to which extent the abundance of antimicrobials-producing pseudomonads is related to the general disease resistance of common agricultural soils. Moreover, virtually nothing is known about the conditions under which pseudomonads express antimicrobial genes in agricultural field soils. We present here results of the first side-by-side comparison of 10 representative Swiss agricultural soils with a cereal-oriented cropping history for (i) the resistance against two soilborne pathogens, (ii) the abundance of *Pseudomonas* bacteria harboring genes involved in the biosynthesis of the antimicrobials DAPG, PHZ, and pyrrolnitrin on roots of wheat, and (iii) the ability to support the expression of these genes on the roots. Our study revealed that the level of soil disease resistance strongly depends on the type of pathogen, e.g., soils that are highly resistant to *Gaeumannomyces tritici* often are highly susceptible to *Pythium ultimum* and vice versa. There was no significant correlation between the disease resistance of the soils, the abundance of *Pseudomonas* bacteria carrying DAPG, PHZ, and pyrrolnitrin biosynthetic genes, and the ability of the soils to support the expression of the antimicrobial genes. Correlation analyses indicated that certain soil factors such as silt, clay, and some macro- and micronutrients influence both the abundance and the expression of the antimicrobial genes. Taken together, the

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results of this study suggests that pseudomonads producing DAPG, PHZ, or pyrrolnitrin are present and abundant in Swiss agricultural soils and that the soils support the expression of the respective biosynthetic genes in these bacteria to various degrees. The precise role that these pseudomonads play in the general disease resistance of the investigated agricultural soils remains elusive.

Keywords: *Pseudomonas*, PGPR, plant-beneficial activity, antimicrobial metabolites, *Pythium ultimum*, *Gaeumannomyces tritici*, soil, disease suppressiveness

INTRODUCTION

The ability of soilborne plant pathogens to attack and damage host plants is influenced by biotic and abiotic soil factors (Weller et al., 2002; Haas and Défago, 2005; Lemanceau et al., 2006; Almario et al., 2014). In some soils, even susceptible crop plants suffer only a little or not at all from specific diseases although soilborne pathogens are present (Weller et al., 2002). In general, two different types of natural pathogen suppression are thought to occur in agricultural soils. First, the general disease suppression, where different soilborne pathogens are controlled to a certain degree depending on the total microbial activity in the soil and/or on abiotic soil factors (Weller et al., 2002; Lemanceau et al., 2006). Second, the specific disease suppression, where the soil restricts the activity of a distinct species of plant pathogen based on its interactions with a specific group of microorganisms (Weller et al., 2002; Haas and Défago, 2005; Lemanceau et al., 2006; Berendsen et al., 2012; Raaijmakers and Mazzola, 2016).

Soils with specific disease suppression have been described worldwide (Cook and Rovira, 1976; Stutz et al., 1986; Weller et al., 2002; Lemanceau et al., 2006; Chng et al., 2015) and for diverse soilborne plant pathogens. They include soils suppressive to *Gaeumannomyces graminis var. tritici* (recently renamed *G. tritici* (Hernández-Restrepo et al., 2016)) causing take-all of wheat (Weller et al., 2002), *Thielaviopsis basicola* causing black root rot of tobacco (Stutz et al., 1986; Almario et al., 2014), *Fusarium oxysporum* causing wilt on tomatoes (Alabouvette, 1986; Tamietti et al., 1993), *Pythium* spp. causing seedling damping-off (Martin and Hancock, 1986), and *Rhizoctonia solani* causing damping-off and root rot on various crop species (Mendes et al., 2011). Such soils are commonly referred-to as *suppressive soils*. By contrast, *conducive soils* do not restrict the development of soilborne diseases (Haas and Défago, 2005).

Suppressive soils have been found to host distinct microbial communities that are thought to be responsible for the natural disease control effect (Weller et al., 2002; Haas and Défago, 2005; Mendes et al., 2011, 2013; Klein et al., 2013; Kyselkova et al., 2014; Cha et al., 2016). In particular, bacteria of the *Pseudomonas fluorescens* group have been isolated from suppressive soils and used as plant or soil inoculants. Several strains proved to be very efficient at colonizing roots, protecting plants from different diseases, and increasing plant productivity (Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009; Höfte and Altier, 2010). Thus, it has been suggested that such pseudomonads contribute to soil suppressiveness (Weller et al., 2002; Haas and Défago, 2005; Garbeva et al., 2006; Lemanceau et al., 2006; Weller, 2007; Mazurier et al., 2009). The capacity of many root-associated pseudomonads to release antimicrobial compounds placed them in the focus of research on the nature of soil disease suppressiveness. Many *P. fluorescens* group strains produce an array of potent antimicrobials, among which 2,4-diacetylphloroglucinol (DAPG), phenazines (PHZ), pyrrolnitrin (PRN), and hydrogen cyanide (HCN) are most prominent (Blumer and Haas, 2000; Haas and Défago, 2005; Raaijmakers and Mazzola, 2012; Mavrodi et al., 2013). All these antimicrobials were shown, mostly in pot and gnotobiotic assays, to play indeed an important role in the *Pseudomonas*-mediated protection of plants from soilborne pathogenic fungi and oomycetes (Thomashow and Weller, 1988; Voisard et al., 1989; Keel et al., 1992; Maurhofer et al., 1992; Pierson and Thomashow, 1992; Hwang et al., 2002; Chin-A-Woeng et al., 2003; Weller et al., 2007; Mavrodi et al., 2013).

The role of these antimicrobial compounds in disease-suppressive soils is still not fully understood. They are indeed produced in some field soils, as demonstrated for DAPG in the Quincy take-all decline soil (Raaijmakers et al., 1999) and for PHZ in wheat fields of the Columbia Plateau, USA (Mavrodi D.V. et al., 2012). Several studies performed during the last 15 years aimed at investigating whether disease-suppressive soils are specifically enriched for *Pseudomonas* genotypes producing antimicrobials compared to conducive soils. In fact, in the Pacific Northwest of the USA, DAPG-producing pseudomonads were found to be more abundant in take-all suppressive soils than in adjacent conducive soils (Raaijmakers et al., 1997). However, DAPG producers were not more abundant in *Fusarium* suppressive soils of Châteaurenard (France) than in adjacent conducive soils, in contrast to PHZ-producing pseudomonads, which were more abundant in the suppressive soils (Mazurier et al., 2009). In some studies, the total abundance of DAPG-producing pseudomonads was found to be similar in suppressive and conducive soils (Ramette et al., 2006; Almario et al., 2013a), but suppressive soils harbored distinct genotypes of DAPG producers (Frapolli et al., 2010). Moreover, abundances of plant-beneficial pseudomonads with antimicrobial activity were mostly investigated in specific disease-suppressive soils, and very little is known about the occurrence of these bacteria in common agricultural soils and on how soil factors might impact these bacteria. In wheat fields of the Pacific Northwest of the USA, it was found that the abundance of DAPG- and PHZ-producing pseudomonads on wheat roots is influenced by irrigation (Mavrodi O.V. et al., 2012).

Based on such observations, the abundance and genotypic diversity of antimicrobials-producing *Pseudomonas* bacteria in

soil seems not a sufficient argument to explain the disease suppressiveness of some soils. It has been suggested that (i) other bacterial species contribute importantly to the disease suppressiveness (Mendes et al., 2011; Kyselkova et al., 2014), and (ii) somehow the expression of antimicrobial genes in *Pseudomonas* bacteria is favored in suppressive soils and hampered in conducive soils (Ramette et al., 2006; Almario et al., 2014). Indeed, studies on the abundance of antimicrobial metabolite-producing pseudomonads do not consider the complex interactions in the rhizosphere that ultimately modulate the production of the antimicrobials in the rhizosphere (Rochat et al., 2010; de Werra et al., 2011). To date, little is known about the biotic and abiotic factors affecting the expression of biosynthetic genes for these metabolites in soil. Studies conducted under gnotobiotic conditions indicate that the expression of DAPG, HCN, and PRN biosynthesis genes is influenced by the crop species and variety (Rochat et al., 2010; de Werra et al., 2011; Latz et al., 2015) and for DAPG also by the interaction with other microorganisms and the iron availability in the rhizosphere (Notz et al., 2001, 2002; Maurhofer et al., 2004; Jousset et al., 2010, 2011; Almario et al., 2013b). How biotic and abiotic soil factors affect antimicrobial gene expression under natural conditions in agricultural soils remains, however, unexplored.

There is a clear lack of studies investigating the link between natural disease resistance and abundance and expression of antimicrobial *Pseudomonas* genes in common agricultural soils. To address this gap, in the present study 10 representative Swiss agricultural soils with a cereal-oriented cropping history and differing in their physical and chemical characteristics were compared for their resistance to two soilborne pathogens of wheat, i.e., *G. tritici* (Gt) and *Pythium ultimum* (Pu). In parallel, the 10 soils were planted with wheat and pseudomonads harboring the biosynthetic genes required for the production of the antimicrobial compounds DAPG, PHZ, and PRN were quantified on roots using qPCR. In addition, the expression of these genes and the HCN biosynthetic genes was monitored by flow cytometry using fluorescent reporter strains of the representative model pseudomonads *P. protegens* CHA0 and *P. chlororaphis* PCL1391. To our best knowledge, this is the first side-by-side comparison using root-associated pseudomonads as bio-indicators to explore relationships between abundance and expression of antimicrobial genes, soil disease resistance and soil physicochemical characteristics in a range of common agricultural soils.

MATERIALS AND METHODS

Sampling and Physicochemical Analysis of Field Soils

Soil samples were collected in 10 farmer's fields across Switzerland (**Figure 1**) in May 2013. The main characteristics of the 10 field soils are listed in **Table 1**. Field sites had a history of multi-year cereal-oriented crop rotation and were chosen to represent predominant Swiss agricultural soil types and climate conditions. All fields were cropped with winter wheat in the year of sampling. For sampling, soil cores of 15–20 cm depth were extracted with disinfected soil recovery augers between the rows of wheat plants at 20 random locations in each field in order to obtain a representative sample. Then, extracted soil samples were sieved (mesh size, 10 by 10 mm) in order to remove stones, plant residues or other larger material, pooled and thoroughly mixed. For each site, approximately 120 kg of sieved soil were collected and stored in barrels for 3 months at 15°C before the start of the experiments in order to equilibrate the soils, i.e., to minimize effects of different environmental conditions (e.g., temperature, soil moisture) prevailing at the different sampling sites at the time of sampling. The storage temperature was chosen because it can be considered as the average temperature in Switzerland during the growing season of wheat from April to September (according to long-term monthly temperature averages recorded by the Swiss Federal Office MeteoSwiss[1]). Soil parameter analyses were carried out by the Labor für Boden- und Umweltanalytik (Eric Schweizer AG, Steffisburg, Switzerland) following standard protocols used in Swiss agriculture (Agroscope, 2006). Concentrations of soluble, readily plant-available macronutrients were determined following H_2O extraction, for which soil samples were suspended in distilled water at a ratio of 1:10 (g mL^{-1}). Reserve macronutrients and micronutrients were extracted with ammonium acetate EDTA, for which soil samples dried at 65°C were suspended at a ratio of 1:10 (g mL^{-1}) in a solution consisting of acetic acid (0.5 mol L^{-1}), ammonium acetate and EDTA (0.02 mol L^{-1}) adjusted to a pH of 4.65. Soil suspensions were then vigorously shaken for 1 h and filtered prior to analysis by mass spectrometry. The soil parameter analyses were carried out (i) directly after sampling and (ii) after the end of the experiments. Except for nitrate, the soil parameters did not change significantly between the two analyses (**Table 1**).

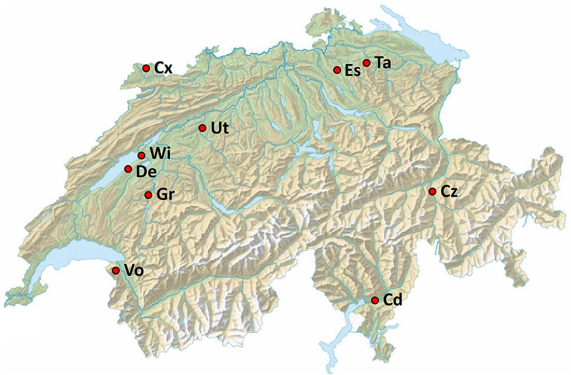


FIGURE 1 | Location of the 10 field sites in Switzerland used for soil sampling. Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil. Empty topographical map of Switzerland courtesy of Federal Office of Topography swisstopo.

[1]http://www.meteoswiss.admin.ch/home/climate/past/homogenous-monthly-data.html

TABLE 1 | Characteristics of agricultural soils sampled at different Swiss farmers' fields cropped with wheat[1].

	Cadenazzo (Cd)	Courtedoux (Cx)	Cazis (Cz)	Delley (De)	Eschikon (Es)	Grangeneuve (Gr)	Tänikon (Ta)	Utzenstorf (Ut)	Vouvry (Vo)	Witzwil (Wi)
Coordinates[2]										
SN	46.160862	47.405785	46.75664	46.918324	47.447086	46.774855	47.482525	47.11437	46.330365	46.983912
EW	8.934138	7.027313	89.423094	6.964087	8.687439	7.112180	8.910636	7.565597	6.904972	7.071365
Basic soil properties										
Organic matter (%)	1.7	2.6	2.2	1.7	2.4	1.7	3.5	4.7	1.5	11.6
Clay (%)	8.4	25.7	7.8	16.8	24.1	19.2	27.0	20.4	7.4	31.0
Silt (%)	42.9	61.4	32.9	26.7	27.9	16.0	24.0	32.7	33.1	30.5
Sand (%)	47.8	10.5	58.4	55.7	46.7	64.3	47.1	44.3	59.0	30.5
pH	6.0	6.7	7.7	7.3	7.0	6.6	6.9	7.0	7.1	7.3
Soluble macronutrients (H_2O, 1:10)[3]										
Nitrate (analysis 1)[4] (mg kg^{-1})	8.2	12.6	26.7	23.1	34.6	29.4	34.3	30.3	10.0	52.0
Nitrate (analysis 2)[4] (mg kg^{-1})	80.9	10.1	153.9	21.8	192.8	9.1	148.0	80.1	77.7	91.9
P (mg kg^{-1})	4.3	2.2	1.9	6.0	1.5	2.5	3.6	7.0	4.2	1.1
K (mg kg^{-1})	3.7	18.3	22.2	30.7	22.3	14.8	43.4	56.8	14.9	35.3
Ca (mg kg^{-1})	36.4	164.8	155.7	144.6	117.4	76.9	118.1	135.6	103.5	332.2
Mg (mg kg^{-1})	5.2	8.2	14.7	10.8	14.0	11.5	21.8	15.9	7.3	14.2
Reserve macronutrients (NH_4-Ac. + EDTA, 1:10)[3]										
P (mg kg^{-1})	41.5	45.9	77.9	211.5	29.5	38.4	206.6	291.7	137.3	33.7
K (mg kg^{-1})	46.6	182.2	50.5	140.8	110.9	98.9	261.1	240.9	50.5	81.6
Ca (mg kg^{-1})	870.1	4608.0	21880.0	3386.0	2611.0	1746.0	4122.0	3927.0	27170.0	69060.0
Mg (mg kg^{-1})	79.7	98.5	232.6	93.9	141.3	116.6	392.5	191.8	256.1	537.3
Micronutrients (NH_4-Ac. + EDTA, 1:10)[3]										
Fe (mg kg^{-1})	248.2	336.5	552.1	836	381.4	372.8	1385.0	1275.0	785.0	1500.0
Cu (mg kg^{-1})	8.0	5.7	34.8	11.3	8.4	6.6	15.4	16.2	23.6	17.7
B (mg kg^{-1})	0.0	0.3	0.4	0.8	0.1	0.0	0.6	0.8	1.0	0.9
Mn (mg kg^{-1})	148.8	790.8	754.9	528.3	686.7	601.2	911.1	682.4	184.9	207.5
Zn (mg kg^{-1})	1.9	6.1	9.25	4.51	7.0	3.3	11.6	14.3	4.3	14.7

[1]*Soils from farmers' fields were sampled in May 2013. All fields were planted with winter wheat at the time of sampling. Soil parameters were analyzed by the Labor für Boden- und Umweltanalytik, Eric Schweizer AG, Steffisburg, Switzerland after sampling in 2013.*

[2]*Geographical coordinates of each field site were measured by GPS. The field site localization was described by the south–north orientation (SN) and the east–west (EW) orientation codes.*

[3]*Souble macronutrients were extracted with water. Reserve macronutrients and micronutrients were extracted with ammonium acetate EDTA. See section "Materials and Methods" for details.*

[4]*Soil parameters were analyzed twice, once immediately after sampling and once at the end of the experiments, i.e., after prolonged storage. Only nitrate levels (shown in the table) were significantly different between the first and the second analysis.*

Assessment of Field Soil Resistance against Root Pathogens

In order to test the natural resistance of the 10 Swiss agricultural soils toward soilborne pathogens, pot experiments were carried out in the greenhouse with cucumber and Pu and wheat and Gt. The Pu inoculum was prepared by inoculating 25 g of autoclaved millet seeds moistened with 10 mL of sterile water with three plugs of a culture of Pu strain ETH-2 (isolated from a Swiss agricultural soil) grown on Oxoid malt agar (Thermo Fisher Scientific, Reinach, Switzerland) for 7 days. Pu millet seed cultures were grown for 7 days at 18°C and then cut into small fragments for soil inoculation. The Gt inoculum was prepared by inoculating 100 g of autoclaved oat seeds without spelts and moistened with 100 mL of sterile, distilled water with 20 plugs of cultures of Gt strain I-17 (Lebreton et al., 2007) grown on Oxoid potato dextrose agar for 14 days. Gt oat cultures were grown for 4 weeks at 24°C in the dark and then dried in a sterile cabinet on sterile filter paper for 3 days. Seeds of cucumber (*Cucumis sativus* cv. Chinese Snake) and spring wheat (*Triticum aestivum* cv. Rubli) were surface-sterilized for 30 min in 1.5% (v/v) NaOCl, rinsed with sterile distilled water and pre-germinated on sterile moist filter paper for 2 days in the dark at 24°C. For the plant experiments, 250-mL plastic pots of were filled with a 4:1 mixture (wt/wt) of the respective field soil and quartz sand (grain size of 0.5–2.2 mm diameter). Pathogen inoculum at different concentrations was thoroughly mixed into soil. Pu inoculum was added at 0.125, 0.25, 0.5, or 1.0 g inoculum per pot, whereas Gt inoculum was added at 0.2, 0.6, 2.0, or 6.0 g per pot. Pots in control treatments contained field soil without pathogen addition. Three seedlings of cucumber or wheat, respectively, were then planted per pot. For each treatment, six replicate pots were prepared. Plants were grown in the greenhouse at 70% relative humidity with light (210 μmol m^{-2} sec^{-1}) for 16 h at 22°C (cucumber) or 18°C (wheat), followed by an 8-h dark period at 18°C (cucumber) or 15°C (wheat). Plants were watered routinely to keep the soil at constant moisture. The position of the pots was changed at random every other day to avoid position effects. After incubation for 10 days (cucumber) or 21 days (wheat), total shoot fresh weights per pot were assessed.

Development of qPCR Methods for Quantification of Pseudomonads Harboring DAPG, PHZ, and PRN Biosynthetic Genes

To quantify the abundance of DAPG and PHZ producing bacteria in soil, we developed quantitative real-time polymerase chain reaction (qPCR) assays targeting *phlD* and *phzF* genes. These genes encode, respectively, a polyketide synthase involved in the synthesis of phloroglucinols from malonyl-CoA (Bangera and Thomashow, 1996; Achkar et al., 2005) and an isomerase involved in the synthesis of phenazine-1-carboxylic acid (Mavrodi et al., 1998; Blankenfeldt et al., 2004). Alignments were created with publicly available *phlD* and *phzF* sequences from GenBank[2] and conserved regions were chosen for the design of primers and probes (**Table 2**), which was carried out with the Primer 3 Plus software (Untergasser et al., 2007). The parameters were amplicon length between 100 and 200 bp, melting temperature (TM) between 50 and 70°C, TM of probe 5°C higher than TM of primers, and the default setting of the program for self-complementarity and 3′-end stability. Partial sequences of *phlD* (GenBank accession CP003190.1| :6563260-6563937) of strain *P. protegens* CHA0 (Jousset et al., 2014) and *phzF* (locus tag, PFLU3_RS28075) of *P. synxantha* 2–79 (Nesemann et al., 2015) were used for primer design. The specificity of the primers was tested *in silico* with Primer-Blast (Ye et al., 2012) and *in vitro* with genomic DNA from 28 DAPG-producing strains and 38 PHZ-producing strains of the *P. fluorescens* group and nine additional PHZ-producing strains (Supplementary Table S1). Results of these tests revealed that our qPCR assays amplify *phlD* and *phzF* genes exclusively from DAPG and PHZ producing species

[2]https://www.ncbi.nlm.nih.gov/genbank/

TABLE 2 | Primers and probes used to quantify antimicrobial genes with qPCR.

Metabolite, target gene	Primers and probes[1]	Sequence (5′–3′)	Annealing temperature (°C)	Reference
DAPG[2], *phlD*	PhlD_65F_DEG	GGT RTG GAA GAT GAA RAA RTC	50	This study; Flury et al., 2017
	PhlD_153P_DEG	FAM-ATG GAG TTC ATS ACV GCY TTG TC-BHQ1		
	PhlD_236R_DEG	GCC YRA BAG YGA GCA YTA C		
Phenazine, *phzF*	PhzF_2Fm	ACC GGC TGT ATC TGG AAA CC	62	This study
	PhzF_2Pm	FAM-GCC GCC AGC ATG GAC CAG CCG AT-BHQ1		
	PhzF_2Rm	TGA TAG ATC TCG ATG GGA AAG GTC		
Pyrrolnitrin, *prnD*	PrnD_F	TGC ACT TCG CGT TCG AGA C	60	Garbeva et al., 2004
	PrnD_P	FAM-CGA CGG CCG TCT TGC GGA TC-BHQ1		
	PrnD_R	GTT GCG CGT CGT AGA AGT TCT		
Internal control, APA9 plasmid[3]	CMV_1F	TCA TCA TTT CCA CTC CAG GCT C	62	Von Felten et al., 2010
	CMV_1R	TCA TCC CTC TGC TCA TAC GAC TG		

[1]*TaqMan probes were labeled with fluorescein (FAM) at the 5′ end and with the black hole quencher 1 (BHQ-1) at the 3′ end.*
[2]*DAPG, 2,4-diacetylphloroglucinol.*
[3]*Plasmid from cassava mosaic virus.*

of the *P. fluorescens* lineage. The PRN biosynthetic genes were quantified on wheat roots by the qPCR method of Garbeva et al. (2004). That assay targets a gene for the class IA oxygenase PrnD that is involved in the final step of PRN biosynthesis (Kirner et al., 1998). In contrast to our *phlD* and *phzF* primers, the primers of Garbeva et al. (2004) have broader specificity and, in addition to *Pseudomonas*, amplify *prnD* from PRN-producing strains of *Burkholderia* and *Serratia*.

The efficiency of *phlD* and *phzF* primers at low gene copy numbers was evaluated using *in vitro* standard curves prepared by serially diluting genomic DNA of *P. protegens* CHA0 and *P. synxantha* 2–79. The genomic DNA was prepared by growing both strains in lysogeny broth (LB) (Bertani, 1951) overnight at 24°C on a rotary shaker at 180 rpm and extracting DNA with the Wizard Genomic DNA Purification Kit (Promega AG, Dübendorf, Switzerland). The concentration of purified DNA was quantified by fluorimetry with Qbit (Thermo Fisher Scientific). We also generated an *in vivo* standard curve for each qPCR assay to quantify the corresponding target genes on wheat roots. To this end, aliquots of 1 g of 21-days-old roots of spring wheat cv. Rubli grown in autoclaved soil were inoculated with decreasing concentrations of a mixture of bacterial cells belonging to different strains carrying the respective target gene. Strains used for *in vivo* standard curves are listed in Supplementary Table S1. Bacterial cells were harvested from overnight cultures in LB, washed and suspended in sterile 0.9% NaCl solution. Cell suspensions from each strain were set to the same optical density at 600 nm (OD_{600}) and then mixed together at equal proportions. The mixed suspensions were adjusted to an OD_{600} of 0.125, corresponding to approximately 10^8 CFU mL^{-1}, serially diluted and inoculated at 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU g^{-1} roots for the preparation of the standard curve. For each concentration and for the control without bacteria, three replicates were performed. The inoculated root samples used for standard curves were processed with the same method as the samples from pot experiments with the different soils (see following chapter). *In vivo* standard curves were prepared as described above for the *phlD*, *phzF* and *prnD* qPCR, using strains listed in Supplementary Table S1. Since all *in vivo* standard curves were prepared with bacterial cells recovered from wheat roots, the CT values can be directly converted to numbers of bacteria harboring *phlD*, *phzF,* or *prnD* per g root. Our qPCR data also directly reflect the abundance of the antimicrobial biosynthesis genes because *phlD, phzF,* or *prnD* are present in single copy in genomes of the *P. fluorescens* group (Flury et al., 2016). A survey of published bacterial genomes revealed that *phzF* and *prnD* are also found as a single copy in other bacterial species such as *Burkholderia* (*phzF* and *prnD*), *Pectobacterium* (*phzF*) or *Serratia* (*prnD*).

qPCR-Based Quantification of Antimicrobial Genes on Roots of Wheat Grown in Field Soils

To standardize the root material for qPCR quantification of DAPG, PHZ and PRN biosynthetic genes, soil samples from the 10 Swiss field sites were planted with spring wheat cv. Rubli in a greenhouse pot experiment. Plastic pots of 8 cm diameter and 30 cm height were part-filled with field soil and three wheat seedlings, prepared as described above, were planted per pot. Six pots per field soil were prepared. Wheat plants were grown for 2.5 months under the conditions described above for the Gt resistance assays. Root samples were collected, rinsed with tap water, incubated overnight in a sterile 0.9% NaCl solution at 3°C, and then vigorously agitated at 350 rpm for 30 min. Roots were separated from the root-wash suspensions and kept for dry weight assessment. Root-wash suspensions were centrifuged at 3500 rpm for 20 min. The supernatant was discarded and aliquots of 0.5 ml of the resulting root-wash pellet were used for DNA extraction. To each sample, 10^9 copies of the APA9 plasmid from a cassava mosaic virus were added as an internal standard (Von Felten et al., 2010). DNA extraction was performed with the MPBio soil kit (MP Biomedicals, Illkirch, France) following the protocol of the manufacturer. The concentration of extracted DNA was measured with Qbit. qPCR reactions consisted of 10 μL TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL of the respective forward and reverse primer solutions (10 μM), 2 μL of the respective probe solution (2.5 μM), 0.5 μL of bovine serum albumin solution (20 mg mL^{-1}), and 2 μL of template DNA in a total reaction volume of 20 μL. Primer and probe sequences are indicated in **Table 2**. Cycling conditions consisted of 2 min at 50°C (to permit uracil-DNA glycosylase activity), an initial denaturation step of 10 min at 95°C, and 40 cycles of 15 s at 95°C, 30 s at annealing temperature (see **Table 2**) and 30 s at 72°C. In all samples, the added APA9 plasmid was quantified with the primers listed in **Table 2**, following the method of Von Felten et al. (2010). The results from the APA9 plasmid quantification were used to normalize DNA extraction.

To compare the abundance of *phlD* and *phzF* measured with qPCR with the abundance measured using a cultivation-dependent terminal endpoint dilution assay, also called most probable number PCR (MPN-PCR) method (Ramette et al., 2006), a greenhouse experiment was carried out with soil samples from the Cazis and Taenikon field sites and spring wheat cv. Rubli cultivated under the same conditions as described above. After 3 weeks, *phzF* and *phlD* qPCR assays were performed on one fraction of each harvested root-wash pellet as described above, while the other fractions were serially diluted into microtiter plate wells (200 μl volume) containing King's medium B (King et al., 1954) broth amended with 100 mg L^{-1} cycloheximide, 13 mg L^{-1} chloramphenicol and 40 mg L^{-1} ampicillin. The microtiter plates were incubated at 24°C for 3 days and MPN-PCR was performed as described by Ramette et al. (2006), except for adapting annealing temperatures to the primers used in the present study (**Table 2**).

Quantification of Resident Pu and Gt by qPCR

Pythium ultimum and Gt populations naturally present on roots of wheat were quantified using the qPCR methods developed by Cullen et al. (2007) and Bithell et al. (2012), respectively. The reaction mix was prepared as described above for antimicrobial

genes, and cycling conditions were set as described previously (Cullen et al., 2007; Bithell et al., 2012). *In vitro* standard curves were performed with genomic DNA of Pu isolate ETH-2 (concentration range from 0.1 ng to 200 ag per reaction) and of Gt isolate I-17 (10 ng to 10^{-4} ng per reaction). Genomic DNA of the two pathogens was extracted with the DNeasy plant mini kit (Qiagen, Hombrechtikon, Switzerland) from lyophilized mycelia prepared from cultures grown in potato dextrose broth (Difco, Becton, Dickinson and Company, Franklin Lakes, USA) for 7 days at 24°C with agitation at 180 rpm.

Construction and Culture of *Pseudomonas* Reporter Strains

Bacterial strains and plasmids used for generation of *Pseudomonas* reporter strains for monitoring antimicrobial gene expression are listed in **Table 3**. *Pseudomonas* and *Escherichia coli* strains were routinely cultured at 30 and 37°C, respectively, on nutrient agar plates, in LB and in nutrient yeast broth (Stanisich and Holloway, 1972). When appropriate, selective antibiotics were added to the media at the following concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 50 μg mL^{-1}; gentamicin, 10 μg mL^{-1}; kanamycin, 25 μg mL^{-1}; and tetracycline, 125 μg mL^{-1}. Genomic DNA from *P. protegens* strain CHA0 and *P. chlororaphis* strain PCL1391 was isolated as previously described (Schnider-Keel et al., 2000). Plasmids were extracted and purified using the QIAprep Spin Miniprep kit (Qiagen) or the JETStar Plasmid Purification Midi kit (Genomed, Basel, Switzerland). PCRs were done using the PrimeSTAR HS DNA polymerase kit (Takara Bio Inc., Shiga, Japan) as described elsewhere (Péchy-Tarr et al., 2005). All DNA digestion and ligation reactions were done using standard techniques (Sambrook and Russell, 2001; Schnider-Keel et al., 2001). DNA extractions from agarose gels were carried out with the QIAquick Gel Extraction kit (Qiagen). Transformations of electro-competent cells with plasmid or purified ligation products were performed by electroporation (Schnider-Keel et al., 2000). To amplify genomic DNA or to detect the presence of recombinant DNA in *E. coli* colonies by screening, 100–200 ng of DNA were amplified using the GoTaq DNA polymerase kit (Promega, Dübendorf, Switzerland). All PCR constructs intended for transformation were verified by sequence analysis. DNA sequencing was carried out by GATC Biotech AG (Konstanz, Germany). Sequences were analyzed using the DNASTAR Lasergene software package version 11.0.

TABLE 3 | Bacterial strains, plasmids, and primers used for construction of reporter strains.

Strain, plasmid, or oligonucleotide	Relevant characteristics[1] or sequences (5′ 3′)	Reference
Pseudomonas protegens		
CHA0	Wild type; biocontrol agent; $DAPG^+$, PRN^+, HCN^+	Stutz et al., 1986
CHA0-*gfp*	CHA0::*att*Tn*7*-*gfp*; Gm^r	Péchy-Tarr et al., 2013
Pseudomonas chlororaphis		
PCL1391	Wild type; biocontrol agent; HCN^+, PHZ^+	Chin-A-Woeng et al., 1998
PCL1391-*gfp*	PCL1391::*att*Tn*7*-*gfp*; Gm^r	This study
Escherichia coli		
DH5α	Laboratory strain	Sambrook and Russell, 2001
Plasmids		
pBK-miniTn*7*-*gfp2*	pUC19-based delivery plasmid for miniTn*7*-*gfp2*; mob^+ Gm^r Cm^r Ap^r	Koch et al., 2001
pME7116	*prnA-gfp* transcriptional fusion; reporter of PRN biosynthetic gene expression in CHA0;Tc^r	Baehler et al., 2005
pME9010	*mCherry*-based promoter-probe vector derived from pPROBE'-*gfp* (AAV); Km^r	Rochat et al., 2010
pME9011	*hcnA-mcherry* transcriptional fusion; reporter of HCN biosynthetic gene expression in CHA0; Km^r	Rochat et al. (2010)
pME9012	*phlA-mcherry* transcriptional fusion; reporter of DAPG biosynthetic gene expression in CHA0; Km^r	Rochat et al., 2010
pME11011	*prnA-mcherry* transcriptional fusion; reporter of PRN biosynthetic gene expression in CHA0; Km^r	This study
pME11017	*phzA-mcherry* transcriptional fusion; reporter of PHZ biosynthetic gene expression in PCL1391; Km^r	This study
pUK21	Cloning vector; Km^r	Vieira and Messing, 1991
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	Bao et al., 1991
Oligonucleotides[2]		
P5 BamHI new	CGGGATCCCGGGCTCAAGGACAGTTGGTTCA, BamHI	This study
P6	GGAATTCCCGAGGTACGAAGCGGCCATC, EcoRI	Baehler et al., 2005
phzAF	CGGGATCCCTAACTCCATTTTGAGCACC, BamHI	This study
phzAR	CGAGCTCGCTCAATCTCCAATGAATAAGGGGGCT, SacI	This study

[1] *Abbreviations: Ap^r, ampicillin; Cm^r, chloramphenicol; Gm^r, gentamicin; Km^r, kanamycin; and Tc^r, tetracycline resistance, respectively.*
[2] *Restriction sites are underlined.*

In order to tag *P. protegens* CHA0 and *P. chlororaphis* PCL1391 with green fluorescent protein (GFP), a single copy of a *gfp* variant gene constitutively expressed from the P_{tac} promoter, was inserted into the chromosome using the pBK-miniTn*7*-*gfp2* delivery plasmid (Koch et al., 2001) and the Tn*7* transposition helper plasmid pUX-BF13 (Bao et al., 1991) as described previously (Péchy-Tarr et al., 2013). For use as reporters to monitor the expression of HCN, DAPG, PRN, and PHZ biosynthetic genes, strain CHA0-*gfp* was transformed with pME9011 (*hcnA-mcherry*), pME9012 (*phlA-mcherry*) or pME11011 (*prnA-mcherry*), and strain PCL1391-*gfp* with pME11017 (*phzA-mcherry*) (**Table 3**). To construct the *prnA-mcherry* reporter plasmid pME11011, a 629-bp fragment containing the CHA0 *prnA* promoter was amplified from pME7116 (Baehler et al., 2005) using primers P5 BamHI new and P6 (**Table 3**). The obtained fragment was digested with BamHI and EcoRI and ligated into the *mcherry*-based promoter-probe vector pME9010 (Rochat et al., 2010) opened with the same enzymes. Similarly, for the construction of the *phzA-mcherry* reporter plasmid pME11017, a 961-bp fragment containing the *phzA* promoter (Chin-A-Woeng et al., 2001) was amplified from genomic DNA of PCL1391 (Flury et al., 2016) using primers phzAF and phzAR (**Table 3**). The PCR product was digested with BamHI-SacI and the resulting fragment was first cloned into pUK21 and, from there, into pME9010, both opened with the same restriction enzymes.

Assay to Monitor Antimicrobial Gene Expression in Field Soils

Antimicrobial gene expression and colonization levels of GFP-marked *P. protegens* CHA0 and *P. chlororaphis* PCL1391 harboring mCherry-based reporter plasmids were monitored on roots of spring wheat grown in the soils sampled at the 10 different Swiss field sites. Untreated seeds of spring wheat cv. Rubli were surface-sterilized for 12 min in 4% NaClO (vol/vol) and then washed with sterile distilled water. Seeds were germinated on soft agar (Agar Agar Serva at 9 g L^{-1}; Serva, Heidelberg, Germany) for 48 h at room temperature in the dark. The wheat seedlings were then transferred to 200-mL Erlenmeyer flasks (5-cm opening; Simax, Czech Republic) containing 60 g of soil. In each flask, three seedlings were placed into the soil and inoculated with 1 mL of a suspension of washed cells of the respective *Pseudomonas* reporter strain adjusted within a range from 3.6 10^7 to 7.8 $\times$ 10^7 cells mL^{-1}. Washed cells were prepared from LB cultures grown without antibiotic addition under the conditions described above to an OD_{600} of 0.8 to 1.5, depending on the reporter strain used, whereby choosing a growth stage at which no significant expression of the antimicrobial genes occurred. This was done by determining, with a fluorimetry assay (Baehler et al., 2005), the time point at which the relative red fluorescence emitted by the *Pseudomonas* strains carrying mCherry-based reporter plasmids was not yet significantly different from the red background fluorescence emitted by control strains carrying the empty vector pME9010. Wild-type and GFP-tagged *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (with and without pME9010) were included as control treatments for properly setting green and red fluorescence backgrounds for the FACS-based flow cytometry analysis described below. Flasks were sealed with cotton wool plugs and incubated in a growth chamber set to 60% relative humidity for 16 h with light (176 $\mu E\ m^{-2}\ s^{-1}$) at 25°C, followed by an 8-h dark period at 20°C. After 5 days of incubation, wheat roots from each flask were removed, washed using distilled water to remove loosely adhering soil particles from roots and pooled in 10 mL of autoclaved, ultrapure water contained in a sterile 50-mL Falcon tube. Tubes were agitated for 20 min at 300 rpm in order to remove the majority of bacteria from the roots. The resulting suspensions were filtered using a 5.0-μm sterile syringe single-use filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany), transferred on ice and immediately analyzed by FACS as described below. Dry weights of wheat roots were recorded and the number of GFP-marked *Pseudomonas* cells present in the root washes were determined by FACS and recorded as cells g^{-1} of root.

FACS Analysis

Green fluorescent protein and mCherry expression levels in *Pseudomonas* reporter cells in natural soils were quantified with a Becton–Dickinson LSRFortessa flow cytometer. Size and granularity of *Pseudomonas* cells and particles were determined by measuring the forward scatter (FSC-A) and side scatter (SSC-A) signals, respectively. FSC-A signals were collected with a photodiode detector (set to 350 V), in the range of 483 to 493 nm (488/10 BP filter), with a threshold set to 200. SSC-A signals were detected with a photomultiplier tube (PMT) (detector G, set to 300 V) in the range of 483 to 493 nm (488/10 BP filter). Green fluorescence signals were collected with the PMT detector E (set at the voltage of 676 V), in the range of 515 to 545 nm (530/30 BP filter, 505 LP mirror). Red fluorescence signals were detected using the PMT detector C (set to 700 V), between 600 and 620 nm (610/20 BP filter, 600 LP mirror). For FACS analysis, aliquots of 300 μL of filtered root-washes were placed into Nunc MaxiSorp flat-bottom 96-well plates (Sigma–Aldrich, Buchs, Switzerland), and each sample was mixed three times. The analyzed volume was standardized to 200 μL, allowing the detection of 500–10,000 GFP events, depending on the analyzed soil. Gating of GFP-marked bacteria was done by delimiting on the FSC-A/FITC-A density plot particles with green fluorescence values above the background fluorescence noise (i.e., autofluorescence emitted by root and soil particles, cell fragments or bacterial cells not expressing GFP). Control samples obtained from soils amended with pure water, wild-type *P. protegens* CHA0 and *P. chlororaphis* PCL1391, and GFP-tagged strains CHA0-*gfp* and PCL1391-*gfp* were used to identify the GFP background fluorescence in soil extraction samples. The red fluorescence emitted by the gated GFP-tagged *Pseudomonas* cells was then analyzed on the PE-Texas Red-A histogram and on the FSC-A/PE-Texas Red-A density plot, allowing to detect and analyze all GFP-marked cells actively expressing their mCherry-based reporter fusion. Control samples including CHA0-*gfp* and PCL1391-*gfp* without mCherry-based vector, or carrying the empty pME9010 vector were used to define the mCherry background fluorescence among the GFP-marked *Pseudomonas* population. The median of red fluorescence emitted by the

Pseudomonas cells was calculated using the BD FACSDiva software version 8.0 (Becton–Dickinson). To determine root colonization levels of *Pseudomonas* reporter strains, the GFP tag was used to count by FACS the number of reporter cells present in the analyzed 200 μL of filtered root-wash and then to calculate their concentration per gram of dry roots.

Data Analysis

Statistical data analysis was carried out with the open source software R version 3.2.3 (R Core Team, 2015). Shoot fresh weights of cucumber and wheat plants obtained from the Pu and Gt infection assays, respectively, were analyzed as a proxy for soil disease resistance. Shoot weights from samples where pathogen inoculum was added were normalized against the shoot weights from non-infested control plants grown in the same soil, to minimize variation due to different nutrient contents in the different soils. Data were checked for normal distribution with the Shapiro–Wilk test and by plotting QQ-Plots. Equality of variance was verified with Bartlett's test. Analysis of variance was carried out with a non-parametric test (Kruskal–Wallis test, significance level $p < 0.05$), followed by a *post hoc* test (kruskalmc, R package 'Pgirmess').

The abundance of *phlD*, *phzF*, and *prnD* harboring bacterial cells on wheat roots was calculated with the *in vivo* standard curves described above. Efficiencies and detection limits of qPCR assays determined by *in vivo* standard curves are indicated in Supplementary Table S2. Cycle threshold values obtained from the *in vivo* standard curves and from the samples were normalized for differences in DNA extractions as described by Von Felten et al. (2010). Normalized values were used for further analysis. The average values obtained from the three technical replicates of each qPCR assay were used for statistical analysis, which was performed as described above for shoot weights.

Data on the expression of reporters of antimicrobial genes in the different soils represent the medians of three independent repetitions of the same experiment, with nine replicates per treatment in each experiment. Significant differences between treatments were calculated with a non-parametric Kruskal–Wallis test (significance level $p < 0.05$), followed by Dunn's test for *post hoc* comparisons.

Data used for the heat map showing rankings of pathogen suppression, abundance of antimicrobial genes, and expression of antimicrobial genes by reporter strains in the different soils were normalized using the function 'scale' (R package 'stats'). Correlations between pathogen suppression, gene abundance, gene expression and abiotic soil parameters were inferred with Spearman's rho rank correlation (significance level $p < 0.05$). Data were displayed in a heat map with the functions 'levelplot' (R package 'lattice') or 'corrplot' (R package 'corrplot').

RESULTS

Resistance of Swiss Agricultural Soils to Soilborne Pathogens

The general resistance of 10 Swiss agricultural soils (**Figure 1** and **Table 1**) to soilborne pathogens was tested in a greenhouse assay in which increasing quantities of Gt or Pu inoculum were added to soil samples planted with spring wheat or cucumber, respectively. Resistance to both pathogens varied between soils. In the Gt-infested soils at 0.6 g inoculum per pot, shoot fresh weights of wheat plants ranged from 31% in Grangeneuve soil to 107% in Taenikon soil of the weights of control plants grown in not artificially infested soils (**Figure 2A**). The shoot fresh weights of wheat grown in infested Taenikon soil were significantly higher (1.3–3.5-fold) than those of plants grown in infested soils from Cazis, Eschikon, Grangeneuve, Vouvry, and Witzwil. Similar trends were observed at other inoculum quantities (Supplementary Figure S1). Plants grown in Taenikon soil had the highest shoot fresh weights at all Gt concentrations used except the highest (6 g of inoculum per pot), at which the fungal pathogen heavily affected plant growth in all the soils and reduced shoot weights by 60–90% (Supplementary Figure S1D).

When the soils were artificially infested with Pu inoculum at 0.125 g per pot, median shoot fresh weights of cucumber plants ranged from 0% in Vouvry soil to 106% in Cazis soil of those of control plants grown in non-infested soils (**Figure 2B**). The shoot fresh weights of cucumber grown in Pu-infested Cazis soil were significantly higher (three–fivefold) than those of plants grown in infested soils from Cadenazzo, Courtedoux, Delley, Taenikon, and Vouvry (**Figure 2B**) and similar trends were observed for other inoculum densities (Supplementary Figure S2). Plants grown in soil from the Cazis field site had the highest shoot weights at all Pu concentrations, while plants grown in soil from the Vouvry field had the lowest shoot weights at all levels of the pathogenic oomycete except at 0.25 g per pot.

Individual soils did not display equal resistance levels to the two soilborne pathogens. While the soils from the Taenikon and Delley field sites were the most resistant against Gt, they were among the least resistant against Pu (**Figure 2** and Supplementary Figures S1, S2). Likewise, the soil from Cazis was the most resistant against Pu, but was only moderately resistant against Gt.

To account for potential effects of resident Gt and Pu populations on the outcome of the soil resistance experiments, a qPCR method targeting the ITS rRNA gene region was used to detect and quantify the two pathogens in the rhizoplane of spring wheat plants grown in the not artificially infested control treatments of all soils. Gt could not be detected in any of the samples. By contrast, Pu was detected on the plant roots in all ten soils, but there was no significant difference in the abundance of the oomycete pathogen among the individual soils (Supplementary Figure S3).

In summary, the 10 agricultural soils strongly varied in their resistance against soilborne pathogens. However, the soil resistance levels observed for the two investigated pathogens in general were different; i.e., some soils displaying high resistance to Pu were highly susceptible to Gt and vice versa, pointing to specificity in the buffering capacity of individual soils toward specific soilborne pathogens.

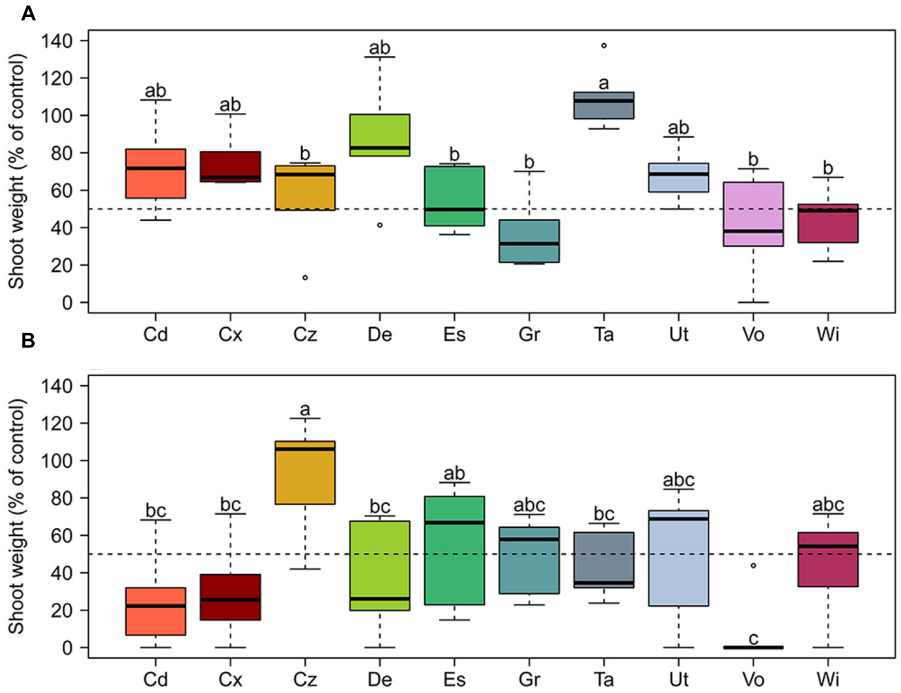


FIGURE 2 | Relative resistance of 10 representative Swiss agricultural soils with a cereal-oriented cropping history to the soil-borne pathogens (A) *Gaeumannomyces tritici* (Gt) and **(B)** *Pythium ultimum* (Pu). Increasing concentrations of pathogen inoculum were added to the soil before planting with spring wheat (Gt experiment) or cucumber (Pu experiment) seedlings. Data shown here are for Gt at 0.6 g and Pu at 0.125 g inoculum per pot. Results for the other inoculum concentrations tested are shown in Supplementary Figures S1, S2. Each pathogen concentration and soil was tested in six replicate pots. Soil resistance is shown as fresh shoot weight of plants in artificially pathogen-infested soil compared to fresh shoot weight of control plants grown in non-infested soil. The dotted line indicates 50% of shoot weight compared to the control. Letters indicate significant differences (Kruskal–Wallis test, $p < 0.05$). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

Abundance of *phlD*$^+$ Pseudomonads, *phzF*$^+$ Pseudomonads, and *prnD*$^+$ Bacteria on Roots of Wheat Grown in Swiss Agricultural Soils

The abundance of bacterial cells harboring *phlD, phzF*, and *prnD* required for the biosynthesis of the antimicrobials DAPG, PHZ, and PRN, respectively, was quantified by qPCR on roots of spring wheat grown in the 10 Swiss agricultural soils. As detailed in section "Materials and Methods," we assume that *phlD*$^+$ and *phzF*$^+$ cells quantified in our assays correspond to cell numbers of DAPG and PHZ producing pseudomonads, whereas *prnD*$^+$ cells correspond to cell numbers of PRN producing bacteria. Since the investigated genes are present as one copy per bacterial cell, we also refer to the abundance of cells harboring an antimicrobial gene as gene abundance.

The abundance of the *phlD*$^+$ pseudomonads in all studied soils in general was higher than the abundance of the *phzF*$^+$ and *prnD*$^+$ bacteria, and in some soils reached 10^7 cells per gram of root dry weight, whereas the abundance of the latter remained below 10^6 gene copies per gram of root dry weight (**Figure 3**). For individual genes, pronounced differences in abundance between soils were observed. The biggest differences were found for *phlD*$^+$ cells with approximately 10^4-fold higher numbers on roots of wheat grown in soil from Taenikon compared to those grown in soil from Vouvry (**Figure 3A**). The abundance of *phlD*$^+$ pseudomonads was significantly higher in soils from Courtedoux, Delley, Eschikon, Grangeneuve, Taenikon, Utzenstorf, and Witzwil than in soils from Cadenazzo, Cazis, and Vouvry. The abundances of *phzF*$^+$ pseudomonads and *prnD*$^+$ bacteria varied at maximum 100-fold between the different soils. The number of pseudomonads harboring the *phzF* gene was significantly higher on roots samples from Courtedoux and Delley soils compared to samples extracted from Vouvry soil (**Figure 3B**). The abundance of *prnD*$^+$ bacteria was significantly higher in soil samples from Cadenazzo, compared to those from Courtedoux, Cazis, and Vouvry (**Figure 3C**). Taken together, for all three investigated antimicrobial genes pronounced differences in the abundances were found between the individual agricultural soils, indicating that the different soils may sustain to different extents specific populations of pseudomonads producing DAPG, PRN, and/or PHZ.

Expression of Antimicrobial Genes in Swiss Agricultural Soils

The relative capacity of the 10 different Swiss agricultural soils to sustain the expression of biosynthetic genes for the antimicrobial compounds DAPG, HCN, PRN, and PHZ on roots was followed using GFP-tagged *Pseudomonas* strains carrying mCherry-based reporter plasmids inoculated into

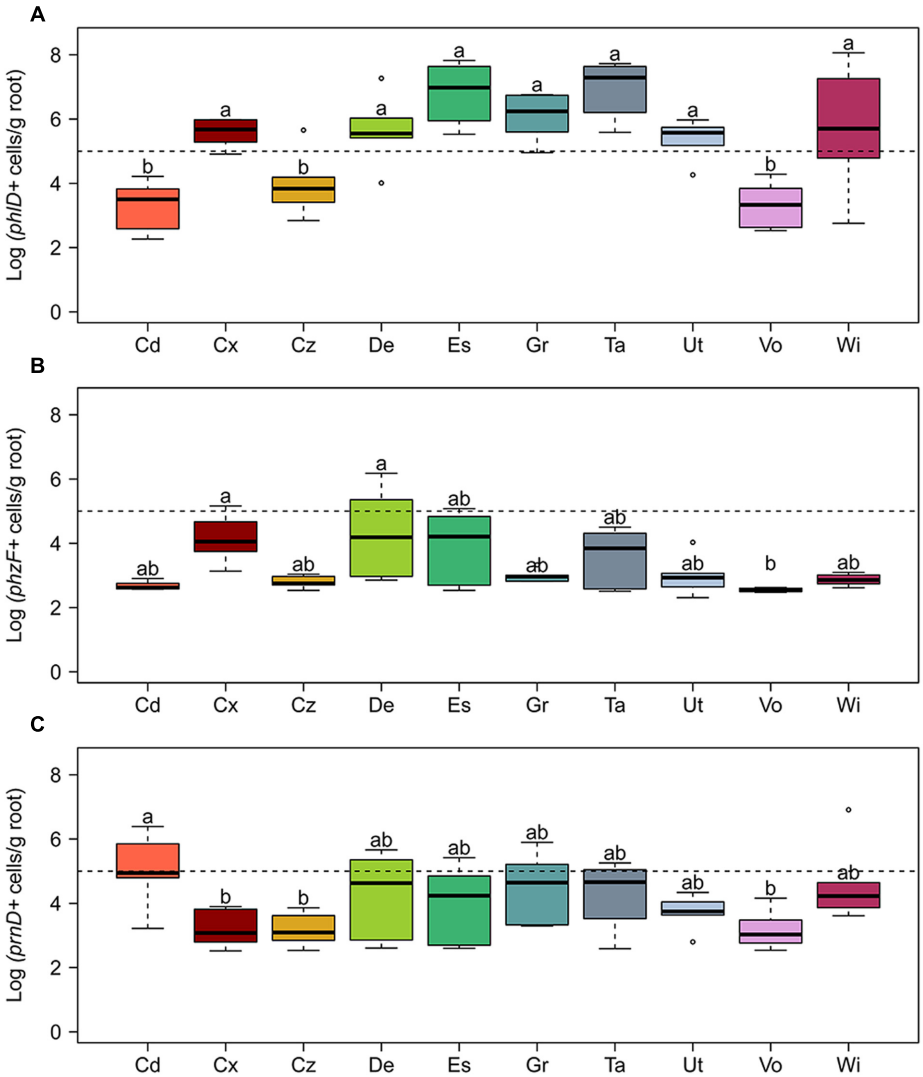


FIGURE 3 | Abundance of bacterial cells harboring genes required for the biosynthesis of the antimicrobial compounds (A) 2,4-diacetylphloroglucinol (*phlD*), **(B)** phenazine (*phzF*), and **(C)** pyrrolnitrin (*prnD*) on roots of spring wheat in 10 Swiss agricultural soils. Cells harboring the antimicrobial genes were quantified by qPCR. The dotted line indicates 10^5 cells per gram of root dry weight. For each soil, six replicates were used. Letters indicate significant differences (Kruskal–Wallis test, $p < 0.05$). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

soil microcosms planted with spring wheat. *P. protegens* CHA0-*gfp* carrying plasmid pME9012 (*phlA-mcherry*), pME9011 (*hcnA-mcherry*), or pME11011 (*prnA-mcherry*), or *P. chlororaphis* PCL1391-*gfp* carrying pME11017 (*phzA-mcherry*) served as reporter strains. GFP fluorescence (identifying the tagged reporter strains) and relative mCherry fluorescence intensities (reporting expression levels of respective antimicrobial genes) of cells in root washes extracted from the different soils were recorded with FACS-based flow cytometry. Data presented as total antimicrobial gene expression by all cells of the respective reporter strain per gram dry weight of roots, and they were calculated by multiplying the median gene expression per cell with the total number of reporter cells per gram dry weight of roots (Supplementary Table S3).

The levels of total expression of all investigated antimicrobial genes varied significantly among the 10 field soils, with the highest variations observed for DAPG and HCN biosynthetic genes (**Figure 4**). The soil from Cadenazzo supported the highest levels of total *phlA* expression on the roots, which were approximately 16-fold higher than those measured on roots growing in soil from Taenikon, which yielded the lowest expression levels (**Figure 4A**). The Cadenazzo soil also supported highest levels of total *hcnA* expression among all 10 soils, and these levels were approximately 20-fold higher than *hcnA* expression levels recorded in the soil from the Grangeneuve

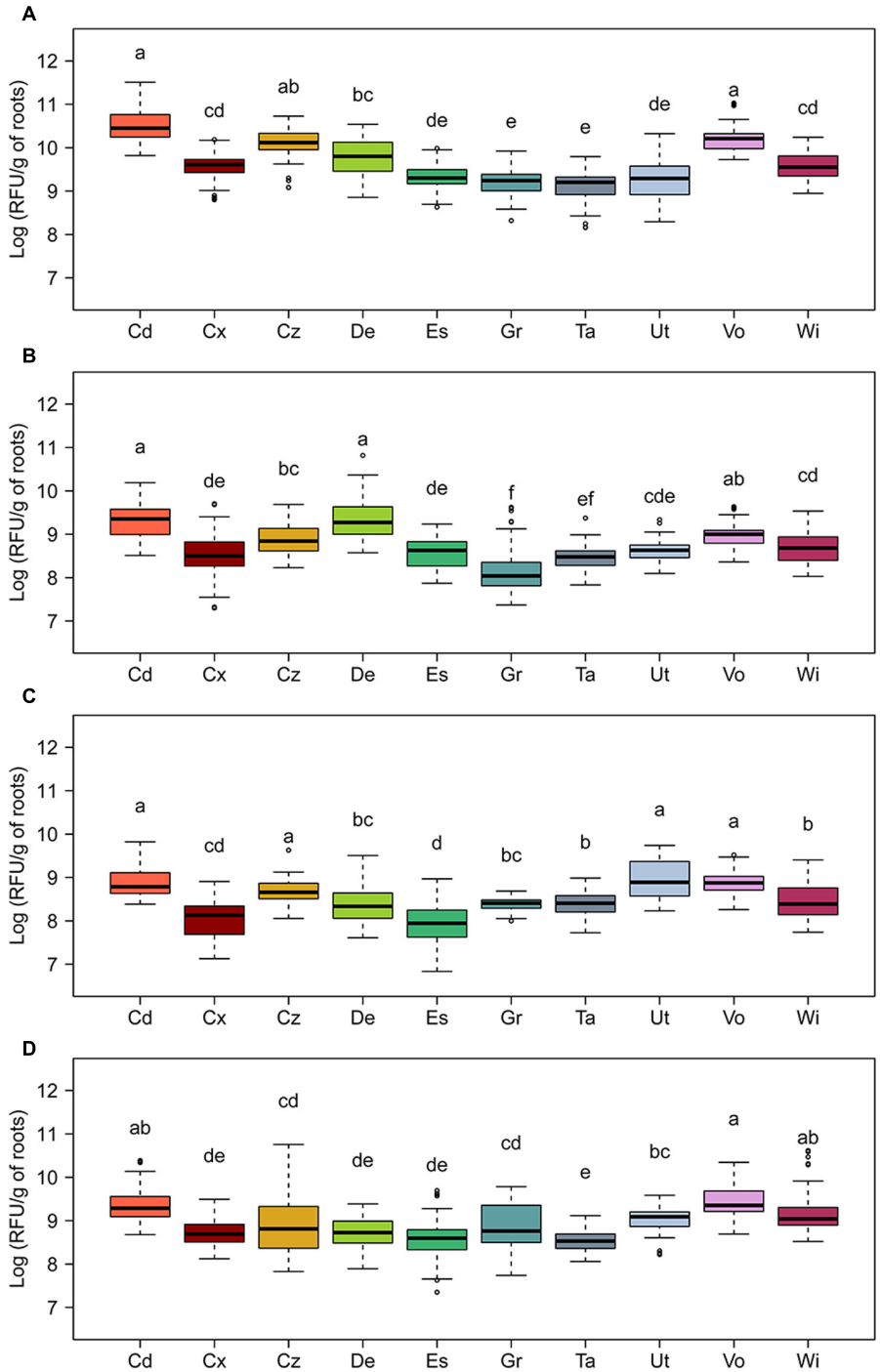


FIGURE 4 | Relative expression of genes required for the biosynthesis of the antimicrobial compounds (A) 2,4-diacetylphloroglucinol (*phlA*), **(B)** hydrogen cyanide (*hcnA*), **(C)** pyrrolnitrin (*prnA*), and **(D)** phenazines (*phzA*) on roots of spring wheat in 10 Swiss agricultural soils. Expression was monitored by fluorescence-activated cell-sorting-based flow cytometry using GFP-tagged strains of *Pseudomonas protegens* (CHA0-*gfp*) carrying reporter plasmids pME9012 (*phlA-mcherry*), pME9011 (*hcnA-mcherry*), or pME11011 (*prnA-mcherry*) or of *P. chlororaphis* (PCL1391-*gfp*) carrying reporter plasmid pME11017 (*phzA-mcherry*). Seedlings inoculated with the reporter strains were grown in soil microcosms for 5 days prior to analysis of bacterial cells in root washes. Data are shown as relative fluorescence units (RFU) per gram of root dry weight, and were calculated as the median mCherry expression per GFP-tagged *Pseudomonas* cell multiplied with the total number of GFP-tagged *Pseudomonas* cells per gram of root. Results from three independent experiments with nine replicates each are presented. Since Kruskal–Wallis analyses did not reveal significant experiment × treatment interactions, data of the three experiments were pooled for statistical analysis. Letters indicate significant differences (Dunn test, $p < 0.05$). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

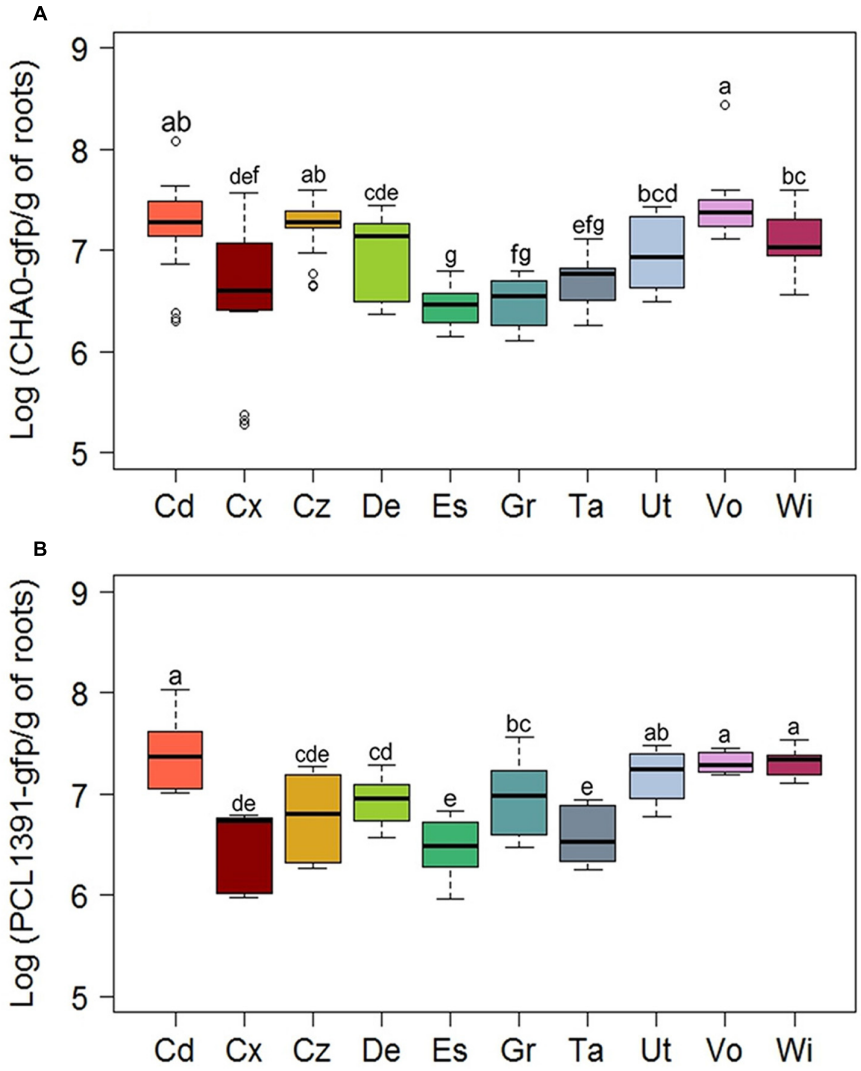


FIGURE 5 | Root colonization levels of *Pseudomonas* reporter strains in 10 Swiss agricultural soils planted with spring wheat. Cells of GFP-tagged strains of **(A)** *P. protegens* (CHA0-*gfp*) or **(B)** *P. chlororaphis* (PCL1391-*gfp*) were monitored by fluorescence-activated cell-sorting-based flow cytometry. Data show numbers of GFP-tagged cells per gram of root dry weight. Results from three independent experiments with nine replicates each are presented. Since Kruskal–Wallis analyses did not reveal significant experiment × treatment interactions, data of the three experiments were pooled for statistical analysis. Letters indicate significant differences (Dunn test, $p < 0.05$). Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

field site, which was the least favorable to the expression of this antimicrobial gene (**Figure 4B**). Levels of total expression of PRN and PHZ biosynthetic genes appeared to be less variable among the 10 soils. In particular, *prnA* expression levels were eightfold higher in the soil from Utzenstorf that supported the highest expression compared to the soil from Eschikon that supported the lowest expression (**Figure 4C**). Levels of overall *phzA* expression varied only about fivefold between the most contrasting soils from the Cadenazzo and Taenikon field sites, respectively (**Figure 4D**). Several individual soils sustained total expression levels for all investigated antimicrobial genes to a similar extent. This was evident notably for soils from Cadenazzo and Vouvry in which all four antimicrobial genes attained high total expression levels, while soils from Eschikon, Grangeneuve and Taenikon supported significantly lower overall expression levels of these genes (**Figure 4**).

The Cadenazzo and Vouvry soils appeared to favor also highest levels of root colonization (1.9×10^7 to 2.4×10^7 cells g^{-1} of dry root weight) by *P. protegens* and *P. chlororaphis* among the 10 soils tested, while the soils from Eschikon, Grangeneuve, and Taenikon were among those supporting lower levels of root colonization (2.9×10^6 to 9.7×10^6 cells g^{-1} of dry root weight) (**Figure 5** and Supplementary Table S3). However, whereas the soil from Cadenazzo was indeed also the field soil supporting highest *phlA*, *hcnA*, *prnA,* and *phzA* expression levels in individual reporter cells, the median single cell expression

of these genes was significantly lower in the soil from Vouvry (Supplementary Table S3). For the Vouvry soil, it thus seems that the high total expression in the reporter population on the roots was mainly due to the higher colonization levels attained in this soil. Likewise, the relatively high single cell expression but low colonization levels in the Eschikon and Taenikon soils (**Figure 5** and Supplementary Table S3) resulted in the significantly lower overall expression as compared to the Cadenazzo and Vouvry soils (**Figure 5**). By contrast, in the soil from Grangeneuve, single cell expression levels of antimicrobial genes as well as colonization levels were consistently relatively low (Supplementary Table S3). Similar overall gene expression levels may therefore reflect contrasting levels of single cell gene expression and colonization in individual soils.

In summary, the expression of the investigated antimicrobial genes strongly varied between the studied agricultural soils. Some soils seem to favor higher levels of overall antimicrobial gene expression whereas others apparently support the expression of these genes much less. The findings suggest that the expression of different antimicrobial genes may be induced by the same soil factors (or similar combinations thereof).

Relationships between Pathogen Resistance and Abundance and Expression of Antimicrobial Genes in Swiss Agricultural Soils

Results obtained from experiments on pathogen resistance of soils, abundance and expression of antimicrobial genes were displayed in a gradient map (**Figure 6**). Soils were grouped in three clusters. The first cluster consisted of soils supporting high abundances but low expression levels of antimicrobial genes (i.e., soils from Eschikon and Taenikon field sites). The second cluster consisted of soils supporting high antimicrobial gene expression levels but low abundances of bacteria harboring antimicrobial genes (i.e., soils from Cadenazzo and Vouvry field sites). The third cluster consisted of soils ranging in between the two other clusters. Two soils displayed high resistance against soilborne plant pathogens, i.e., the soil from Taenikon against Gt and the soil from Cazis against Pu. Although the soil from Taenikon was the one supporting the highest abundance of pseudomonads harboring the DAPG biosynthetic gene *phlD*, resistance to the soilborne pathogens did not generally cluster with high abundances or expression levels of antimicrobial genes (**Figure 6**).

Correlation analysis revealed that the expression of the four studied antimicrobial genes was positively correlated (**Figure 7B**). A similar trend could be observed for the abundance of the antimicrobial genes (**Figure 7A**). Resistance to soilborne pathogens was not significantly correlated to the abundance of antimicrobial genes, although a weak positive correlation could be observed between the abundance of bacteria harboring individual antimicrobial genes and resistance to pathogens (**Figure 7A**). These results suggest that the expression of the investigated antimicrobial genes is similarly influenced by biotic and abiotic factors prevailing in the respective soils. The same could be true for the abundance of bacteria harboring these antimicrobial genes. Our results further indicate that the abundance of such bacteria probably plays only a limited role in the resistance of the investigated cereal crop-oriented agricultural soils to the soilborne pathogens Pu and Gt.

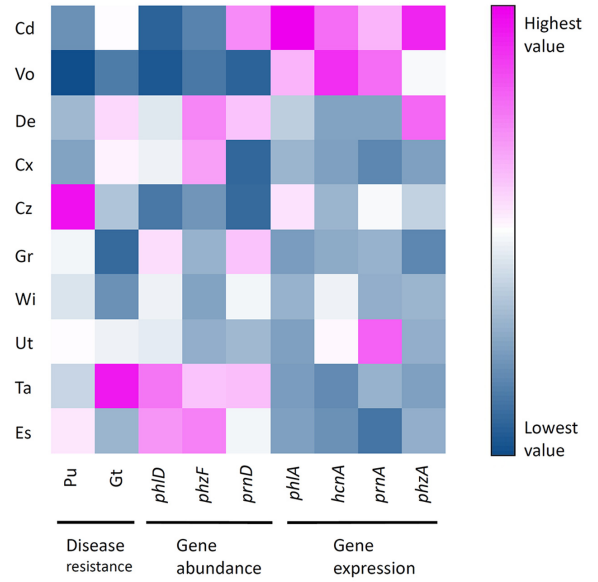


FIGURE 6 | Heatmap showing normalized-values of disease resistance, antimicrobial gene abundance and antimicrobial gene expression measured in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. The color scale depicts highest (fuchsia) via intermediate (white) to lowest (blue) values for each variable. Sampling sites: Cd, Cadenazzo; Cx, Courtedoux; Cz, Cazis; De, Delley; Es, Eschikon; Gr, Grangeneuve; Ta, Taenikon; Ut, Utzenstorf; Vo, Vouvry; Wi, Witzwil.

Relationships between Soil Parameters, Pathogen Resistance, Abundance and Expression of Antimicrobial Genes

The physical and chemical properties of the 10 Swiss agricultural soils investigated in this study were analyzed (**Figure 1** and **Table 1**) and correlated with their resistance to pathogens, the abundance of antimicrobial genes and the expression of antimicrobial genes (**Figure 8**). Macronutrients were extracted from soils with the water or ammonium acetate-EDTA (AA-EDTA) soil extraction procedures routinely used in Swiss agriculture to account for, respectively, soluble, i.e., readily plant-available macronutrients and bound, reserve macronutrients that become available to plants at mid or long term (Agroscope, 2006; Stünzi, 2007). Plant micronutrients were extracted with AA-EDTA.

Organic carbon and clay, and silt and sand inversely influenced antimicrobial gene abundance and expression. Abundance of DAPG biosynthesis genes (recorded for *phlD*) was significantly positively correlated with clay and significantly negatively correlated with silt, while expression of these genes (recorded for *phlA*) was significantly negatively correlated

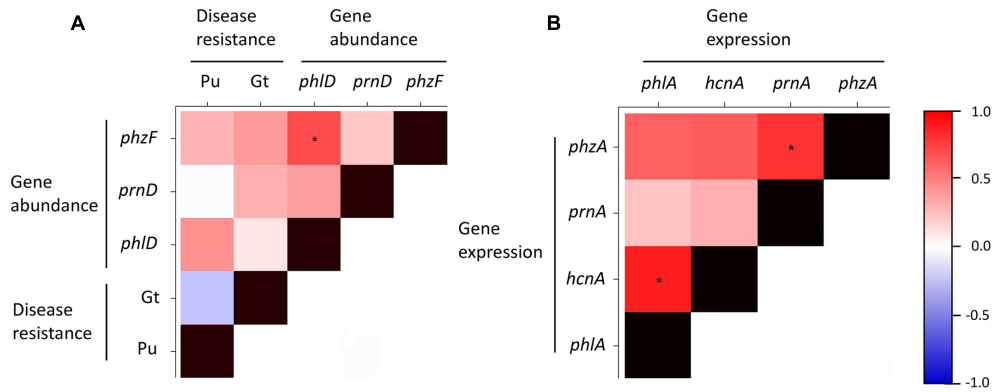


FIGURE 7 | Heatmap showing Spearman's rank correlations for disease resistance and abundance of antimicrobial genes (A) and for expression levels of antimicrobial genes **(B)** in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. The abundance of antimicrobial genes is defined as the number of bacterial cells harboring the indicated gene. Significant correlations ($p < 0.05$) are highlighted with asterisks. The color scale to the right of the matrix indicates rho correlation coefficients.

with organic carbon and clay and significantly positively correlated with silt (**Figure 8**). Similar trends were also observed for the pyrrolnitrin and phenazine biosynthetic genes *prnA* and *phzF*, respectively. No clear positive or negative correlation was found between pH and antimicrobial gene abundance or expression (**Figure 8**). Nitrate concentration in soil was positively correlated with the abundance of bacteria harboring the investigated antimicrobial genes; in particular, it was significantly positively correlated with the abundance of $phlD^+$ pseudomonads (**Figure 8**). Antimicrobial gene expression was neither clearly positively nor clearly negatively correlated with nitrate. Of the other macronutrients, potassium inversely influenced abundance and expression of antimicrobial genes. Reserve potassium extracted with AA-EDTA was significantly positively correlated with the abundance of $phlD^+$ and $phzF^+$ pseudomonads and significantly negatively with *phlA* and *phzA* expression (**Figure 8**). A similar trend was also observed for water-extracted, i.e., readily plant available magnesium. Among the measured micronutrients, a significant effect was only observed for manganese, which was significantly negatively correlated with *phzA* and *hcnA* expression (**Figure 8**).

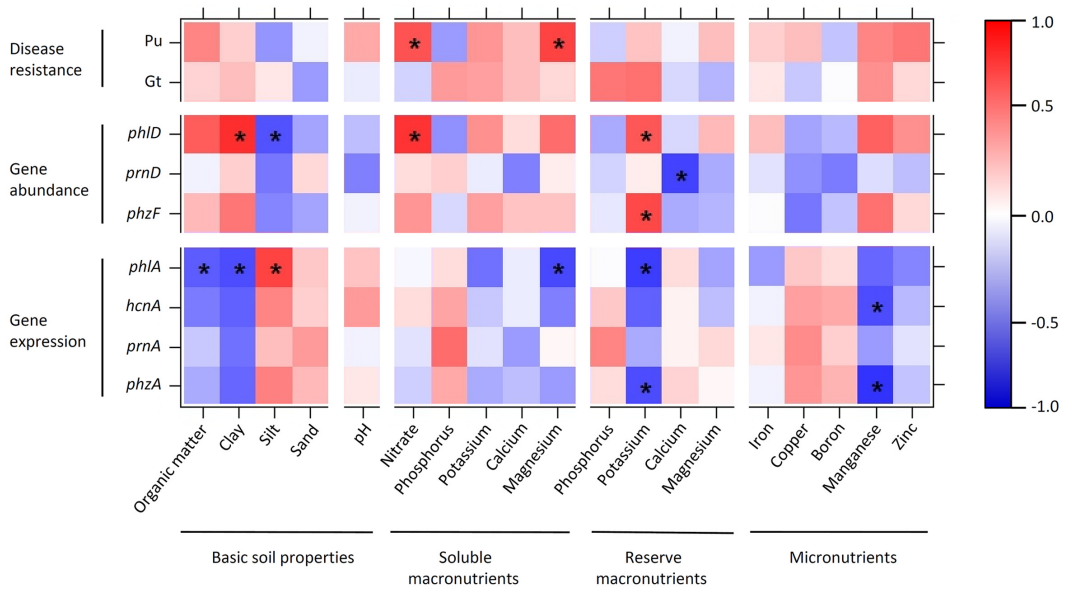


FIGURE 8 | Heatmap showing Spearman's rank correlations between soil parameters, disease resistance, and abundance and expression of antimicrobial genes in 10 representative Swiss agricultural soils with a cereal-oriented cropping history. The abundance of antimicrobial genes is defined as the number of bacterial cells harboring the indicated gene. Significant correlations ($p < 0.05$) are highlighted with asterisks. The color scale to the right of the matrix indicates rho correlation coefficients.

Resistance to soilborne pathogens was not significantly correlated to any soil factor in the case of Gt, and significantly positively correlated to nitrate and magnesium extracted with water in the case of Pu (**Figure 8**). The resistance to both pathogens was positively, but not significantly correlated to organic carbon, clay, potassium, manganese, and zinc.

Taken together, these analyses indicate that soil physical and chemical properties have contrasting and subtle effects on the abundance and expression of antimicrobial genes. No pronounced correlations between soil properties and general disease resistance of soils could be observed for the 10 agricultural field sites investigated. Remarkably, all soil factors that were positively correlated with the abundance of *phlD*$^+$ and *phzF*$^+$ pseudomonads were also positively correlated with Pu resistance.

DISCUSSION

Resistance to soilborne diseases and beneficial microbial populations involved have been studied extensively in soils specifically suppressive to one particular pathogen species (Weller et al., 2002; Mendes et al., 2011; Raaijmakers and Mazzola, 2016). However, virtually nothing is known about interactions between soilborne pathogens and beneficial microorganisms in common agricultural soils, i.e., in soils that lack specific disease suppressiveness. Similarly, it is not clear whether a particular soil may simultaneously exhibit suppressiveness toward multiple soilborne pathogens. To our best knowledge, the present study is the first to compare side-by-side a range of common agricultural soils for their resistance toward two soilborne pathogens, *P. ultimum* (Pu) and *G. tritici* (Gt), as well as for the abundance and expression of biosynthetic genes required for the production of antimicrobial compounds by plant-beneficial pseudomonads.

First, we investigated the capacity of different Swiss agricultural soils to buffer the attack of two soilborne pathogens by testing the growth of crop plants in these soils after amendment with increasing quantities of pathogens up to very high concentrations. Individual soils differed markedly in their respective resistance to the two pathogens. Several soils that showed comparatively high suppressiveness toward Pu were poorly or only moderately resistant to Gt and vice versa. These results indicate that conventional agricultural soils do not necessarily exhibit a general level of resistance toward a range of soilborne pathogens, but rather display variable resistance levels toward specific pathogens, which are likely modulated by different microbial and abiotic soil factors. Nevertheless, soils suppressive to more than one pathogen have been reported, notably the *Fusarium* wilt of pea suppressive soils in Mt. Vernon, WA, USA (Landa et al., 2002; Weller et al., 2007), which are also suppressive to take-all of wheat (Allende Molar, 2006; Allende-Molar and Weller, personal communication).

Soil resistance to soilborne pathogens has often been linked to the abundance of pseudomonads producing antimicrobial compounds (Stutz et al., 1986; Raaijmakers et al., 1997; Weller et al., 2002, 2007; Haas and Défago, 2005; Raaijmakers et al., 2008; Mazurier et al., 2009; Almario et al., 2014). For this reason, we have used a qPCR approach targeting biosynthetic genes for DAPG, PHZ, and PRN in bacterial cells present on roots of wheat grown in the agricultural soils that we tested for disease resistance. While the qPCR assays used in this study to quantify *phlD*$^+$ and *phzF*$^+$ bacteria are specific for fluorescent pseudomonads (see Supplementary Table S1), the assay used to quantify *prnD*$^+$ cells additionally detects *Burkholderia* and *Serratia* (Garbeva et al., 2004). However, 16S rRNA amplicon sequencing performed on root samples from wheat grown in the same soils (Dennert et al., unpublished results) showed that the relative abundance of *Pseudomonas* (4.4–25.2%) was markedly higher than the relative abundances of *Burkholderia* (0.003–0.53%) and *Serratia* (0.003–1.21%). Therefore, we assume that the *prnD* genes detected in the present study predominantly derive from pseudomonads. Most studies that quantified pseudomonads harboring antimicrobial genes so far were carried out using cultivation-dependent approaches, e.g., colony plating/colony hybridization assays or endpoint dilution assays followed by PCR (Raaijmakers et al., 1997; Meyer et al., 2010; Mavrodi D.V. et al., 2012; Mavrodi O.V. et al., 2012). An examination of genome sequences published by Flury et al. (2016) and other investigators indicated that fluorescent pseudomonads harbor only one copy of *phlD*, *phzF*, or *prnD* per cell. Accordingly, we did not find any significant difference between the abundances of *phlD* and *phzF* quantified by qPCR or a cultivation-dependent endpoint dilution assay in samples from Taenikon (Supplementary Figure S4). Still, qPCR assays can potentially detect viable but non-culturable or even dead cells, thus caution is required when comparing our findings with results of cultivation-dependent experiments.

Studies simultaneously investigating the abundance of several antimicrobial genes in soil are rare. Raaijmakers et al. (1997) used a colony-hybridization assay to quantify pseudomonads harboring DAPG or PHZ genes in different take-all suppressive and conducive US soils. They found pseudomonads harboring DAPG genes to be enriched in suppressive soils, but much less abundant or below the detection limit in conducive soils and they did not detect pseudomonads harboring PHZ genes in any of the tested soils. In our study, we found *phzF*$^+$ pseudomonads to be present in all investigated agricultural soils. However, in general their abundance was quite low, ranging from 2.5 to 5.3 log cells/g root, compared to another US study on soils of the Columbia Plateau of the Pacific Northwest, in which the abundance of PHZ-producing pseudomonads detected by endpoint-dilution assays coupled with *phzF*-specific PCR was up to 100-fold higher in certain soils (Mavrodi D.V. et al., 2012; Mavrodi O.V. et al., 2012). It has been suggested that PHZ producing pseudomonads are more abundant in dryland fields without irrigation, where yearly rainfall ranges from 150 to 300 mm, compared to irrigated fields (Mavrodi O.V. et al., 2012). At the sampling sites of the present study, average annual rainfall was high, ranging from 610 mm per year to 1780 mm per year, which could be a possible reason for

the rather low abundance of *phzF*$^+$ pseudomonads detected here.

The abundance of DAPG producing pseudomonads in the investigated soils varied strongly, from 2.2 to 8.0 log cells/g root. We detected higher but also lower numbers compared to previous studies, which may be explained by the very different types of soils we investigated (**Table 1**). Two studies using terminal endpoint dilution assays followed by *phlD*-specific PCR, one by Meyer et al. (2010) on two Swiss agricultural soils and one by Mavrodi O.V. et al. (2012) on irrigated fields of the Pacific Northwest, detected *phlD*$^+$ pseudomonads at levels ranging from 4.5 to 6.5 log CFU/g on roots of wheat. The population levels of pseudomonads on roots of wheat grown in the Delley soil reported by Meyer et al. (2010) were similar to the numbers of *phlD*$^+$ pseudomonads detected by qPCR in our study. Bacteria harboring *prnD* were detected in the 10 Swiss soils investigated here at abundances comparable to those found in different types of agricultural soils in a previous study (Garbeva et al., 2004).

The abundance of antimicrobial genes, respectively, of the bacteria harboring these genes, does only reflect the bacterial population that potentially could produce a particular antimicrobial compound but is not useful to identify the conditions that favor the proliferation and the consequent niche domination for a given bacterial species, as well as the production of specific antimicrobials *in situ*. The present study is the first to monitor side-by-side the expression of several biosynthetic genes for antimicrobial compounds in different agricultural soils, i.e., in particular *phlA*, *hcnA*, *prnA,* and *phzA*, reflecting the biosynthesis of DAPG, HCN, pyrrolnitrin, and phenazines (Baehler et al., 2005; Chin-A-Woeng et al., 2005; Rochat et al., 2010). This was done by FACS-based monitoring of dual-labeled *Pseudomonas* reporter strains that carry a GFP cell tag and a mCherry-based reporter allowing to record the relative expression of a specific antimicrobial gene in individual cells of a *Pseudomonas* population. A similar technique was used by de Werra et al. (2008) and Rochat et al. (2010) for measuring the expression of *phlA*, *hcnA,* and *prnA* genes by *Pseudomonas* reporter strains on roots of different plant varieties in soilless systems. However, this is the first time that the combination of FACS and fluorescent reporter strains was applied to measure antimicrobial gene expression on plant roots grown in different natural soils. Moreover, to our best knowledge, there were no reports so far on phenazine gene expression in soil or on plant roots. To date, only a handful of studies attempted to determine the expression of antimicrobial genes of pseudomonads in soil (DeCoste et al., 2011; Novinscak and Filion, 2011; Almario et al., 2013b), mainly because of practical challenges. In our study, our initial attempts of tracking fluorescence of reporter strains extracted from roots and soil with microscopy revealed impracticable because of the strong autofluorescence of the investigated soil and root material. To address this problem, we modified the FACS approach of Rochat et al. (2010) and used GFP as a constitutively expressed cell marker and mCherry-based reporters for monitoring antimicrobial gene expression. This approach allowed to specifically track the *Pseudomonas* reporter cells and their expression of select antimicrobial genes in natural soil. However, due to the lack of suitable fluorescent proteins allowing reliable visualization of bacteria cells and gene expression in natural soil, a major limitation of this method is that it is impossible to monitor the expression of two or more antimicrobial genes in the same bacterial cell simultaneously. An alternative method of measuring gene expression in soils involves the extraction of total RNA followed by quantitative reverse transcription PCR of bacterial transcripts of interest. This method was used to quantify the expression of DAPG and HCN biosynthetic genes in natural and artificial soils (DeCoste et al., 2011; Novinscak and Filion, 2011; Almario et al., 2013b). However, the main problem with this approach is the inefficiency of RNA extraction from a complex material such as soil along with the limited abundance of transcripts for antimicrobial genes.

We observed an interesting trend where in soils from Cadenazzo and Vouvry the expression of the four studied genes was markedly enhanced, while lower levels of gene expression occurred in soils from Eschikon, Grangeneuve, and Taenikon. The observation that a specific group of soils is capable of promoting or hampering the expression of four distinct antimicrobial genes in pseudomonads suggests that something in the abiotic or biotic composition of these soils can modulate the production of corresponding plant-beneficial compounds in the *P. protegens* and *P. chlororaphis* reporter strains. Furthermore, we observed that the soil type also influenced the capacity of both pseudomonads to colonize wheat, e.g., plants grown in soils from Cadenazzo and Vouvry supported higher populations than those grown in the Eschikon soil (**Figure 5**). However, the differences in root colonization by the two pseudomonads do not explain entirely the difference in gene expression observed in the different soils (**Figures 4, 5** and Supplementary Table S3).

The relative importance of the abundance of *Pseudomonas* spp. for suppression of soilborne pathogens was questioned recently (Kyselkova et al., 2014). Indeed, DAPG and HCN producing pseudomonads could be isolated not only from suppressive but also from conducive soils, leading to the hypothesis that differential environmental factors prevailing in the two types of soils may shape the expression of relevant biocontrol genes in *Pseudomonas* bacteria and thus disease suppression (Ramette et al., 2006). Moreover, the abundance of the DAPG biosynthesis gene *phlD* was not indicative of the resistance of soils to black root rot of tobacco (Almario et al., 2013b; Kyselkova et al., 2014). Our results support the hypothesis that the abundance of pseudomonads producing antimicrobial metabolites may, in fact, be less important for disease suppression in the rhizosphere than previously hypothesized, at least for certain soils. We failed to detect any significant positive correlation between the resistances to the two soilborne pathogens Pu or Gt and the abundance of *Pseudomonas* harboring antimicrobial genes in the investigated agricultural soils (**Figure 7**). The lack of a significant positive correlation between disease resistance and abundance of antimicrobial pseudomonads could also be because only the *phlD*$^+$ bacteria were detected at more than 10^5 cells/g root, which is close to the threshold considered to be relevant for biocontrol activity

(Raaijmakers et al., 1999; Haas and Défago, 2005) in many soils. By analogy, the populations of PHZ and PRN producing pseudomonads might therefore also be too low to contribute to efficient pathogen suppression in the soils of our study. Although correlations between gene abundances and disease resistance were never significant, it is still worth to note that for both pathogens they were mostly positive (**Figure 7A**). The expression of antimicrobial genes by reporter strains cannot be directly correlated to pathogen suppression data in the present study. Nevertheless, our findings suggest that high expression levels of antimicrobial genes in a particular soil may not necessarily be indicative of high levels of pathogen suppression (**Figure 6**). Furthermore, soils supporting the high expression of antimicrobial genes mostly harbored lower numbers of antimicrobials-producing pseudomonads (**Figure 6**). We thus speculate that the specific biotic and abiotic factors operating in the distinct agricultural soils might differently influence the abundance and expression of antimicrobial genes. At the present stage, it remains therefore elusive to which extent and how exactly pseudomonads producing DAPG, PHZ, and PRN contribute to the disease resistance of the investigated agricultural soils. Moreover, only two soilborne pathogens were investigated here and individual soils strongly differed in their response to Pu and Gt. It is likely that different relations to gene abundances and expression would be found for other pathogen and plant species.

Soil nutrients are known to be important factors influencing resistance to pathogens (Martin and Hancock, 1986; Löbmann et al., 2016) and the abundance of pseudomonads producing antimicrobial compounds (Meyer et al., 2010; Kim et al., 2013). Recently, Löbmann et al. (2016) found that soils can influence the resistance to Pu in two ways: through abiotic effects that inhibit pathogen growth, which was the case in soils with a high pH, high calcium, and high clay content; or through balanced nutrient contents, which were hypothesized to stimulate the proliferation of plant-beneficial microorganisms. Löbmann et al. (2016) postulated this effect in soils with high P, K, Mg, sand and organic matter contents, although the involved plant-beneficial microorganisms were not identified in their study. In our study, we found significant positive correlations between Pu resistance and the contents of nitrate and Mg in soil. However, the same two nutrients were also positively correlated with the abundance of the DAPG biosynthesis gene *phlD,* so we cannot conclude on how exactly these nutrients have a positive impact on soil resistance. They could have a negative effect on the pathogen, have a positive effect on the growth of the plants and increase their pathogen resistance, or have an indirect positive effect on soil resistance by promoting beneficial bacteria.

We found no significant correlation between pH and abundance of *Pseudomonas* harboring antimicrobial genes, probably because the soils of this study all had a pH close to neutral and ranged from 6.0 (Cadenazzo) to 7.7 (Cazis) (**Table 1**). The influence of pH of agricultural soils on the population sizes of pseudomonads producing antimicrobial compounds is poorly understood. Previously, indigenous pseudomonads were found to be more abundant in soils with a neutral pH than in soils with an acidic pH (Kim et al., 2013). Likewise, *P. protegens* CHA0 inoculated into soils with an acidic pH reached lower population sizes compared to when it was inoculated into soils with neutral or basic pH (Mascher et al., 2013).

We obtained contrasting results for clay and silt, where clay was positively and silt negatively correlated with gene abundance (**Figure 8**). For gene expression, the opposite was the case. The precise relation between *Pseudomonas* abundance and clay content is not known. However, high clay content in soil was previously shown to reduce the expression of the HCN biosynthetic gene *hcnC* (Novinscak and Filion, 2011) and the biocontrol activity of phenazine-producing strains (Ownley et al., 2003). The pronounced effect of clay on biocontrol activity of pseudomonads was also observed in studies carried out in artificial soils (Keel et al., 1989; Almario et al., 2013b, 2014). In particular, vermiculitic clay supported a higher level of biocontrol activity and HCN production than illitic clay (Keel et al., 1989), and the expression of *phlA* was greater in the presence of vermiculite than in the presence of illite (Almario et al., 2013b).

The plant macronutrients nitrate, potassium and magnesium also inversely influenced abundance and expression of antimicrobial genes. To the best of our knowledge, it has not been investigated if high macronutrient contents in the soil directly stimulate the growth of antimicrobial pseudomonads. We hypothesize that the positive correlation between abundance of antimicrobial pseudomonads and certain macronutrients such as nitrate could also be due to an indirect effect, e.g., via stimulation of plant growth and increased root exudation. Support for this hypothesis comes from a recent study on maize roots, where nitrogen concentration in soil was found to be positively correlated with plant root exudation and abundance of rhizosphere bacteria (Zhu et al., 2016). For the micronutrients, a significant, though negative, correlation was found only for manganese and the expression of the HCN and PHZ biosynthetic genes (**Figure 8**). However, in previous studies, several micronutrients were described as factors that affect abundance, gene expression, metabolite production and biocontrol activity in pseudomonads. For instance, copper negatively influenced the abundance of *Pseudomonas* spp. in agricultural soils (Brandt et al., 2006), while the bioavailability of iron affected the expression of *phlA* and the production of HCN in artificial soils (Keel et al., 1989; Almario et al., 2013b). The biocontrol activity of phenazine-producing strains was positively correlated to zinc and negatively to iron and manganese levels in soil (Ownley et al., 2003). Moreover, the production of several antimicrobial metabolites, notably DAPG, pyoluteorin and PRN by strain *P. protegens* CHA0 was stimulated by zinc, but these experiments were performed in culture media (Duffy and Défago, 1997, 1999). The lack of significant correlations between micronutrient contents in soil and abundance and expression of antimicrobial genes in our study could be due to the relatively high micronutrient content in the sampled soils. In fact, all studied soils had sufficient amounts of micronutrients according to the classification for farmers approved by the Swiss government (Flisch et al., 2009).

Boron concentration was an exception, as three out of 10 sampled soils tested as poor (**Table 1**).

CONCLUSION

Results of this study suggest that resistance of soils to pathogens, and abundance and expression of antimicrobial genes are not generally positively or negatively correlated in a wide range of diverse agricultural soils. Complex interactions depending on the host–pathogen system and the soil composition determine pathogen resistance of soils and the abundance and expression of antimicrobial genes. The abundance of antimicrobial metabolites producing pseudomonads in the investigated agricultural soils and the expression of biosynthetic genes for these compounds as studied here using reporter strains seem to be differentially shaped by multiple soil factors. This could explain, at least in part, why soils that sustain high numbers of these bacteria, often support only low levels of antimicrobial gene expression and vice versa. Therefore, to better understand the links between soil characteristics and abundance and expression of antimicrobial genes in pseudomonads, future studies should include extreme soils (i.e., highly acidic or alkaline soils or soils enriched in or depleted of specific nutrients). Pseudomonads are probably only one among many microbial groups determining the natural pathogen tolerance or resistance of agricultural soils (Mendes et al., 2011; Kyselkova et al., 2014; Cha et al., 2016; Raaijmakers and Mazzola, 2016). In future work, the potential of *Pseudomonas* bacteria as bio-indicators of soil resistance has to be re-evaluated with care. It will probably be difficult to identify specific groups of microorganisms as general indicators of soil health and disease resistance, since natural disease suppression likely requires individual compositions of the beneficial microbiota depending on the soil type, the crop species, the soilborne disease and maybe even the cropping system.

AUTHOR CONTRIBUTIONS

NI, FD, MM, and CK designed the research. MM and CK supervised the study. MF and FM organized the soil sampling and soil analysis. FD performed pathogen resistance greenhouse experiments. FD, DM, and OM developed the qPCR assays. FD performed the DNA extractions and qPCR assays. NI developed the reporter strains and performed the gene expression experiments. FD and NI analyzed the data. TL, JS, MW, and CV assisted with experiments and/or data evaluation. FD, NI, MM, and CK wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00427/full#supplementary-material

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Potential of Finger Millet Indigenous Rhizobacterium *Pseudomonas* sp. MSSRFD41 in Blast Disease Management—Growth Promotion and Compatibility With the Resident Rhizomicrobiome

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Finger millet [*Eleusine coracona* (L). Gaertner] "Ragi" is a nutri-cereal with potential health benefits, and is utilized solely for human consumption in semi-arid regions of Asia and Africa. It is highly vulnerable to blast disease caused by *Pyricularia grisea*, resulting in 50–100% yield loss. Chemical fungicides are used for the management of blast disease, but with great safety concern. Alternatively, bioinoculants are widely used in promoting seedling efficiency, plant biomass, and disease control. Little is known about the impact of introduced indigenous beneficial rhizobacteria on the rhizosphere microbiota and growth promotion in finger millet. Strain MSSRFD41 exhibited a 22.35 mm zone of inhibition against *P. grisea,* produces antifungal metabolites, siderophores, hydrolytic enzymes, and IAA, and solubilizes phosphate. Environmental SEM analysis indicated the potential of MSSRFD41 to inhibit the growth of *P. grisea* by affecting cellular functions, which caused deformation in fungal hyphae. Bioprimed finger millet seeds exhibited significantly higher levels of germination, seedling vigor index, and enhanced shoot and root length compared to control seeds. Cross streaking and RAPD analysis showed that MSSRFD41 is compatible with different groups of rhizobacteria and survived in the rhizosphere. In addition, PLFA analysis revealed no significant difference in microbial biomass between the treated and control rhizosphere samples. Field trials showed that MSSRFD41 treatment significantly reduced blast infestation and enhanced plant growth compared to other treatments. A liquid formulated MSSRFD41 product maintained shelf life at an average of 10^8 CFU ml^{-1} over 150 days of storage at 25°C. Overall, results from this study demonstrated that *Pseudomonas* sp. MSSRFD41, an indigenous rhizobacterial strain, is an alternative, effective, and sustainable resource for the management of *P. grisea* infestation and growth promotion of finger millet.

Keywords: pseudomonads, bioinoculants, indigenous, compatibility, 2, 4-DAPG, finger millet, formulation, *P. grisea*

INTRODUCTION

The problems associated with indiscriminate use of chemical pesticides in agriculture have led to increasing interest in the use of native and non-native beneficial microorganisms to improve plant health and to increase crop productivity while ensuring food safety and environmental protection (Verma et al., 2013; Santhanam et al., 2015; Souza et al., 2015; Sharma et al., 2017; Schütz et al., 2018). The use of bioinoculants and the exploitation of novel beneficial plant microbes offer promising, sustainable and eco-friendly strategies in conventional and organic agriculture systems worldwide (Negi et al., 2015; Gopalakrishnan et al., 2016; Zhou et al., 2016). Reports have shown that both conventional and organic growers indicate an interest in using microbial inoculants. Approximately 43 million hectares of land have been devoted to organic agriculture using inputs like plant growth promoting bacteria, biocontrol agents, and arbuscular mycorrhizal fungi (AMF) across the globe, and these practices have had positive impacts on health and the quality of food, the environment, and soil (Lernoud and Willer, 2016). The growing demand for bioinoculants is reflected by the fact that in 2016, bioproducts accounted for $1.50 billion and are expected to increase at a compound annual growth rate (CAGR) of 14.1% to reach $3.79 billion by 2023 (Souza et al., 2015; Sekar et al., 2016; Lori et al., 2017; Stratistics Market Research Consulting, 2017).

Among the plant-associated microbes, pseudomonads are dominant bacteria known to protect plants against several phytopathogens (Radjacommare et al., 2005; Waghunde et al., 2013; Yin et al., 2013; Sekar and Prabavathy, 2014; Wang et al., 2015), and to promote plant growth under several abiotic stress conditions (Wang et al., 2015; Jegan et al., 2016). The biocontrol ability of pseudomonads is directly correlated with production of various antibiotics such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines, pyrrolnitrin (PRN), pyoluteorin (PLT), hydrogen cyanide (HCN), and lytic enzymes (Radjacommare et al., 2005; Sekar and Prabavathy, 2014; Wang et al., 2015; Ganga et al., 2016). Among these, 2,4-diacetylphloroglucinol (DAPG) exhibits a broad range of antagonistic activity against phytopathogens, and can induce host plant systemic resistance (Weller et al., 2012; Jegan et al., 2016; Viswanath et al., 2016; Yan et al., 2017). Numerous studies on the biocontrol of phytopathogens and plant growth promotion by pseudomonads are available (Yin et al., 2013; Gopalakrishnan et al., 2016; Zhou et al., 2016; Yan et al., 2017), but very little research has been carried out on the control of *P. grisea* by pseudomonads in a highly nutritive crop like millet (Radjacommare et al., 2005; Senthil et al., 2012; Waghunde et al., 2013; Negi et al., 2015).

Nutri-cereals like millets have increasingly gained attention across the world for its health benefits. Finger millet [*Eleusine coracona* (L). Gaertner], also known as "Ragi," is one of the minor millet cereals which is solely utilized for human consumption in the semi-arid tropics of Asia and Africa (Waghunde et al., 2013; Jegan, 2015; Negi et al., 2015; Kumar et al., 2016; Gupta et al., 2017). This "nutritious millet" offers several health benefits, as it is rich in calcium (0.38%), dietary fiber (18%), and phenolic compounds (0.3–3%). It is also recognized for its beneficial health effects including antidiabetic, antitumorigenic, and atherosclerogenic effects, and antioxidant and antimicrobial properties (Kumar et al., 2016). India is the major producer of finger millet in Asia and it is the staple food for millions of people in the states of Karnataka, Tamil Nadu, Andhra Pradesh, Orissa, Maharashtra, and Bihar, with annual production of 2.2 million tons over an area of 1.6 million ha (Jegan, 2015; Kumar et al., 2016; Gupta et al., 2017).

Although finger millet is considered a hardy crop, it is affected by more than 20 diseases, of which blast disease caused by *Pyricularia grisea* is the most devastating (Prajapati et al., 2013; Waghunde et al., 2013; Magar et al., 2015; Cruz and Valent, 2017). The pathogen infects different parts and stages of the plants from seedling to grain formation, causing diamond-shaped lesions and premature drying of young leaves, affecting the panicle and causing neck and/or finger blast leading to yield loss up to 100% that result in economic loss to farmers and ultimately food crisis (Senthil et al., 2012; Prajapati et al., 2013; Negi et al., 2015). Control of blast disease is a serious and challenging issue relying heavily on chemical pesticides like organophosphorus fungicides which have been reported to be highly effective (Kumar and Kumar, 2011; Magar et al., 2015). However, extended use of chemical pesticides has resulted in the development of pesticide-resistant fungal pathogens, with negative effects on the ecosystem, soil fertility, and water quality, leading to serious health problems including birth defects (Hawkins et al., 2014; Hollomon, 2016). Hence globally, there is a huge demand for pesticide-free food which is safe and nutritious.

Bioinoculants, an alternative to synthetic chemical pesticides, offer multiple beneficial traits; they can ensure the production of quality grains, protect plants against biotic and abiotic stresses; enhance soil fertility, and are sustainable and environmental safe. The development and application of indigenous bioinoculants products has gained momentum among researchers, because they can play a vital role in plant growth promotion and crop protection in sustainable farming systems, and also for their economic value (Schreiter et al., 2014; Santhanam et al., 2015; Sekar et al., 2016; Cai et al., 2017). However, the performance of bioinoculants in the field depends highly on their survival and ability to express key traits in the soil without adversely impacting the native soil microbial community (Gupta et al., 2015; Thomas and Sekhar, 2016; Sharma et al., 2017). Many studies have reported a gradual reduction of introduced bioinoculants in the soil over the period of plant growth, but fewer have addressed the effects of the bioinoculants on the rhizosphere bacterial community (Chowdhury et al., 2013; Yin et al., 2013; Kröber et al., 2014; Schreiter et al., 2014; Thomas and Sekhar, 2016; Sharma et al., 2017). In addition, many bioinoculants have shown promising biocontrol and plant growth promotion under *in vitro* conditions, but exhibit variable performance in greenhouse and field trials (Bulgarelli et al., 2013; Mahmood et al., 2016; Gouda et al., 2018). Hence, identifying suitable crop specific bioinoculants is critical for growth promotion

Abbreviations: 2,4-DAPG, 2,4-diacetylphloroglucinol; PGPR, plant growth-promoting rhizobacteria; Biopriming; IAA, Indole-3-acetic acid; *P. grisea*; Blast; ESEM, Environmental scanning electron microscopy.

and disease suppression under variable ecological conditions. Therefore, this study attempted to investigate the efficiency of indigenous rhizosphere *Pseudomonas* sp. MSSRFD41 for plant growth promotion and protection against the *P. grisea* blast pathogen under *in vitro* and *in vivo* conditions. *Pseudomonas* sp. MSSRFD41, isolated from the rhizosphere of finger millet in India, is a novel 2,4-DAPG-producing strain with potential biocontrol and plant growth promoting traits (Sekar and Prabavathy, 2014). The present investigation aims (i) to assess the potential traits of MSSRFD41 involved in the inhibition of *P. grisea, in vitro*; (ii) to determine the survival of MSSRFD41 in the rhizosphere and its impact on the rhizosphere microbial community; and (iii) to determine the efficacy of MSSRFD41 in blast disease control and growth promotion in finger millet under field conditions.

MATERIALS AND METHODS

Source and Growth Conditions of Strain MSSRFD41 and *P. grisea*

Pseudomonas sp. MSSRFD41 was isolated from the rhizosphere of finger millet cultivated in the Dharmapuri district of Tamil Nadu, India (Sekar and Prabavathy, 2014). The active culture was maintained in King's B agar (KBA) medium (King et al., 1954) at 28°C and stored at −80°C in phosphate-buffered 20% (v/v) glycerol. *P. grisea* TN508 was obtained from Tamil Nadu Agricultural University culture collection center—Coimbatore and maintained in Oat meal agar and sterile finger millet seeds.

In vitro Screening for Antifungal Activity of MSSRFD41

The antagonistic activity of MSSRFD41 was assayed against the blast pathogen, *P. grisea* TN508 by the dual plate method in KBA agar medium. A 6 mm diameter plug of actively growing 10 days old fungal culture was inoculated in the middle of the agar plates and incubated at 28°C for 48 h. Later, a loopful of an actively growing culture of the *Pseudomonas* was inoculated 3.5 cm away from the fungal disc on two sides. Plates without MSSRFD41 served as control. The plates were incubated at 28°C for 10 days and examined for inhibition of fungal growth by MSSRFD41.

Profiling of Crude Metabolite by GC-MS

Crude metabolites were extracted from 100 ml broth of MSSRFD41 grown in King's B medium at 28°C for 48 h. The supernatant was collected by centrifuging at 9,300 g for 10 min and the pellet was discarded. The supernatant was acidified with concentrated HCl to pH 2.0 and then extracted twice with an equal volume of ethyl acetate. The extract was pooled and concentrated using a rotary evaporator at 35°C and then stored at −20°C for further use. The concentrated crude metabolites obtained from the culture broth were dissolved in 1 ml methanol: chloroform mixture (1:1) and analyzed by GC-MS (MassHunter GC/MS, Agilent Technologies, US). The compounds were identified by comparison of mass spectra with the National Institute of Standards and Technology (NIST database) library and by direct comparison with published data (Dheepa et al., 2016).

Environmental Scanning Electron Microscope Analysis of *P. grisea* Mycelium

Morphological changes of the hyphae of *P. grisea* in control cultures and cultures co-inoculated with MSSRFD41 were determined through Environmental Scanning Electron Microscopy (ESEM). Mycelial bits were cut from the actively growing edge of the fungal cultures and directly subjected to ESEM analysis. Images of samples were taken by FEI Quanta 200—High-Resolution Scanning Electron Microscope at a voltage of 8 kV and a pressure from 500 to 600 Pa.

Determination of Hydrolytic Enzyme Activity

Strain MSSRFD41 were tested for the production of chitinase as described by Kole and Altosaar (1985) in Dworkin-Foster (DF) salts minimal medium containing 2.5% (w/v) colloidal chitin. Cellulase activity was determined in carboxymethyl cellulose (CMC) agar containing 5% (w/v) CMC (Sigma Aldrich, USA) (Ariffin et al., 2008). Proteolytic activity was assessed using skimmed milk agar (Hi Media, India) (Wikström, 1983).

A overnight grown culture of MSSRFD41 was inoculated into media containing peptone 10 g l^{-1}, NaCl 5 g l^{-1}, $CaCl_2$ $2H_2O$ 0.1 g l^{-1}, agar 18 g l^{-1}, and 1% of sterilized Tween 80 (Hi Media, India) for detection of esterase activity or Tween 20 (Hi Media, India) for lipolytic activity as described by Sierra (1957). After incubation at 28°C for 48 h, a clear halo zone around the colony was considered as positive.

Amylase activity of MSSRFD41 was determined by inoculating in starch agar (Hi Media, India) plates containing starch as the only carbon source. After incubation at 28°C for 48 h, plates were stained with Gram's iodine solution and the formation of a clear halo zone in the starch agar around the colony indicated amylase production (Cappuccino and Sherman, 1992).

Strain MSSRFD41 was assessed for inorganic phosphate solubilization by inoculating a freshly grown culture in the National Botanical Research Institute's phosphate (NBRIP) agar medium contained glucose, 10 g l^{-1}, $Ca_3(PO_4)_2$, 5 g l^{-1}, $MgCl_2{\cdot}6H_2O$, 5 g l^{-1}, $MgSO_4{\cdot}7H_2O$, 0.25 g l^{-1}, KCl, 0.2 g l^{-1}, $(NH_4)_2SO_4$, 0.1 g l^{-1}, and agar 18 g l^{-1} (Nautiyal, 1999). The plates were incubated at 28°C for 5 days and the diameter of a clear halo zone around the bacterial colony indicating solubilization of mineral phosphate was measured.

Siderophores Production

Strain MSSRFD41 was grown overnight at 28°C in King's B broth and spotted on Blue Agar Chromeazurol "S" plates and incubated at 28°C. After incubation, production of siderophores was detected by the appearance of orange-halo zones against a blue background (Louden et al., 2011).

Quantification of Indole-3-Acetic Acid

An overnight culture of strain MSSRFD41 grown on Luria-Bertani (LB) medium was inoculated into tubes containing 5 ml LB broth supplemented with 100 μg ml^{-1} L-tryptophan and incubated at 28°C. Samples were collected after 72 h of incubation and the bacterial cells were removed by centrifugation

at 6,500 g for 10 min. A 1 ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent and incubated in the dark for 20 min at room temperature. IAA production was observed as the development of a pink-red color and absorbance was measured at 535 nm using a spectrophotometer. The concentration of IAA was quantified by comparison with a standard curve prepared with 5–100 μg ml^{-1}of pure IAA (Sigma Chemicals, India) (Patten and Glick, 2002).

Ammonia Production

Ammonia production was assessed qualitatively by inoculating 10 ml of peptone broth with strain MSSRFD41 and incubating the tubes at 28°C for 48 h. After incubation, drops of Nessler's reagent (Hi Media, India) were added and development of a yellowish brown color indicated the production of ammonia (Cappuccino and Sherman, 1992).

Impact of MSSRFD41 on Soil and Rhizobacterial Isolates

The compatibility and impact of strain MSSRFD41 on 129 isolates from indigenous and non-indigenous rhizosphere and soil sources were assessed by cross-streaking. The isolates were obtained from the M.S. Swaminathan Research Foundation (MSSRF) culture collection and are described in Table S1. A fresh culture of MSSRFD41 grown overnight at 28°C in King's B broth was streaked across the center of a KBA plate and the test isolates were streaked at right angles to MSSRFD41. The plates were incubated at 28°C for 72 h and observed for growth inhibition.

In Vitro and *in Vivo* Plant Growth Stimulation by MSSRFD41

Cultures of strain MSSRFD41 were grown overnight in 50 ml KB broth at 28°C on a rotary shaker at 180 rpm. The bacterial culture was pelleted by centrifugation at 6,500 × g for 10 min and the supernatant was discarded. Bacterial pellets were washed twice with 5 ml of sterile 0.03 M $MgSO_4$, and the final suspension was adjusted spectrophotometrically to an OD_{600} of 0.5 with 0.03 M $MgSO_4$, corresponding to a cell density of 10^8 cells ml^{-1}.

To determine colonization efficiency, finger millet seeds were surface sterilized and inoculated with a bacterial suspension following the method described by Patten and Glick (2002). Approximately 50 g seeds were surface sterilized by washing in 10 ml of 70% ethanol for 1 min and residues was removed by washing the seeds five times with sterile distilled water. The sterilized millet seeds were incubated with 5 ml of bacterial suspension (OD_{600} of 0.5) at room temperature for 4 h to allow the bacteria to bind to the seed, and control seeds were incubated in sterile 0.03 M $MgSO_4$ under the same conditions. The treated and control seeds were spread on KBA medium and incubated overnight at room temperature to check for contamination.

The impact of seed priming with strain MSSRFD41 was assessed with ten seeds per treatment that were transferred aseptically to presoaked sterile germination paper, aseptically rolled, and placed inside a test tube containing sterilized half-strength Hoagland's solution (ISTA, 1993). Tubes were incubated in a growth chamber with an 8 h of darkness and 16 h of light and 65% humidity at 25°C. On the 4, 6, and 8th day of incubation, 3 tubes per treatment were removed from the growth chamber and biometric parameters *viz*., seed germination, root and shoot length, and fresh and dry biomass were measured. The seed vigor index between treatments was calculated by using the formula: Vigor index = (Mean root length + Mean shoot length) X Germination (%) (Abdul-Baki and Anderson, 1973).

For pot experiments, millet seeds were surface sterilized, as described above and germinated in a seedling tray filled with vermiculite. Fifteen days after sowing, seedlings were dipped in an inoculum of MSSRFD41 (10^8 CFU ml^{-1}) and control seedlings were dipped into water. After 1 h of incubation, two seedlings were transplanted from the treatment to the respective pots (17 × 17 × 16 cm) filled with 2.5 kg of agricultural soil. The treatments were arranged in a completely randomized block design with four pots of each replication and three replications per treatment. Pots were maintained under greenhouse conditions at 35 ± 2°C with a photoperiod of 12/12 h (light/ dark) and watered periodically. Plant growth response biometric parameters including shoot and root length, straw fresh and dry weight, the fresh and dry weight of root were measured at 20, 60, and 100 days after transplantation (DAT).

Rhizosphere Colonization and Population Dynamics of Strain MSSRFD41

Root adherent soils were collected by uprooting the control and MSSRFD41 treated seedlings of 20, 60, and 100 days old. The cultivable rhizobacterial population dynamics were determined using Kings B agar—KBA, and pseudomonads community was assessed using selective media like Kings B agar + sodium lauroyl sarcosine + Trimethoprim (20 mg^{-1})—KBA+SLST (Gould et al., 1985) and Kings B agar + Cetrimide + nalidixic acid (15 μg ml^{-1})—KBA+CN (Goto and Enomoto, 1970). The rhizosphere soil samples were diluted up to 10^{-6} in 1% (v/v) PBS (phosphate buffered saline—pH 7.2) and plated onto the above media in triplicates. The plates were incubated at 28°C for 3 to 4 days and log CFU g^{-1} fresh weight of rhizosphere soils were determined.

To monitor populations of strain MSSRFD41 over time, bacterial colonies were picked from the dilution plates of selective media from both the control and treated samples and streaked on fresh KBA medium. DNA was extracted from isolated rhizobacteria and DNA fingerprinting analysis was carried out with BOX primer 5′-ACG GCA AGG CGA CGC TGA CG-3′ and compared to reference DNA from strain MSSRFD41 as described by Jegan (2015). The BOX-PCR genetic patterns were visualized by UV illumination at 365 nm and documented using a Bio-Rad Gel Doc system (Bio-Rad, USA). Normalization, recognition, and assignment of bands on the gel were performed with the GelJ analysis DNA fingerprint tool by the Dice coefficient (Heras et al., 2015). The unweighted pair group with mathematical average (UPGMA) algorithm was performed for cluster analysis with similarity matrices for generation of a dendrogram. Rhizobacterial isolates showed similar fingerprinting pattern to reference DNA were identified using 16S rRNA sequencing as described by Sekar and Prabavathy (2014).

Phospholipid Fatty Acid (PLFA) Analysis

Rhizosphere soil samples were collected from 100-day-old control and MSSRFD41-treated plants, were pooled according to treatment, and outsourced to the Royal Research Laboratories (Secunderabad, India) for PLFA analysis and to calculate the biomass (nmol g^{-1} of dry rhizosphere soil) for each microbial type present. Samples were extracted as described by (Buyer and Sasser, 2012) and analyzed by gas chromatography and the MIDI Sherlock Software v.6.2B PLFA Package (Agilent 6890N Series). Sherlock PLFA Tools software was used to calculate the biomass, mole percent, and key PLFA ratios nmol g^{-1} of rhizosphere soil dry weight.

Field Trial

A field trial was conducted in a farmer's field located in Ramiyanahalli, Pappireddipatti taluk Dharmapuri district, Tamil Nadu (12°02′16.7″ N 78°22′21.5″ E). The trial consisted of five treatments T1–Control; T2–chemical (Carbendazim−2 g^{-1}); T3–C-PF (commercial Ecomonas *P. fluorescens*−10 g^{-1}); T4–Bio + 50% chemical (*Pseudomonas* sp. MSSRFD41–5 ml^{-1} + Carbendazim−1 g^{-1}); and T5–MSSRFD41 (5 ml^{-1}). Farmyard manure at the rate of 7.5 t per ha were applied to the field and plot layouts were prepared in Randomized Block Design (RBD) in triplicates with an individual plot size of 6 × 4 m (Figure S1). One kg of seeds for each treatment were treated with the above as described dose, dried in the shade for 3 to 4 h, and sown in the respective seed beds. After 25 days, seedlings were uprooted, dipped into the respective treatments, incubated for 1−2 h and planted, two seedlings per hill, at a depth of 4−5 cm with a distance of 10 cm between seedlings in the respective treatments plots. Foliar sprays were given separately to the plots according to the treatment after 30 and 60th DAT. The percentage of natural disease incidence was assessed at 90th day by monitoring blast infestation in plants using the following formula:

$$\text{Percent disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

After 90 days randomly 20 plant samples were harvested from each plot and the root, shoot length, and 1,000 seed weight were measured.

Preparation of Liquid-Based Formulation of MSSRFD41

An overnight culture of MSSRFD41 grown in 100 ml KB broth with 5.08 log CFU ml^{-1} was pelleted and suspended in 1 liter of sterilized KB broth with 5% polyvinylpyrrolidone (PVP), 3% glycerol and 1.5% polyethylene glycol 6000 (PEG). The formulated product was incubated at 4, 8, 27, and 37°C in static conditions in triplicate. The viable population in the formulated product was determined at 3, 7, 15, 30, 60, 90, 120, and 150 days of storage by serial dilution and plating on nutrient agar medium (Goljanian-Tabrizi et al., 2016).

Data Analysis

Data were analyzed using SPSS (Version 22) and GraphPad Prism 6. Significant differences among treatments were determined by applying a one-way ANOVA, and Tukey's Honestly Significant Difference (Tukey's HSD) *post-hoc* tests were used for mean separation when ANOVA results were significant ($P < 0.05$).

RESULTS

Antifungal Effect of MSSRFD41

P. grisea TN508 inoculated in control KBA plates showed regular growth without any inhibition of mycelial growth (**Figure 1A**). In dual culture plates, strain MSSRFD41 showed significant inhibition of *P. grisea* TN508 mycelial growth, producing a 22.35 mm zone of inhibition (**Figure 1B**). GC-MS of crude metabolite revealed the presence of 47 different metabolites which included bioactive compounds in the groups of acids, esters, alcohols, nitrogenous compounds, aldehydes, and volatiles. Based on the retention time and comparison with NIST database presence of potential antifungal compounds including 2,4-DAPG derivatives, octasiloxane, pyrrolo, 2,5-piperazinedione, 1,2-benzenedicarboxylic acid, hexadecanoic acid, octadecenoic acid, pyran, propenoic acid, and dasycarpidan was detected (**Table 1**). SEM analysis was employed to examine the structural changes of *P. grisea* in control and dual culture plates with strain MSSRFD41. Mycelia obtained from the edges of the fungus grown in control plates showed hyphae with typical net structure with smooth surfaces lacking visible damage (**Figure 1C**). Co-culturing with strain MSSRFD41 resulted in the presence of damaged hyphae showing loss of smoothness, aberrant shape, and forming unusual bulges in the hyphal network (**Figure 1D**).

Physiological Traits Detected *in Vitro*

MSSRFD41 formed clear zones around colonies grown in the presence of chitinase (8 mm), protease (25 mm), cellulase (11 mm), esterase (13 mm), lipase (10 mm), and amylase (21 mm) (**Table 2**). Phosphate solubilization was observed in NBRIP medium as a 7.08 mm clearance zone around the colony. Siderophore production was detected on the basis of a 12.8 mm zone of color change from blue to orange in CAS medium of 12.8 mm. In the presence of tryptophan, strain MSSRFD41 produced IAA at a concentration of 29 μg ml^{-1} after 72 h, and 29 μg ml^{-1} of ammonia was detected from peptone broth. Dual cultures of different rhizobacterial isolates and strain MSSRFD41 revealed no growth inhibition (Figure S2), indicating that the strain was compatible with groups of indigenous and non-indigenous rhizobacteria. This pilot scale assay revealed strain MSSRFD41 is compatible in the rhizosphere without impairment to other rhizobacterial isolates.

Effect of MSSRFD41 Biopriming on Finger Millet Germination and Vigor

Priming of finger millet seeds with a suspension of MSSRFD41 had a significant effect on the germination percentage, plant biomass and vigor index compared to the control at different time intervals. Treated millet seeds showed significantly increased germination by 7.44% compared to control on the 8th day of incubation, while the vigor index was increased by 21.58%. Priming of millet seeds with MSSRFD41 significantly improved

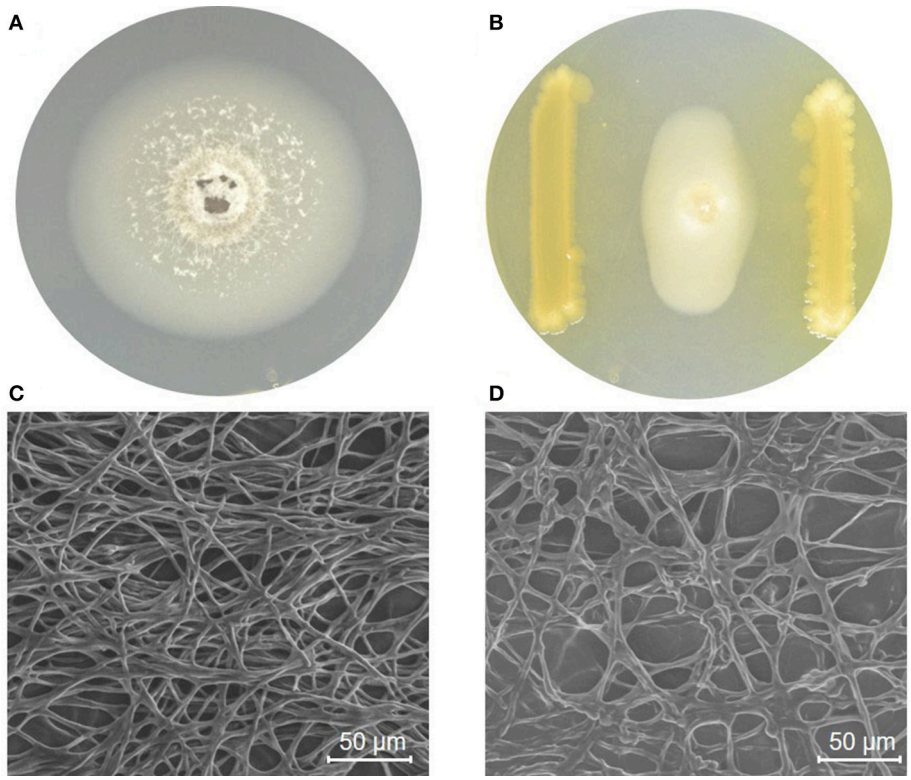


FIGURE 1 | MSSRFD41 inhibiting mycelial growth of *P. grisea* in a dual plate culture. **(A)** *P. grisea* control; **(B)** *P. grisea* with MSSRFD41. Scanning electron microscopic studies on the impact of MSSRFD41 on hyphae of *P. grisea,* **(C)** Control hyphae without MSSRFD41; **(D)** Antagonized hyphae with MSSRFD41.

TABLE 1 | Antimicrobial compounds identified from *Pseudomonas* sp. MSSRFD41 strain through GC/MS.

S. No	Name of the compound	Peak Number	Peak Area %	Retention time
1.	3,3,3-Trifluoro-2-(2-methoxy-benzoylamino)-2-(4-methyl-thiazol-2-ylamino)-propionic acid methyl ester	8	9.24	4.895
2.	Z-10-Methyl-11-tetradecen-1-ol propionate	13	9.9	5.164
3.	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans	16	16.55	5.39
4.	2-Propenoic acid, 3-phenyl-	21	30.34	5.755
5.	1-Heptatriacotanol	27	5.45	6.269
6.	Phenol, 2,5-bis(1,1-dimethylethyl)-	28	54.03	6.346
7.	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-	30	41.1	6.485
8.	2H-Pyran, 2-(7-dodecynyloxy)tetrahydro-	33	10.52	6.793
9.	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	37	13.22	7.549
10.	(3S,6S)-3-Butyl-6-methylpiperazine-2,5-dione	40	29.33	8.508
11.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	49	5.43	10.129
12.	n-Hexadecanoic acid	54	42.46	11.376
13.	7-Methyl-Z-tetradecen-1-ol acetate	57	26.95	12.364
14.	Piperidine-3-carboxylic acid, 1-(5,8a-dimethyl-2-oxo-2,3,3a,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-3-ylmethyl)-, ethyl ester	61	43.21	14.234
15.	tert-Hexadecanethiol	65	24.17	14.878
16.	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	69	44.19	17.706
17.	Dasycarpidan-1-methanol, acetate (ester)	70	34.02	17.99

the length of shoots (19.58%) and roots (11.65%) compared to the control (Figure S3). Increased germination rate, plant length and vigor index were observed in the MSSRFD41 primed seedlings (**Table 3**). At all-time points, the treated seeds performed better than the control, suggesting that priming could enhance the germination, biomass, and vigor of millet seeds.

Impact of MSSRFD41 on Growth Promotion of Finger Millet in Pots

Under greenhouse conditions, seedlings treated with strain MSSRFD41 had increased shoot (61.54 cm) and root length (17.30 cm) that were significantly greater than those of control seedlings, with differences of 13–26% among treatments and days after transplanting (DAT). The maximum straw fresh weight (8.43 g), straw dry weight (3.53 g); root fresh weight (4.01 g) and root dry weight (2.58 g) were observed from samples of 100 DAT. Values for straw fresh weight (6.84 g), dry weight (2.42 g), root fresh weight (3.07 g), and dry weight (1.78 g) were lower for the control seedlings (Figure S5). At 20 DAT, treated and control seedlings did not differ significantly by biometric analysis, but the 60 and 100 day biometric data were significant between the treatments (**Table 4**).

Survival of MSSRFD41 and Dynamics of Rhizobacterial Populations

Root colonization and establishment in the rhizosphere are vital for any potential bioinoculants. Rhizobacterial populations from 20-day roots of treated millet seedlings grown in pots harbored cultivable bacterial population of 9.06 log CFU g^{-1} (KB); and in pseudomonads selective media 4.82 log CFU g^{-1} in KBA+SLT; and 4.71 log CFU g^{-1} in KBA+CN were recorded. In control seedlings, the populations were 9.00 log CFU g^{-1} (KB); 4.68 log CFU g^{-1} (KBA+SLT); 4.58 log CFU g^{-1} (KBA+CN). The 60-day roots collected from treated seedlings harbored 8.39 log CFU g^{-1} (KB); 4.38 log CFU g^{-1} (KBA+SLT); 4.07 log CFU g^{-1} (KBA+CN); in control seedlings the cultural populations of 8.21 log CFU g^{-1} (KB); 4.15 log CFU g^{-1} (KBA+SLT); 4.04 log CFU g^{-1} (KBA+CN) were observed. At 100 days, the rhizobacterial population from the roots decreased slightly in treated and control plants. Overall, among the treatments no significant difference in total rhizobacterial population was detected ($P = 0.503$), but was highly significant in CFU g^{-1} of the samples collected at different days ($P = <0.0001$) and media ($P = <0.0001$) (**Figure 2**). At all-time points and on all the media, greater numbers of CFU were observed on roots from treated than from control plants. These results confirm that the introduced MSSRFD41 had no significant impact on rhizobacterial population dynamics.

TABLE 2 | Biocontrol and plant growth promoting traits of *Pseudomonas* sp. MSSRFD41.

S. No	Traits	Zone of Inhibition (or) Production
1.	*P. grisea*	22.35 ± 0.88 mm
2.	Chitinase	8.02 ± 0.53 mm
3.	Protease	24.98 ± 0.78 mm
4.	Cellulase	10.96 ± 0.65 mm
5.	Esterase	13.12 ± 0.48 mm
6.	Lipase	10.28 ± 0.68 mm
7.	Amylase	21.34 ± 0.39 mm
8.	Phosphate solubilization	7.04 ± 0.48 mm
9.	Siderophore production	12.8 ± 0.86 mm
10.	IAA production	29.09 ± 0.30 μg/ml
11.	Ammonia production	13.20 ± 0.57 μg/ml

Each value represents the mean ± SD (n = 5).

Monitoring of MSSRFD41 in the Rhizosphere via BOX-PCR

Based on the rhizobacterial colony morphotypes, total of 37 rhizobacteria were isolated of which 19 isolates from treated and 18 from control samples were recovered from the 100-day plant rhizosphere soil plated on KB, KBA+SLT, and KBA+CN media. BOX-PCR profiles of the amplified products ranged from 200 to 6,000 bp. BOX fingerprinting revealed that isolates 3 and 8 obtained from samples that had been treated with strain MSSRFD41 showed a banding pattern similar to that of MSSRFD41 reference DNA and formed a monophyletic cluster at a similarity coefficient value of 100% (**Figure 3**). In addition, the diversity among the total rhizobacterial isolates from the treated and control samples were identical at a similarity coefficient value of 80%. This strongly confirms the colonization of MSSRFD41 in the rhizosphere of the plant at harvest without any impact on the existing rhizobacterial community.

16S rRNA gene (∼1450 bp) sequencing of the rhizobacterial isolates MSSRF treated 3 (Accession no. MH071151) and MSSRF treated 8 (Accession no. MH071151) showed 100% similarity index to the reference MSSRFD41 (Accession no. MG738708) and *Pseudomonas* sp in the BLAST analysis. Phylogenetic relationship among the isolates showed that rhizobacterial isolates MSSRF treated 3 and 8 forms clade with MSSRFD41,

TABLE 3 | Biopriming effect of MSSRFD41 on finger millet seedling growth.

Days	Treatments	Germination %	Shoot Length (cm)	Root length (cm)	Plant biomass (g)	Vigor Index
4	Control	82.11 ± 2.1^{a}	1.53 ± 0.09^{a}	3.47 ± 0.24^{a}	0.10 ± 0.001^{a}	411.70 ± 3.04^{a}
	Treated	88.89 ± 2.8^{b}	1.65 ± 0.13^{a}	4.56 ± 0.17^{b}	0.12 ± 0.001^{b}	552.73 ± 3.14^{b}
6	Control	87.44 ± 2.3^{ab}	2.26 ± 0.49^{b}	5.94 ± 0.29^{c}	0.14 ± 0.001^{c}	718.13 ± 3.01^{c}
	Treated	96.77 ± 2.0^{c}	2.93 ± 0.69^{c}	6.87 ± 0.22^{d}	0.16 ± 0.005^{d}	949.47 ± 2.87^{d}
8	Control	90.07 ± 2.0^{b}	3.04 ± 0.75^{c}	8.73 ± 0.46^{e}	0.17 ± 0.002^{d}	1060.67 ± 3.66^{e}
	Treated	97.44 ± 2.6^{c}	3.70 ± 0.81^{d}	9.81 ± 0.18^{f}	0.21 ± 0.003^{e}	1316.66 ± 2.95^{f}

Each value represents the mean ± SD (n = 30) and within a column different letters were assigned when values were significantly different according to the HSD Tukey test (P < 0.05).

TABLE 4 | Effect of MSSRFD41 priming on growth of finger millet seedling in pots.

DAT	Treatments	Shoot length (cm)	Root length (cm)	Straw fresh weight (g)	Straw dry weight (g)	Root fresh weight (g)	Root dry weight (g)
20	Control	6.64 ± 0.98^{a}	2.99 ± 0.63^{a}	0.73 ± 0.16^{a}	0.47 ± 0.20^{a}	0.17 ± 0.06^{a}	0.10 ± 0.02^{a}
	Treated	7.94 ± 0.78^{a}	3.50 ± 0.43^{a}	1.02 ± 0.27^{a}	0.76 ± 0.22^{a}	0.29 ± 0.11^{a}	0.20 ± 0.07^{ab}
60	Control	48.27 ± 1.80^{b}	8.98 ± 2.7^{b}	3.18 ± 0.81^{b}	1.51 ± 0.33^{b}	1.97 ± 0.43^{b}	1.17 ± 0.49^{bc}
	Treated	54.60 ± 2.95^{c}	12.46 ± 1.53^{c}	4.84 ± 0.88^{c}	2.77 ± 0.522^{c}	2.89 ± 1.13^{c}	1.89 ± 0.69^{cd}
100	Control	53.70 ± 2.53^{c}	13.37 ± 2.49^{c}	6.84 ± 1.17^{d}	2.42 ± 0.23^{c}	3.07 ± 0.23^{c}	1.78 ± 0.42^{c}
	Treated	61.54 ± 2.98^{d}	17.30 ± 2.87^{d}	8.43 ± 1.66^{e}	3.53 ± 0.41^{d}	4.01 ± 0.56^{d}	2.58 ± 1.04^{d}

Each value is the mean ± SD (n = 5) and within a column different letters were assigned when differences were significant according to a HSD Tukey test (P < 0.05).

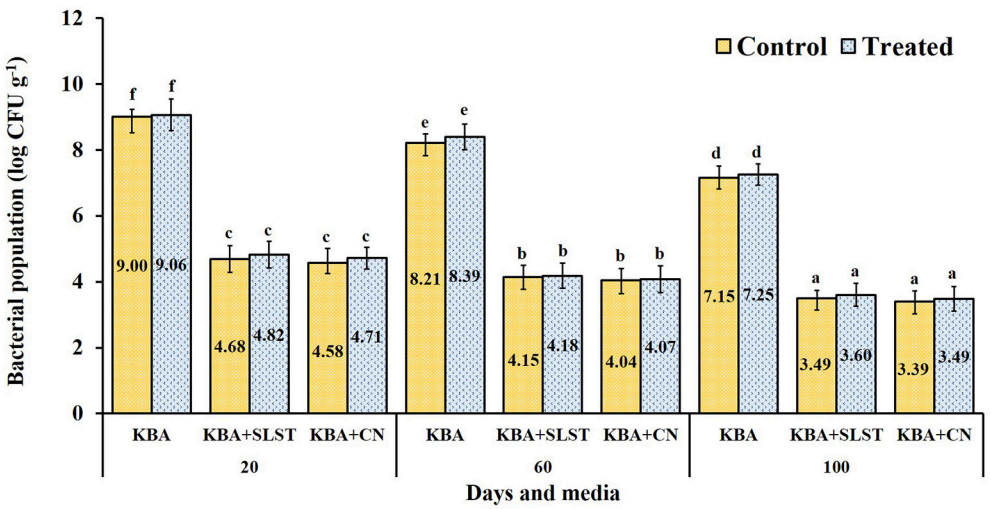


FIGURE 2 | Cultivable rhizobacteria isolated from rhizosphere samples of treated and control finger millet. KBA–Kings B agar; KBA+SLST–Kings B agar + sodium lauroyl sarcosine + Trimethoprim (20 mg^{-1}); KBA+CN–Kings B agar + Cetrimide + nalidixic acid (15 μg ml^{-1}). Each value represents the means ± *SD* of 5 replicates and within a column, different letters were assigned when significant according to HSD Tukey test ($P < 0.05$).

it clearly indicates that both isolates are similar to the reference MSSRFD41 (Figure S4).

PLFA Analysis

PLFA profiles of the rhizosphere samples of 100-day-old plants indicated total PLFA biomass content in control and treated samples, respectively, of 1,451 and 1,459 nmol g^{-1}. Measures of the treated and control rhizosphere samples indicated no significant differences in biomass of Gram-positive/Gram-negative bacteria, anaerobes, actinomycetes, fungi, eukaryotes, not assigned, and total PLFA groups (**Table 5**). These results indicate that strain MSSRFD41 did not inhibit (or) significantly alter the normal flora of rhizosphere microbial community, consistent with the results of the culturable population analysis.

Efficacy of MSSRFD41 in Control of Blast Disease and Growth Promotion Under Field Conditions

A field trial revealed the potential ability of MSSRFD41 to control the blast disease caused by *P. grisea* as well as to promote growth of finger millet compared to other treatments (**Table 6**). All the treatments showed a significant difference in the suppression of disease occurrence, but plots treated with strain MSSRFD41 showed lower incidence of 8.39%, three-fold lower than that of the control treatment. In the Bio + 50% chemical treatment plots, 11.42% disease incidence was observed, which was significantly lesser than the chemical (13.26%), C-PF (16.73%), and control (26.87%) treatments. Plant growth assessment showed maximum root length (19.05 cm) and shoot length (64.68 cm) in plants that had received the MSSRFD41 treatment which was significantly greater than values observed for other treatments including the Bio + 50% chemical treatment (root length 18.15 cm and shoot length 63.42 cm). All the four treatments showed significantly higher root and shoot lengths than the control treatment, but between the chemical and C-PF treatments, no significant difference was observed. Among the treatments, MSSRFD41-treated plots had 1,000 seed weight (3.23 g) that was significantly higher than the results from the other treatments. But no critical difference in 1,000 seed weight was detected among chemical, C-PF, and Bio + 50% chemical treatments. Overall the MSSRFD41 treatment evidenced control of blast disease incidence and enhancement of millet growth greater than that of the chemical treatment, which supports the

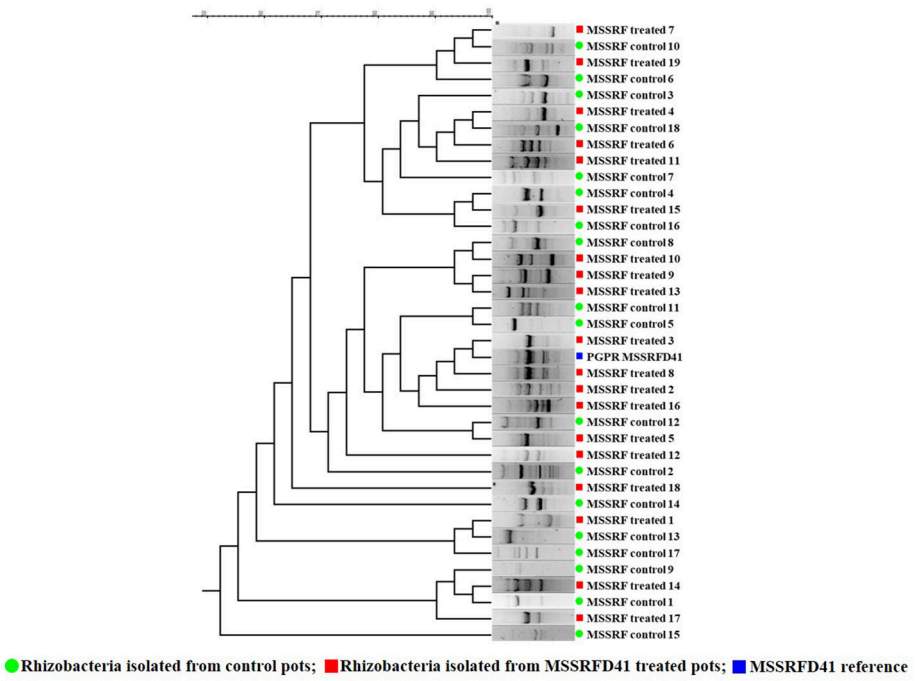


FIGURE 3 | Genomic fingerprinting to evaluate MSSRFD41 colonization and survival.

TABLE 5 | Soil microbial biomass analysis in treated pots by PLFA.

PLFAs	Biomass content in control samples (nmol g^{-1} soil)	Biomass content in treated samples (nmol g^{-1} soil)
Gram Positive	301.11 ± 2.55	303.66 ± 2.52
Gram Negative	530.72 ± 3.79	535.12 ± 3.06
Anaerobe	9.06 ± 0.87	9.12 ± 0.87
Actinomycetes	17.59 ± 1.48	17.97 ± 1.15
Fungi	7.47 ± 0.45	7.25 ± 0.60
Eukaryote	333.39 ± 3.25	334.30 ± 2.65
Not Assigned	251.71 ± 2.51	251.59 ± 2.01
Total PLFA	**1451.05 ± 3.14**	**1459.02 ± 2.15**

Each value represents the mean ± SD (n = 3) and no significant difference was observed between the treatments.

idea that it can be used as a potential bioinoculant for finger millet crop protection and growth promotion.

Preparation of Liquid-Based Formulation of MSSRFD41

The formulated product incubated at different temperatures showed a gradual increase in population of from 4.5 to 12.1 log CFU ml^{-1}. The shelf life of the formulated product incubated at 25°C showed the highest average population of 9.12 log CFU ml^{-1} compared to other tested temperatures, followed by 8°C with an average of 8.67 log CFU ml^{-1} with the highest survival rate at 150 days of incubation (8.2 log CFU ml^{-1}). At 37°C the formulated product had an average population level of 8.01 log CFU ml^{-1}, which showed higher CFU initially (8.9 log CFU ml^{-1}), but a reduction in the population level that occurred from the 30th day of incubation (4.5 log CFU ml^{-1}). In products stored at 4 and 8 °C the population level gradually increased (5.4 log CFU ml^{-1}) and declined at by the 90th day of incubation (**Figure 4**). Overall, the product incubated at 8 and 25°C temperature maintained the recommended dosage level till 150th days of incubation without any contamination.

DISCUSSION

Control of blast disease employing biological agents can enhance agricultural productivity in crops such as wheat, rice, millet, and barley, and is reported to protect yield loss (Kumar and Kumar, 2011; Prajapati et al., 2013; Cruz and Valent, 2017). Bioinoculants are sustainable natural resources with a wide range of disease control strategies and multiple beneficial plant growth promoting traits (Negi et al., 2015; Santhanam et al., 2015; Souza et al., 2015; Cruz and Valent, 2017; Deketelaere et al., 2017; GutiéRrez-GarcíA et al., 2017). Many studies have reported pseudomonads as bioinoculants with the potential to manage phytopathogens and promote the growth of crops cultivated under different agro-climatic conditions (Yin et al., 2013; Selvaraj et al., 2014; Wang et al., 2015). In this study, we have demonstrated the potential of the indigenous rhizospheric *Pseudomonas* sp. strain MSSRFD41 to control blast disease and promote the growth of finger millet.

Strain MSSRFD41 significantly suppressed mycelial growth of *P. grisea* in dual plate culture assays, consistent with antifungal action through the production of secondary metabolites,

TABLE 6 | Field efficacy of *Pseudomonas* sp. MSSRFD41 in the control of *P. grisea* and growth promotion of finger millet.

Treatments	Percent disease incidence	Shoot length (cm)	Root length (cm)	1000 seed weight (g)
Control	26.87 ± 1.99^{e}	56.05 ± 0.50^{a}	15.47 ± 0.57^{a}	2.83 ± 0.09^{a}
Chemical	13.26 ± 1.97^{c}	57.89 ± 0.53^{b}	16.31 ± 1.25^{b}	2.98 ± 0.18^{ab}
C-PF	16.73 ± 1.82^{d}	57.87 ± 0.43^{b}	16.79 ± 0.78^{b}	2.93 ± 0.15^{ab}
Bio + 50% Chemical	11.42 ± 1.55^{b}	63.42 ± 0.62^{c}	18.15 ± 0.56^{c}	3.02 ± 0.11^{ab}
MSSRFD41	8.39 ± 1.63^{a}	64.68 ± 0.77^{d}	19.05 ± 0.98^{d}	3.23 ± 0.12^{b}

Each value represents the means ± SD (n = 20) and within a column different letters were assigned when differences were significant according to the HSD Tukey test (P < 0.05).

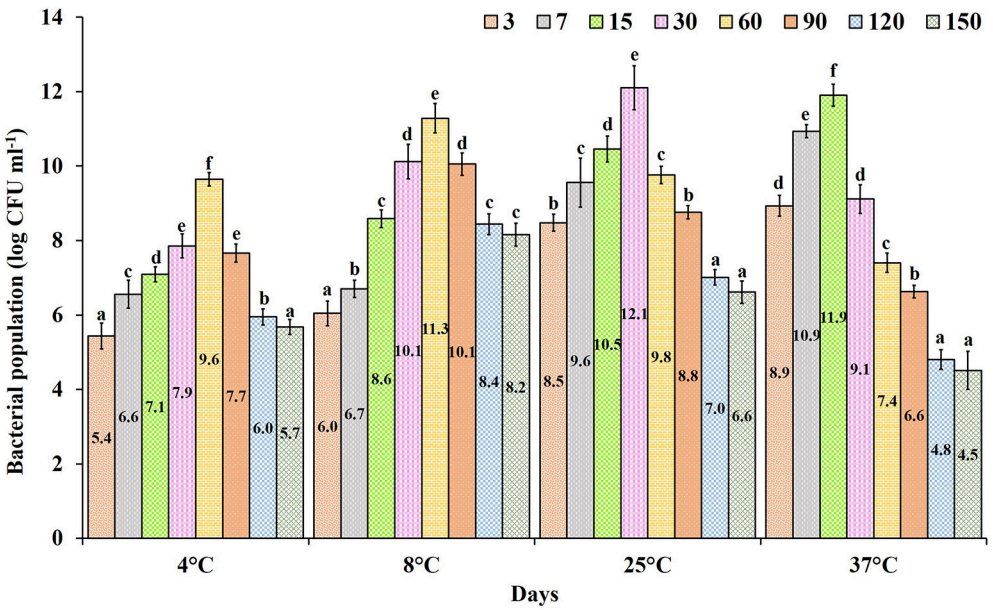


FIGURE 4 | Shelf life of *Pseudomonas* sp. MSSRFD41 in liquid formulated product of different ages incubated at various temperatures. Each value is the mean± *SD* of 3 replicates. Within a column, different letters were assigned when significant according to the HSD Tukey test ($P < 0.05$).

antibiotics, volatiles and hydrolytic enzymes (Sekar and Prabavathy, 2014). Pseudomonads are known to produce a wide range of metabolites including antibiotics (2,4-DAPG, HCN, PLT, and PCA) and enzymes that exhibit antagonistic activity against phytopathogens (Jegan, 2015; Müller et al., 2016; Vacheron et al., 2017; Yan et al., 2017). A GC/MS profile of MSSRFD41 crude extract showed the presence of reported potential antimicrobial metabolites including pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) a major antimicrobial metabolite (Melo et al., 2014), H-pyran, 2-(7-heptadecynyloxy)tetrahydro (Devi and Muthu, 2014); piperidine-3-carboxylic acid (Santiago et al., 2016); tert-hexadecanethiol (Giri et al., 2017); 1-heptatriacotanol (Kalaiarasan et al., 2011); octadecadienoic acid (Ganesh and Mohankumar, 2017); pyrazine and 2,4-DAPG derivatives (GutiéRrez-GarcíA et al., 2017). Production of these groups of potential antifungal secondary metabolites correlates to the efficacy of MSSRFD41 in the inhibition of *P. grisea*. In addition, numerous studies have correlated the antagonistic activity of pseudomonads with production of cell wall lytic enzymes such as chitinase and protease, which are reported to suppress the growth of fungal phytopathogens by degrading the cell wall (Radjacommare et al., 2004; Sekar and Prabavathy, 2014; Negi et al., 2015; Giri et al., 2017).

In this study, ESEM analysis demonstrated that the hyphae of *P. grisea* exposed to soluble substances produced by strain MSSRFD41 were damaged, forming clumps and exuding cellular material outside the cell wall. These images are consistent with the impact of metabolites, volatiles and hydrolytic enzymes from MSSRFD41. Similarly, Li et al. (2014) observed that the natural product citral caused damage to hyphal cell walls and membrane structures of *P. grisea*. The cell walls of *P. grisea* are strongly influenced by hydrophilic and aldehyde groups which damage or rupture cell walls and membranes (Xiong et al., 2013). A few studies also have shown that 2,4-DAPG plays an important role in the control of phytopathogens by damaging hyphal tips, causing vacuolization and cell content disintegration, and disrupting multiple basic cellular pathways including membrane function, reactive oxygen regulation, and cell homeostasis (de Souza et al., 2003; Kwak et al., 2011). Strain MSSRFD41 secretes hydrolytic enzymes including cellulase, chitinase, lipase, protease, and esterase, all of which are capable of causing the damage

and lysis of fungal cell walls leading to death (Han et al., 2006; Ariffin et al., 2008; Arunachalam Palaniyandi et al., 2013; Sarma et al., 2014; Negi et al., 2015). Strain MSSRFD41 exhibits multiple modes of action against *P. grisea,* all of which may be a key traits contributing to its efficacy and consistency of performance.

Bioinoculants can enhance the rate of seed germination, plant biomass and vigor index, protect from disease, and exhibit synergistic interactions with the seed and soil microfloral community through direct and indirect traits (Patten and Glick, 2002; Ariffin et al., 2008; Wang et al., 2015; Vacheron et al., 2017). Multiple growth promoting factors have been reported to be responsible for significant responses other than biocontrol in seeds treated with bioinoculants (Raudales et al., 2009; Manikandan et al., 2010; Negi et al., 2015). Finger millet seeds primed with MSSRFD41 were significantly enhanced in germination, seedling emergence, and biomass than control seeds. The ability to produce amylase plays a role in enhancing the rate of seed germination and early growth of millet seeds. It hydrolyzes endosperm starch into sugars, which acts as a source for the growth of roots and shoots in the germinating seedling (Duarah et al., 2011). MSSRFD41 produces the phytohormone IAA, a product which is widely produced by plant-associated bacteria, especially bioinoculants (Patten and Glick, 2002; Zahid et al., 2015). IAA plays significant role in plant signaling pathways to coordinate the physiological and morphological responses. IAA-mediated induction of primary and adventitious root development in young seedlings will augment their establishment in soil and facilitate the uptake of nutrients and water (Patten and Glick, 2002; Verma et al., 2013; Zahid et al., 2015). In addition, IAA plays an important role in the regulation of plant abiotic stresses induced by ethylene. Enhancing the availability of plant-utilizable forms of phosphate, iron, potassium and zinc via solubilisation and mobilization by bioinoculants directly increases nutrient availability in soil, thus increasing soil fertility, plant growth, crop productivity and also nutrient content of the grain (Gupta et al., 2015; Souza et al., 2015; Gopalakrishnan et al., 2016; Jegan et al., 2016; Kamran et al., 2017). The direct and indirect mechanisms of plant growth promotion mediated by strain MSSRFD41 reflect its ability to increase the nutrient content of the seed, soil, and plant biomass.

Currently, seed biopriming is reported as an efficient method of bioinoculant application to protect from seed and soil-borne phytopathogens, increase the speed and uniformity of germination, and plant growth and yield (Patten and Glick, 2002; Raudales et al., 2009; Mahmood et al., 2016). Gopalakrishnan et al. (2016) reported the efficacy of bioinoculants in growth promotion of cereals and legumes with biofortification of mineral nutrients in the grains. In this study, seed treatment with strain MSSRFD41 significantly enhanced biometric observations other than blast control *in vivo*. Pseudomonads are reported widely for their ability to enhance the availability and uptake of plant macro and micronutrients by either mobilization or solubilization from complex resources (Raudales et al., 2009; Goljanian-Tabrizi et al., 2016; Mahmood et al., 2016; Vacheron et al., 2017).

The soil microbiome is involved in the establishment of a stable ecosystem through synergistic interactions of compatible microbes. Rhizobiome communities are often referred to as the plant's second genome (Berendsen et al., 2012). Bioinoculants should be compatible with rhizobiome without adversely impacting the existing microbial community and should significantly improve crop yield, plant growth, and soil fertility (Pandey et al., 2012; Singh et al., 2013; Kröber et al., 2014). Several studies have attempted to determine the compatibility among co-bioinoculants for the development of beneficial consortia, but these studies did not explore the compatibility of bioinoculants against a wide range of rhizobacteria (Pandey et al., 2012; Singh et al., 2013; Zhang et al., 2016; Vacheron et al., 2017). In this study, our cross-streaking assay revealed the compatibility of MSSRFD41 with different native and other rhizobacterial groups without any detectable growth inhibition at the intersection of two colonies. Prior to the application of bioinoculants in the field, the cross streaking assay can strongly suggest that the introduced isolate will be compatible with the indigenous rhizobiome and soil microbiome.

Potential bioinoculants are determined by their efficacy in crop protection, growth promotion, survival in the rhizosphere, and compatibility with the native microbiome (Selvaraj et al., 2014; Gupta et al., 2015; Mahmood et al., 2016; Zhang et al., 2016; Deketelaere et al., 2017). The MSSRFD41 treated finger millet rhizosphere holds higher CFU g^{-1} than control samples but without any significant difference of CFU g^{-1} in both KBA+SLT and KB+CN media. These selective media are reported to use for the selection of pseudomonads from different soil samples and it recover significantly higher number of pseudomonas CFU g^{-1} than normal heterotrophic media (Goto and Enomoto, 1970; Gould et al., 1985; Picard et al., 2000; Thomas and Sekhar, 2016). Difference in colonies number between the treatment and control samples and BOX-PCR analysis may represent survival of MSSRFD41 in the rhizosphere soil of the treated samples. Thomas and Sekhar (2016) used a similar approach with selective media to detect the survival of endophytic *Pseudomonas aeruginosa* and its impact in soil from the banana rhizosphere. Recently, Sharma et al. (2017) described bioinoculants that survived in the rhizosphere, were compatible with other groups of rhizobacteria, and enhanced pigeon pea yield. Yin et al. (2013) also reported no detectable alteration of the native cucumber rhizobacterial community treated with *Pseudomonas fluorescens* 2P24 and CPF10 bioinoculants by using a culturable approach, T-RFLP, and DGGE. Bazhanov et al. (2017) used BOX-PCR approach to determine the colonization of inoculated atrazine-degrading strain *Arthrobacter ureafaciens* DnL1-1 from the roots of treated plants. Along with the results of culturable analysis, the RAPD fingerprinting pattern in our study also indicated the survival of strain MSSRFD41 in treated rhizosphere samples by forming a monomorphic fingerprinting pattern with MSSRFD41 reference DNA, but absent in control samples. In addition, this fingerprinting analysis revealed the existence of similar rhizobacterial communities in both the treated and control rhizosphere soils without a detectable change in the existing bacterial community. Phylogenetic analysis of 16S rRNA also evidenced the existence of MSSRFD41 in the rhizosphere of treated plants. These results support the idea that strain MSSRFD41 survived in the rhizosphere by establishing

a symbiotic relationship with the native rhizobiome and soil microbial communities. Similar to our results, Gupta et al. (2015) reported that a consortium of bioinoculants including *Bacillus megaterium*, *Pseudomonas fluorescens,* and *Trichoderma harzianum* influenced the yield of pigeon pea without a negative impact on the native rhizospheric microbial community. PLFA analysis also indicated that the total biomass, including contents associated with Gram-positive and Gram-negative bacteria, actinomycetes, fungi, and eukaryotes, was not significantly decreased by treatment with strain MSSRFD41. In many studies, PLFA biomarkers provide an indicator for assessing living microbial biomass to detect the effects of treatments on soil microbial community composition (Paterson et al., 2007; Buyer and Sasser, 2012; Zhang et al., 2016).

Several studies have reported a gradual reduction of bioinoculant populations and the native rhizobiome community in soil (Schreiter et al., 2014; Thomas and Sekhar, 2016). On the other hand, studies have shown that bioinoculants maintain a more stable soil microbiome, enhance plant growth, and control phytopathogenic infections more effectively than chemical pesticides and mineral fertilizer applications (Chowdhury et al., 2013; Kröber et al., 2014; Souza et al., 2015; Cai et al., 2017). In this study MSSRFD41 established in the rhizosphere and might survive through its communication signaling system as it is reported to produce AHL quorum sensing (QS) molecules (Sekar and Prabavathy, 2014). Several studies reported QS signaling system plays an important role in the establishment, survival, and gene regulation of bacterial community in the rhizosphere region (Boyer and Wisniewski-Dyé, 2009; Hartmann et al., 2014; Ganga et al., 2016).

Our results from cross-streaking, and culturable, RAPD, and PLFA analyses are all consistent with the hypothesis that strain MSSRFD41 survived, is compatible with the rhizobiome, has no detectable effect on the soil microbial community, and enhanced plant growth. Though all the analyses provided preliminary qualitative data on the differences between the control and treated rhizosphere samples, but does not provide quantitative data on the composition of microbial community. Approaches like next-generation sequencing, markers, fluorescent *in situ* hybridization (FISH), and molecular fingerprinting methods will be necessary to strongly prove the survivability and also for further investigation. In particular, marker approaches will play a significant role in determination of the fate of bioinoculants in the rhizosphere. Mosimann et al. (2016) demonstrated the colonization rate and quantitatively assessed the persistence of a bioinoculants in the maize root and rhizoplane through TaqMan-based quantitative PCR.

Studies to evaluate the impact of bioinoculants for the control of blast disease in the finger millet have shown disease suppression in the range of 16–54% (Radjacommare et al., 2004; Kumar and Kumar, 2011; Waghunde et al., 2013; Negi et al., 2015). A field experiment in this study revealed that inoculation with the native finger millet strain MSSRFD41 resulted in 8.39% disease incidence, which was significantly better than other treatments including a chemical fungicide. Several studies reported that foliar application of pseudomonads had an ability to act at the site of pathogenic infestation by damaging the fungal cell wall and retarded growth through a network of interconnecting signal leading to accumulation of defense-related enzymes and proteins via, induced systemic resistance (ISR) and systemic acquired resistance (SAR) systems (Bahadur et al., 2007; De Vleesschauwer et al., 2008; Spence et al., 2014; Negi et al., 2015; Fatima and Anjum, 2017; Yasmin et al., 2017). In addition, studies also showed that extracellular metabolites such as 2,4-DAPG, HCN, cyclic lipopeptides, defense proteins, peroxidase (POD), catalase (CAT), phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) produced by bioinoculants have been found to be responsible for antifungal activity during aerial infestation (Waghunde et al., 2013; Yin et al., 2013; Negi et al., 2015; Wang et al., 2015; Mahmood et al., 2016; Müller et al., 2016).

The combination of bioinoculants and chemical treatment to minimize the application of fungicide was less effective in controlling blast disease than treatment with strain MSSRFD41. In addition, strain MSSRFD41 was more effective against blast and in promoting growth than were chemical, C-PF and control treatments. Studies have observed similar impacts in different crops with a combination of multiple bioinoculants and chemical fungicides (Senthil et al., 2012; Waghunde et al., 2013; Magar et al., 2015).

Though bioinoculants protect plants from pathogens and promote growth significantly under lab conditions, efficacy often is less under greenhouse and field conditions (Lugtenberg and Kamilova, 2009). The use of a bioinoculant indigenous to the host plant is promising because of its reported high success rate in the establishment, significant growth promotion, and disease control (Verma et al., 2013; Santhanam et al., 2015; Zahid et al., 2015). Strain MSSRFD41 improved plant shoot length of 19.58% in paper towel assay; 13.60% and 14.29% in pot; and field conditions; root length of 11.65% in paper towel assay; 25.62% in pot; 21.12% under field conditions as compared to control plants.

The efficacy of MSSRFD41 was achieved by establishing in the rhizosphere through seed priming and root dipping which provided protection to the finger millet against blast disease in the initial growth stage and foliar spray at later growth stage through induced systematic resistance. In our earlier work we reported MSSRFD41 as a novel 2,4-DAPG producing genotype G with the production of 40 $\mu g\ ml^{-1}$ (Sekar and Prabavathy, 2014). There are strong evidence that 2,4-DAPG and elicitors plays an vital role in the control of phytopathogenic infections by lysis the fungal hyphae and induction of plant immune system under greenhouse and field conditions (Weller et al., 2012; Negi et al., 2015; Wang et al., 2015; Ganga et al., 2016; Vacheron et al., 2017; Yan et al., 2017).

The consistency and efficacy of strain MSSRFD41 in crop protection and growth promotion is critical if it is to be developed as a bioinoculants. Such bioinoculants must exhibit a prolonged shelf life in a viable form. Several reports have stated that liquid formulation is more efficient than other types of formulation such as talc, encapsulation, and jelly, because of its easy application, higher efficacy, potential shelf life, low level of contamination, and higher field performance (Hedge, 2002; Selvaraj et al., 2014). Most research on shelf life has focused on the stability of liquid

formulations for different lengths of time at constant temperature (Manikandan et al., 2010; Selvaraj et al., 2014; Goljanian-Tabrizi et al., 2016). In this study, the impact of both the duration of storage and the temperature of a formulated preparation of strain MSSRFD41 revealed significant changes in the population over time. At 25°C an average of 10^7 CFU ml^{-1} was maintained over 150 days, strongly suggesting the suitability of the liquid formulation for storage at room temperature. Manikandan et al. (2010) also reported that the shelf life of *P. fluorescens* in a glycerol-amended liquid formulation was maintained for up to 6 months at 25°C.

CONCLUSION

The present study systematically investigated the potential of an indigenous rhizobacterium of finger millet, *Pseudomonas* sp. MSSRFD41, for control of blast disease and growth promotion in finger millet. Our results suggest MSSRFD41 exhibited multiple beneficial traits to the host plants and results of compatibility and survival assessment revealed that MSSRFD41 could be a substitute and sustainable resource to reduce the usage of chemical fungicides for control of blast disease on finger millet. Further, the efficacy of the formulated product remains to be determined by conducting multi-location trials at different seasons. In addition, a biosafety assessment is needed before the formulated product can be developed commercially.

AUTHOR CONTRIBUTIONS

JS designed and performed experiments, field trial, data collection, statistical analysis, and manuscript write-up. KR and PD were involved in sample collection, contributed to *in vitro* experiments, analysis tools, and manuscript correction. PR supervised and supported the study, and revised and approved the final version of the manuscript to be published. All authors proofread and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01029/full#supplementary-material

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Indigenous *Pseudomonas* spp. Strains from the Olive (*Olea europaea* L.) Rhizosphere as Effective Biocontrol Agents against *Verticillium dahliae*: From the Host Roots to the Bacterial Genomes

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The use of biological control agents (BCA), alone or in combination with other management measures, has gained attention over the past decades, driven by the need to seek for sustainable and eco-friendly alternatives to confront plant pathogens. The rhizosphere of olive (*Olea europaea* L.) plants is a source of bacteria with potential as biocontrol tools against Verticillium wilt of olive (VWO) caused by *Verticillium dahliae* Kleb. A collection of bacterial isolates from healthy nursery-produced olive (cultivar Picual, susceptible to VWO) plants was generated based on morphological, biochemical and metabolic characteristics, chemical sensitivities, and on their *in vitro* antagonistic activity against several olive pathogens. Three strains (PIC25, PIC105, and PICF141) showing high *in vitro* inhibition ability of pathogens' growth, particularly against *V. dahliae*, were eventually selected. Their effectiveness against VWO caused by the defoliating pathotype of *V. dahliae* was also demonstrated, strain PICF141 being the rhizobacteria showing the best performance as BCA. Genotypic and phenotypic traits traditionally associated with plant growth promotion and/or biocontrol abilities were evaluated as well (e.g., phytase, xylanase, catalase, cellulase, chitinase, glucanase activities, and siderophore and HCN production). Multi-locus sequence analyses of conserved genes enabled the identification of these strains as *Pseudomonas* spp. Strain PICF141 was affiliated to the "*Pseudomonas mandelii* subgroup," within the "*Pseudomonas fluorescens* group," *Pseudomonas lini* being the closest species. Strains PIC25 and PIC105 were affiliated to the "*Pseudomonas aeruginosa* group," *Pseudomonas indica* being the closest relative. Moreover, we identified *P. indica* (PIC105) for the first time as a BCA. Genome sequencing and *in silico* analyses allowed the identification of traits commonly associated with

plant-bacteria interactions. Finally, the root colonization ability of these olive rhizobacteria was assessed, providing valuable information for the future development of formulations based on these strains. A set of actions, from rhizosphere isolation to genome analysis, is proposed and discussed for selecting indigenous rhizobacteria as effective BCAs.

Keywords: *Olea europaea*, verticillium wilt, biocontrol, *Pseudomonas,* rhizobacteria, *Pseudomonas indica*

INTRODUCTION

Cultivated olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the most important oil crops in the world. It constitutes an agro-ecosystem of major relevance for the Mediterranean Basin since 90% of the global olive oil and table olive production is concentrated in this area (FAOSTAT, 2016) [1]. Severe losses, and even tree death, are caused by a range of olive pathogens. Among them, the soilborne fungus *Verticillium dahliae* Kleb., causing Verticillium wilt of olive (VWO), represents a major threat in many regions where this tree is cultivated. Currently, however, no individual measure has proven effective to control VWO, and an integrated disease management strategy is therefore highly recommended (López-Escudero and Mercado-Blanco, 2011). Within this holistic framework, the development and implementation of sustainable and eco-friendly disease control measures is essential.

Plants have co-evolved with specific communities of microorganisms (i.e., the plant microbiome) that play crucial roles for the host's development and health (Berg et al., 2017). Moreover, many components of the plant-associated microbiome, particularly at the rhizosphere level, may constitute the first line of defense against soilborne pathogens (Weller et al., 2007). Hence, the plant rhizosphere constitutes an important, yet insufficiently explored, reservoir of microorganisms with antagonist ability against pathogens. In this sense, the isolation, identification, and characterization of microorganisms with biocontrol potential and able of colonize, endure, and be adapted to a complex niche such as the rhizosphere constitute an interesting disease management strategy. The utilization of biological control agents (BCAs) to suppress pathogens has been studied in several pathosystems involving woody plants (e.g., Pliego and Cazorla, 2012; Kalai-Grami et al., 2014), although it has been implemented to a lesser extent compared to herbaceous species, annual crops, and seedlings (Cazorla and Mercado-Blanco, 2016). Moreover, available information on the diversity and structure of microbial communities associated with woody plants is scant (e.g., Aranda et al., 2011; Zarraonaindia et al., 2015), particularly at the nursery propagation stage (Sun et al., 2014). Likewise, our understanding on woody plant-BCA-pathogen interactions are still limited, i.e., mechanisms underlying biological control, influence of environmental factors, effectiveness of BCAs, interaction between a BCA and the plant microbiome once the former is released, BCA colonization ability, or plant responses to the BCA are issues that still need to be studied in more detail (Cazorla and Mercado-Blanco, 2016). In this sense, omics technologies are contributing to enhance our understanding of these tripartite interactions (Massart et al., 2015). However, their implementation in woody plants is considerably lower than in herbaceous species (e.g., Mgbeahuruike et al., 2013; Gómez-Lama Cabanás et al., 2014; Martínez-García et al., 2015b).

Several studies have shown the potential, or even the true efficacy, of diverse beneficial microorganisms to suppress Verticillium wilts in different hosts (revised by Deketelaere et al., 2017), including olive (e.g., Aranda et al., 2011; Carrero-Carrón et al., 2016; Markakis et al., 2016). Among them, some *Pseudomonas* spp. strains are highly competent in colonizing the rhizosphere (Lugtenberg et al., 2001) and able to suppress the deleterious effects caused by different pathogens (Haas and Défago, 2005; Mercado-Blanco, 2015). Some strains of *P. fluorescens* and *P. putida* have thus been demonstrated as effective BCA against VWO (Mercado-Blanco et al., 2004; Prieto et al., 2009; Maldonado-González et al., 2015b) under different experimental conditions. Nevertheless, most of the studies related with biological control of Verticillium wilts have been conducted under controlled or gnotobiotic conditions. Indeed, BCA performance under field conditions is a scenario not frequently explored in biocontrol research, particularly with woody plants (Markakis et al., 2016). Related to this, it is crucial to understand the complex trophic interactions taking place between a newly-introduced BCA and the indigenous microbial community present in a target site, as well as the influence that diverse biotic and abiotic factors can exert to them, thereby conditioning the performance and effectiveness of the BCA. It seems therefore reasonably to isolate, identify, and characterize beneficial microorganisms from the niche where they will be eventually deployed, since they are theoretically adapted to the environmental conditions they will confront upon release.

Performing *in planta* assays under non-gnotobiotic conditions is therefore crucial to assess the effectiveness of a potential BCA since parameters such as competition for nutrients and space, colonization ability, time of inoculation, mode and site of application, etc., can be evaluated in a near-natural scenario. This is particularly relevant in plants with large root systems as olive. For instance, *P. fluorescens* PICF7 (Martínez-García et al., 2015b), a natural root endophyte of olive roots (Prieto and Mercado-Blanco, 2008), has been demonstrated to be an efficient BCA against *V. dahliae* (Maldonado-González et al., 2015b). However, strain PICF7 seems to require a direct contact with the pathogen to display effective biocontrol, as it was recently demonstrated using a split-root study system (Gómez-Lama Cabanás et al., 2017).

Selection of novel effective BCAs is mostly dependent on the pathosystem under study. The combination of diverse screening methods and the inclusion of the host plant into the

[1] http://www.fao.org/faostat/en/#data/QC

screening assay is essential to select BCAs acting through diverse modes of action that are not mutually exclusive (i.e., induced resistance, antibiosis, competition, mycoparasitism, etcetera). In addition, it is necessary to have a comprehensive knowledge of potential traits involved in colonization efficiency of the target site, biocontrol performance, and plant growth promotion efficacy. This key information is currently aided by implementing genomics approaches which, in addition, also assist to discard the presence of potential undesirable traits (i.e., pathogenicity and/or virulence factors) for plants, animals, and/or humans, phenotypes that must be ruled out when aiming to novel BCA-based formulations.

Considering this framework, the main objective of this study was to implement a strategy to isolate, identify, and in-depth characterize novel BCAs from the olive root/rhizosphere. This approach is based on the following major steps: (i) assessment of both *in vitro* and *in planta* effectiveness against diverse pathogens, (ii) genotypic and phenotypic characterization including features associated with biological control and plant growth promotion, (iii) metabolic profiling to obtain useful information related to microhabitat adaptation as well as to assist in the future development of bioformulations, (iv) genome sequencing and *in silico* analyses to allow taxonomic identification and to acquire essential knowledge on both the presence of beneficial traits and the absence of undesirable and/or potentially-harmful genotypes, and (v) evaluation of their root adhesion and colonization abilities. We test the hypothesis that the root/rhizosphere from young olive plants produced at nurseries is already an important source of beneficial bacteria which are also adapted to the target niche where they display biocontrol activity against VWO.

MATERIALS AND METHODS

Assessment of the *in Vitro* Antagonism Ability of Selected Olive Rhizobacteria against Diverse Olive Pathogens

A collection of olive (cv. Picual susceptible to *V. dahliae*, 1-year-old) rhizobacteria originating from different commercial nurseries located in Córdoba province (southern Spain) was previously generated and preliminary characterized (Ruano-Rosa et al., 2017). Bacterial isolates were tested against several olive pathogens, namely *Rosellinia necatrix* Rn320 and Rn400 (López Herrera and Zea Bonilla, 2007), *Phytophthora cinnamomi* CH1100 (kindly provided by Dr. C. López-Herrera, Institute for Sustainable Agriculture, IAS), *Pseudomonas savastanoi* pv. savastanoi (Psv) PSS-3 (IAS culture collection) and NCPPB 3335 (Pérez-Martínez et al., 2007), *Colletotrichum nymphaeae* Col.114 (Moral and Trapero, 2012), *C. godetiae* Col.516 (kindly provided by Dr. A. Trapero-Casas, University of Córdoba, UCO), and *V. dahliae* Lebrija 1 (defoliating [D] pathotype) (kindly provided by Dr. F.J. López-Escudero, UCO) and *V. dahliae* V-249I (non-defoliating pathotype) (Collado-Romero et al., 2006). To determine the *in vitro* antagonist activity of olive rhizobacteria, dual cultures were performed in potato dextrose agar (PDA) and nutrient agar (NA) media plates. For pathogenic fungi-olive rhizobacteria interactions, microorganisms (i.e., 10 μl drops of either fungal propagules or bacterial cell suspensions) were inoculated simultaneously on agar plates at a distance of 2.8 cm one from each other, and then incubated at 25°C in the dark until the pathogen covered the distance between both microorganisms in the control plates (i.e., only inoculated with the pathogen). For Psv-olive rhizobacteria interactions, experiments were carried out by inoculating the potential antagonists over Psv lawns ($OD_{600} = 0.1$) and incubated at 28°C in the dark for 48 h. In the case of *in vitro* antagonism against *V. dahliae* isolates, olive rhizobacteria yielding the best results against this pathogen were further reassessed against the same D isolate. Individual drops of *V. dahliae* biomass (i.e., mycelium plus conidia) were plated in the middle of PDA plates, and four equidistant 10-μl drops of each tested bacteria were inoculated. One control plate with just a suspension of the pathogen biomass was included on each experiment. Antagonist activity (i.e., halos or inhibition zones) was then scored. The relative inhibition index was calculated according to the equation (Rc-Ra)/Rc, where Rc is the average radius of *V. dahliae* colony in the absence of antagonist bacterium and Ra is the average radius of *V. dahliae* colony in the presence of antagonist bacterium (four equidistant points). These experiments were performed twice with three biological replicates per each interaction and used media. Analysis of variance (ANOVA) for this parameter was carried out. Mean values were compared by the Tukey HSD Test at $P = 0.05$ using Statistix program (Version 10.0 for Windows. Analytical software 1985-2013).

Verticillium Wilt of Olive Biocontrol Assays

Two independent experiments (I and II) were carried out under non-gnotobiotic conditions to test the biocontrol performance of three (PIC25, PIC105, and PICF141) selected olive rhizobacteria showing the highest *in vitro* inhibitory ability against *V. dahliae*. *Pseudomonas fluorescens* PICF7 was included as a reference since this strain is a well-known BCA against *V. dahliae* (Prieto et al., 2009; Maldonado-González et al., 2015b; Martínez-García et al., 2015b). One hundred and fifty olive plants (cv. Picual, 5-month old) originating from a commercial nursery located in Córdoba province were grown in pots (11 × 11 × 12 cm, one plant per pot), each containing 300 g of the potting substrate used in the nursery. Pots were randomly distributed in three blocks (15 plants per treatment) in a greenhouse under natural lighting and day/night temperature of 27/21°C. Bacteria were grown on Luria-Bertani (LB) agar plates at 28°C during 24–48 h. Bacterial cells were then scraped off from plates with 5 ml of $MgSO_4 \cdot 7H_2O$ 10 mM. For each bacterial treatment, inoculation was performed by adding 150 ml of a suspension of bacterial cells ($1 \cdot 10^8$ cfu·ml^{-1} in sterile $MgSO_4 \cdot 7H_2O$ 10 mM) per pot. Non-bacterized plants (control) were just drenched with 150 ml of sterile $MgSO_4 \cdot 7H_2O$ 10 mM. One week after inoculation with the bacteria, plants were challenged with *V. dahliae* V937I, an isolate representative of the D pathotype (Collado-Romero et al., 2006), by adding 150 ml per pot of a conidia suspension ($1 \cdot 10^6$ conidial/ml) prepared as previously described (Gómez-Lama Cabanás et al., 2017). Non-inoculated plants (control) were watered just with 150 ml of water. Disease development was assessed by scoring the severity of symptoms on a 0–4 scale according to the percentage of affected leaves and twigs (0, no symptoms; 1, 1–33%; 2, 34–66%;

3, 67–100%; 4, dead plant) (Maldonado-González et al., 2015b) twice a week during 90 day after pathogen inoculation. Areas under the disease progress curve (AUDPC) were calculated and analysis of variance (ANOVA) for this parameter carried out. Mean values were compared by the Fisher's protected LSD at $P = 0.05$ using Statistix program (Version 10.0 for Windows. Analytical software 1985-2013). Other parameters as Severity (S), Disease incidence (DI), Mortality (M), and Disease intensity index (DII) were also calculated for each treatment.

Phenotypic Characterization of Olive Rhizobacteria

In order to identify phenotypes associated with biological control and/or plant growth promotion in the three olive rhizobacteria eventually selected, assays aimed to evaluate protease (Naik et al., 2008), catalase (Holt et al., 1994), phosphatase (Katznelson and Bose, 1959; Naik et al., 2008), chitinase (Murthy and Bleakley, 2012), phytase (Hosseinkhani et al., 2009), celulase, xylanase, β-glucosidase, glucanase (Gong et al., 2012), pectinase (McKay, 1988), and amylase (Tindall et al., 2007) activities, as well as HCN (Bano and Musarrat, 2003), indole acetic acid (IAA) (Naik et al., 2008), 2,3-butanediol (MRVP medium, instructions according to Micro Media, Nebotrade Ltd.; Budapest, Hungary), and siderophore (Alexander and Zuberer, 1991) production were carried out. Production of the major siderophore pyoverdine was also investigated in liquid succinate mimimal medium (SSM; Meyer and Abdallah, 1978). Production of the typical green-yellow fluorescence in culture supernatants, related to the production of the siderophore pyoverdine (Dimkpa, 2016), was examined at 24, 48, and 72 h under UV light at 360 nm and by measuring the absorbance at 360–480 nm using a spectrophotometer. Finally, nutritional requirements of the selected strains were assessed by determining their ability to metabolize 71 different carbon sources, as well as their sensitivity to 23 chemicals, using the GEN III MicroPlate™ (Biolog, Hayward, CA) system according to the manufacturer's instructions. All these tests were repeated at least once.

Molecular Identification of Genes Commonly Associated with Biological Control Activity

Presence of *Pseudomonas* spp. specific genes involved in the biosynthesis of antibiosis-related compounds or associated with biocontrol phenotypes was performed by PCR analyses. Thus, gene-specific primers for the production of 2,4-diacetylphloroglucinol [DAPG], pyrrolnitrin [PRN], pyoluteorin [PLT], hydrogen cyanide [*hcnBC*], rhizoxin [*rzxB*], pyocin, and the insecticidal toxin protein [*FitD*] were used. Details on primer pair sequences as well as on amplification conditions are summarized in Supplementary Table S1. Amplifications were performed in a total volume of 50 μl containing 5 μl of 10× PCR buffer (50 μM KCl, 10 mM Tris-HCl pH 9 [25°C], 1% v/v Triton X- 100), 1.5 mM $MgCl_2$, 50 pmol each primer, 200 μM each dNTP, 0.5 U of Taq DNA polymerase (Roche®, Mannheim, Germany), and 25 ng of bacterial DNA. Amplification was performed in a DNA thermal cycler (Bio-Rad, Hercules, CA). Amplification products were separated by electrophoresis on a 1% agarose gels using 1x TAE buffer (Sambrook et al., 1989). For each PCR reaction, the reference strain *P. protegens* Pf5 was used as positive control for amplification of selected genes (Supplementary Table S1) (Kraus and Loper, 1992; Nowak-Thompson et al., 1994; Parret et al., 2005; Paulsen et al., 2005; Loper et al., 2008, 2016).

Sequencing and Bioinformatics Analyses of Olive Rhizobacteria Genomes Effective against VWO

Bacterial DNA was obtained by using "JETFLEX Genomic DNA Purification Kit" (Genomed, Löhne, Germany) according to the manufacturer's instructions. The genomes of strains PIC25, PIC105, and PICF141 were sequenced following a high-throughput sequencing strategy by using an Illumina MiSeq (2015 Illumina, Inc.) system, paired-end technology and *de-novo* sequencing protocol at Sistemas Genómicos S.L. (Paterna, Valencia, Spain). The read size was 150 bp (300 bp for the paired reads) and the total initial reads were from 6,291,558 (lowest) to 9,873,454 (highest), giving a fold coverage of 157.06 to 255.05. The quality of the raw data was checked using FASTQC tools (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All the adaptors were removed with the Fastq mcf (v1.04.803) (Aronesty, 2011) tool, and then a quality filter was made with Cutadapt (v1.9.1) (Martin, 2011) using a quality window value of 30. After the reads cleaning process the paired-end reads were merged using Flash (v1.2.11) (Magoč and Salzberg, 2011). With a masking step for the low quality bases, reads were ready to be assembled. Different assemblers were used but the main were Megahit (v1.0.3-29-g707d683) (Li et al., 2016) and Velvet (v1.2.10) (Zerbino and Birney, 2008). A list of several k-mers was used, from 71 to 99. Once the best assemblies were selected using the best N50 criteria, the annotation process started using Glimmer3 (Delcher et al., 1999; Kurtz et al., 2004) for the ORF detection and Blast V.2.2.30+ (Altschul et al., 1997) with an E-value cutoff of $1e^{-3}$ against the latest version (UniProtKB/Swiss-Prot Release 2015_08) of the Uniprot Swissprot protein curated database for bacteria (http://www.uniprot.org/). All the small local alignments were removed applying a filter requiring an alignment size of at least half size of the smallest sequence. All the sequences without a hit after removing the small local alignments were annotated using BLAST V.2.2.30+ (Altschul et al., 1997) against the last version (January 12, 2015) of the NT database (non-redundant nucleotide sequences from all traditional divisions of GenBank, EMBL, and DDBJ excluding GSS, STS, PAT, EST, HTG, and WGS) from the NCBI. Again, all the small local alignments were removed. Identified genes were functionally annotated using functional annotation of Uniprot (The UniProt Consortium, 2008) database from the previous step according to three different functional categories (biological process, molecular function, and cellular component). To allow a better understanding of the obtained genomes and their arrangements, each sample was aligned to the closest relative species available at NCBI (https://www.ncbi.nlm.nih.gov/) using Projector2 (Van Hijum et al., 2005).

The new obtained pseudo assembly was processed again using Glimmer3 (Delcher et al., 1999) and Blast V.2.2.30+ (Altschul et al., 1997) against Uniprot (http://www.uniprot.org/). Genome sequences were deposited at Genbank under the accession IDs SAMN06276402, SAMN06276401, and SAMN06276230 for PICF141, PIC105, and PIC25, respectively.

In order to localize bacterial secretion systems (TSSs) machineries (T3SS, T4SS, and T6SS), a BLASTp and HMMER search was performed by using T346Hunter web application (Martínez-García et al., 2015a). Moreover, bioinformatics identification of genetic factors (i.e., adhesion, antibiotics, biofilm, detoxification, synthesis, and secretion of exopolysaccharides [EPSs], microbe-associated molecular patterns [MAMPs], multidrug resistance [MDRs], bacterial lipopolysaccharides [LPSs], plant cell wall-degrading enzymes [PCWDEs], phytohormones, phytotoxins, pigmentation, proteases, siderophores, etc.) involved in plant-bacteria interaction was performed by implementing the PIFAR open-access, web-based tool (Martínez-García et al., 2016).

Since strains PIC25 and PIC105 showed as highly similar and closest to *P. indica* species (see below), the data set containing ortholog alignments obtained by Glimmer3 as described above was used to compare the genomes of strains PIC25 and PIC105 and two recently-released, unpublished genomes of *P. indica* strains (i.e., JCM21544; Varghese, 2016, and NBRC 103045; Hosoyama, 2017) available in the NCBI database. A genome level comparison was performed in order to obtain the putative pangenome and coregenome of *P. indica*, as well as to identify exclusive genes present in each analyzed *P. indica* strains. Cd-hit-est (Fu et al., 2012) tool was used over the ORFs obtained from each sample with a homology level of 90%. The new clusters were annotated against Uniprot. After the annotation, pangenome, coregenome, and exclusive genes were extracted for our strains, and associated KEGG Ontology pathways (Kanehisa et al., 2017). Additionally, gene Ontology database (Ashburner et al., 2000) and PFam (Finn et al., 2009) terms for PIC25 and PIC105 were obtained to count and sort them in order to create a functional view of the differences.

Verification on the Absence of Undesirable Traits in the Olive Rhizobacteria of the *P. aeruginosa* Group

To exclude the presence of pathogenicity/virulence-related genes in the genomes of strains PIC25 and PIC105, which clustered within the *P. aeruginosa* group (see below) that includes a number of pathogenic representatives, a genome level comparison was performed in order to obtain the pangenome, coregenome, and specific genes of PIC25 and PIC105 and two *P. aeruginosa* strains, one pathogenic, LESB58 (Winstanley et al., 2009) and another non-pathogenic, M18 (Wu et al., 2011). Besides, several virulence-related genes against mammals were also searched in the genomes of the selected olive rhizobacteria. These genes were present in the pathogenic *P. aeruginosa* strain LESB58 and absent, truncated or sharing less than 70% identity in the non-pathogenic strain *P. aeruginosa* strain M18 (Wu et al., 2011). Finally, we assessed pyocyanin (the major phenazine secreted by *P. aeruginosa*) production at 30/37°C after 30 days in liquid SSM, using *P. aeruginosa* BIRD-69 (Bio-Ilíberis collection) as positive control. Moreover, presence/absence of genes related to the synthesis of pyocyanin was *in silico* checked.

Phylogenetic Analysis of Selected Olive Rhizobacteria

A multi-locus sequence analysis (MLSA) (7610 nt positions) was carried out using partial sequences of the following housekeeping genes: *16S rDNA* (1405 nt), *gyrB* (1566 nt), *atpA* (1496 nt), *nusA* (1349 nt), *recA* (995 nt), and *dnaJ* (799 nt) in order to assess the taxonomic position of *Pseudomonas* spp. strains PIC25, PIC105, and PICF141. Gene sequences were obtained from the genomes of the three olive rhizobacteria here sequenced, and compared with the corresponding sequences of 18 selected *Pseudomonas* spp. type strains (retrieved from different public databases, i.e., NCBI, EMBL, KEGG, etc.,) and phylogenetically related to the three strains under studied. A dendrogram was generated with TREECON for Windows software (Van de Peer and De Wachter, 1994), using the Neighbor-Joining algorithm. *P. entomophila* L48 was used as out-group species.

A second analysis was performed (1096 nt positions) using the partial sequences of the *gyrB* (496 nt) and *rpoD* (600 nt) genes, in order to identify the three newly-identified strains at the species level. To achieve this goal, sequences were compared with those of 33 *Pseudomonas* spp. type strains belonging to the closest *Pseudomonas* groups, and according to the results obtained from the above-mentioned analysis. For both trees, bootstrap analyses (1,000 replications) were performed. Distance matrixes were generated using the Maximum Composite Likelihood model (Tamura et al., 2004) with Molecular Evolutionary Genetics Analysis version 5 (Mega5) (Tamura et al., 2011) software, and percentages identity of the concatenated sequences were inferred with the Clustal 2.1 version.

Root Colonization Assay

In order to evaluate the root colonization ability of the newly-selected olive rhizobacteria, bioassays were carried out using maize (*Zea mays* L.) seedlings as model plant and an experimental set-up previously described (Roca et al., 2013) with minor modifications. Bacterial strains were cultured overnight at 30°C in LB broth and culture turbidity was adjusted to $OD_{660} = 1$ in a final volume of 1 ml of M9 minimal medium (Sambrook et al., 1989). Maize seeds were surface sterilized by washing them twice with 70% (vol:vol) ethanol for 10 min, rinsed and washed again twice with 1% (vol:vol) bleach for 15 min and, finally, thoroughly rinsed with sterile deionized water. Seeds were then germinated on water-agar plates at 30°C for 2 days and overnight cultures that had been previously grown in LB broth were diluted in M9 to $OD_{660} = 1$. Next, germinated seeds were added to 2 ml of bacterial suspensions (5µl/ml of $OD_{660} = 1$ culture) of each strain tested. Seeds were then introduced in 50 ml skirted-base sterile tubes containing 35 ml of sterile sand, watered with 5ml of sterile deionized water and kept at room temperature for 2 weeks. To recover bacteria from the rhizosphere, stems were cut off, and roots were weighed and placed in sterile 50 ml screw-cap conical tubes containing 20 ml of M9 salts and 10–20 glass beads (2-mm

diameter). The tubes were mixed by vortex for 2 min and the number of CFU per gram of root was determined for each plant by plating serial dilutions on LB medium. Bacterial colonization of maize roots over time was assessed by sampling roots at 3, 7, and 15 days. This assay was performed twice with three replicates per treatment and time-point.

Descriptive statistical analysis was performed. Mean and absolute errors of the data were calculated. Also inferential statistical analysis was done, specifically, analysis of variance (ANOVA), assuming a normal distribution of the data and homocedastacity. For *post-hoc* analysis, the Tukey test ($P < 0.05$) was used to determine differences in the ability of colonizing seeds. Statistical analysis was performed in R language for statistical computing (R Development Core Team, 2014).

RESULTS AND DISCUSSION

Assessment of Olive Rhizosphere Bacterial Strains as Effective *in Vitro* Antagonists of Relevant Olive Pathogens

In a preliminary study, a collection of bacterial isolates originating from the rhizosphere of nursery-produced olive plants showed *in vitro* antagonism against several relevant olive pathogens, including *V. dahliae* (Ruano-Rosa et al., 2017). Based on these results three of the most promising strains (namely PIC25, PIC105, and PICF141) were selected, and reproducibility and consistency of their antagonist behavior were corroborated (**Table 1**). Results showed that two strains (PIC25 and PIC105) antagonized the six pathogens tested (i.e., *V. dahliae, R. necatrix, P. cinnamomi, C. nymphaeae*, *C. godetiae,* and Psv). The only exception was *P. savastanoi* pv. savastanoi strain NCPPB 3335, that showed no growth inhibition regardless the tested medium or the olive rhizobacteria. In contrast, strain PICF141 (and the reference BCA *P. fluorescens* PICF7) only showed growth inhibition of three out of the six pathogens tested (*V. dahliae*, *P. cinnamomi,* and *C. godetiae*; **Table 1**). Differences were found depending on the culture medium used (PDA or NA), particularly for strain PIC105 for which differences were detected in five out of the nine assays performed (**Table 1**). Inhibition of pathogens' growth was more frequent in PDA for strains PIC25, PIC105, and PICF7, whereas strain PICF141 displayed higher antagonist activity in NA. Influence of culture media on results from *in vitro* antagonism tests has been previously reported by Trivedi et al. (2008), who showed that biomass reduction of pathogenic fungi *Alternaria alternata* (Fr.) Keissl. and *Fusarium oxysporum* Schltdl. by *Pseudomonas corrugata* Roberts and Scarlett. was dependent on the culture medium used. Thus, variable responses in reduction of fungal biomass were a consequence of the effect of specific nutritional factors on the antagonisms exerted by *P. corrugata*.

The three olive rhizobacteria selected were able to significantly ($P < 0.05$) inhibit the growth of *V. dahliae* D pathotype, strain PIC105 showing the highest antagonistic capacity (relative inhibition index 0.428), followed by strains PIC25 (0.320) and PICF141 (0.174). These values were also significantly different among them. According to these results, the most promising strain to be used as BCA against VWO would be PIC105. Nevertheless, this potential benefit must be confirmed by conducting appropriate *in planta* experiments, since a range of biotic and abiotic factors present in the ecological niche where a BCA is deployed may greatly condition biocontrol effectiveness (Mercado-Blanco and Bakker, 2007). For instance, *P. fluorescens* PICF7 is an effective BCA against *V. dahliae* under different experimental conditions (Prieto et al., 2009; Maldonado-González et al., 2015a,b), although its *in vitro* antagonism ability was lower compared to *P. putida* strains also originating from the olive rhizosphere which, in contrast, did not show optimal VWO biocontrol performance (Mercado-Blanco et al., 2004). Also, Martín et al. (2015) reported that environmental conditions could affect the efficacy of preventive treatments with indigenous xylem endophytes in elm trees under field conditions, despite the fact these endophytes showed strong *in vitro* antagonism against the vascular pathogen *Ophiostoma novo-ulmi* Brasier. Moreover, biocontrol effectiveness can be critically influenced by the resident microbial communities (Errakhi et al., 2007; Goudjal et al., 2014). The *in vitro* tests performed only show the interaction between two microorganisms under very specific and artificial conditions. Since results could lead to erroneous assumptions concerning its true potential as BCA, effectiveness of a given BCA candidate must be demonstrated *in planta* and using experimental conditions resembling a natural scenario as much as possible. Thus, further VWO biocontrol experiments with selected olive rhizosphere strains were conducted under non-gnotobiotic conditions, avoiding major disturbance of the host rhizosphere.

Strains PIC25, PIC105, and PICF141 Are Effective Biocontrol Agents of Verticillium Wilt of Olive

Non-inoculated plants (control treatment) and plants inoculated only with bacterial strains showed normal development and growth in the two independent experiments performed (I and II). Neither abnormal appearance nor unexpected symptoms were observed during the bioassays. Five (experiment I) and three (experiment II) weeks after inoculation, first characteristic disease symptoms (including defoliation of green leaves) were observed in plants inoculated only with *V. dahliae* V937-I (disease control treatment). A more severe VWO syndrome was scored in experiment II than in bioassay I (**Table 2**). Approximately 1 week after the onset of the first symptoms in the disease control treatment, BCA/*V. dahliae* (Vd) treatments began to show the first VWO symptoms. Overall, pretreatment with the selected olive rhizobacteria reduced disease onset and development, either by reducing final DII or AUDPC. At the end of the experiment I all BCA/*Vd* treatments but PIC105/*Vd* showed a significantly ($P < 0.05$) lower AUDPC compared with the disease control treatment, though to a lesser extent than the disease suppressive effect exerted by strains PICF7 and PICF141. Compared with disease control plants, treatments with selected strains reduced all VWO parameters evaluated (final DI and DII, mortality and severity; **Table 2**). Regarding to experiment II, pretreatment with each of the strains significantly ($P <$

TABLE 1 | *In vitro* antagonism assays against *Verticillium dahliae* and other olive pathogens.

Pathogens	Vd Lebrija 1		Vd V-249I		Rn320		Rn400		Pc CH1100[A5]		Psv PSS3		Psv NCPPB 3335		Col.114		Col.516	
Media	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**	**PDA**	**NA**
***PSEUDOMONAS* STRAIN**																		
PIC25	+	+	+	+	+	+	+	+	+	−	+	−	−	−	+	+	−	+
PIC105	+	−	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	+
PICF141	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+
PICF7	+	+	+	+	−	−	−	−	−	−	+	−	+	−	+	−	−	−

Vd, Verticillium dahliae; Rn, Rosellinia necatrix; Pc, Phytophthora cinnamomi; Psv, Pseudomonas savastanoi pv. savastanoi; Col.114, Colletotrichum nymphaeae; Col.516, Colletotrichum godetiae. +, positive antagonism against the pathogen; – no antagonism. At least two biological replicates for each antagonism assay and culture medium were performed. PDA, Potato Dextrose Agar; NA, Nutrient Agar.

TABLE 2 | Assessment of biocontrol activity of *Pseudomonas* spp. strains against Verticillium wilt of olive (defoliating pathotype).

Treatments	Experiment I					Experiment II				
	Disease parameters[a]					Disease parameters[a]				
	AUDPC	**Final DI (%)**	**Final DII**	**M (%)**	**S**	**AUDPC**	**Final DI (%)**	**Final DII**	**M (%)**	**S**
V. dahliae/PIC25	44.4**bc**	40	0.36	30	1.45	134.4**b**	100	0.89	80	3.58
V.dahliae /PIC105	53.8**abc**	50	0.49	40	1.95	97.1**bcd**	93	0.83	73.3	3.3
V. dahliae /PICF141	24.4**c**	26.6	0.24	13.3	0.96	65.1**cd**	73.3	0.59	40	2.36
V. dahliae /PICF7	19.9**c**	40	0.23	40	0.92	64.3**d**	66.6	0.56	40	2.25
V. dahliae	112.3**a**	70	0.61	60	2.42	174**a**	100	0.99	93.3	3.98

[a]*AUDPC, area under the disease progress curve over time. Final DI, final disease incidence (%). Final DII, disease intensity index ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 90 days. M, dead plants at the end of the experiment (%) (90 days). S, mean of disease severity symptoms at the end of the experiment (from 0 to 4). Data are the average of three randomly-distributed blocks each with five pots per treatment. Control (non-inoculated) plants did not show any disease symptoms and were not included in the statistical analysis. Means in a column followed by different letters are significantly different according to Fisher's protected LSD test (P = 0.05).*

0.05) controlled VWO, even though disease pressure was much higher than in experiment I. Besides a significant reduction of the AUDPC, parameters such as final DII, mortality, and severity were also reduced in all BCA treatments compared to those observed in control treatment. The only exception was strain PIC105 that showed the same final DI than the disease control treatment (**Table 2**). Variability for some disease parameters observed between experiments highlights the need to conduct several independent assays to claim sound conclusions about the consistency of the suppressive effect of any BCA candidate. This is particularly relevant for tripartite interactions taking place in a complex niche as the rhizosphere. Moreover, in our opinion, results from each experiment should be shown separately (Mercado-Blanco et al., 2004; Maldonado-González et al., 2015a,b).

The three newly-identified indigenous olive rhizobacteria were thus able to reduce significantly disease severity, highlighting strain PICF141 with a comparable biocontrol performance to that previously demonstrated for the reference strain *P. fluorescens* PICF7 (Prieto et al., 2009; Maldonado-González et al., 2015b). Since these results do not correlate with *in vitro* antagonist assays, *in planta* experiments must be always performed to unequivocally demonstrate biocontrol activity. Indeed, while these tests provide useful information related to antibiosis and/or competition for nutrients as mode of actions of a given BCA, they clearly overlook other mechanisms only operating in the target niches, such as induction of resistance and/or competition for space. Considering this outcome these newly-isolated olive rhizobacteria can be postulated as effective BCA against VWO and be further studied at phenotypic and genomic levels. Basic knowledge from these analyses will be instrumental for the future development of single- or consortia-based bioformulations.

Identification of Plant Growth Promotion and Biocontrol Activities

All strains were positive for phytase and catalase activities, as well as for siderophore(s) production although only one of the three strains tested (strain PICF141) was positive for the production of the siderophore pyoverdine, in contrast to data from *in silico* analysis (see below). Phytases are phosphatases catalyzing the hydrolysis of phytic acid, thereby releasing a usable form of inorganic P for the plants. Bacteria with phytase activity have been isolated from the rhizosphere and proposed to promote plant growth in soils with high content of organic P (Singh et al., 2014). Studies have revealed that phytase-producing rhizobacteria not only harbor the ability to mineralize phytate but also harbor other PGPR activities, such as the production of indole acetic acid, siderophore, volatiles, and ammonia (Saharan and Nehra, 2011). None of them were positive for production of the volatile 2,3-butanediol nor for pectinase and chitinase activities (**Table 3**). Very slight xylanase and glucanase activities were detected only for *P. fluorescens* PICF7. Strain PICF141 was the only strain showing β-glucosidase activity and HCN

TABLE 3 | Phenotypic characterization of *Pseudomonas* spp. strains of properties associated with plant growth promotion and/or biocontrol.

Isolates	Activities													
	Siderophores	Fosfatase	Phytase	AIA	Protease	Cellulase	Pectinase	Chitinase	B-Glucosidase	Amilase	Xilanase	Glucanase	HCN	Catalase	2,3-Butanediol
PIC25	+	−	+	+	−	+	−	−	−	+	−	−	−•	+	−
PIC105	+	±	+	+	−	+	−	−	−	+	−	−	−•	+	−
PICF141	+	±	+	+	+	−	−	−	+	−	−	−	+	+	−
PICF7	+	+	+	−	+	+	−	−	−	−	±	±	−	+	−

+, presence of activity; −, absence of activity; ±, ambiguous result; •, isolate did not growth in King's B medium. Results shown were obtained from LB cultures amended with L-glycine. At least two biological replicates for each activity/strain were performed.

production (**Table 3**). The production and release of HCN by beneficial rhizobacteria has been studied as a biocontrol mechanism (i.e., antibiosis) displayed by a number of BCAs. Examples of the inhibitory effect of bacterially-produced HCN have been shown for fungi (Voisard et al., 1989), plants (Alström and Burns, 1989), nematodes (Gallagher and Manoil, 2001), insects (Devi et al., 2007), and other bacteria (Rudrappa et al., 2008). Strains of different *Pseudomonas* spp. are known to be cyanogenic, exerting toxic effects against various prokaryotes and eukaryotes even if they are not in close contact (Cernava et al., 2015). In addition, a role in increased phosphate availability for rhizobacteria and plant hosts has also been proposed for HCN (Rijavec and Lapanje, 2016). Production of HCN by PICF141 further supports its use as a broad-range BCA, antibiosis being one of the predicted modes of action for this rhizobacteria. While this fact correlated with the performance of strain PICF141 as BCA of VWO, PICF141 did not show broad *in vitro* antagonism against olive pathogens, in contrast with strains PIC25 and PIC105 which inhibited most of the pathogens analyzed (**Table 1**).

Results from the Biolog GENE III microplates showed that strains PIC25 and PIC105 shared most of the properties tested (89/95) (i.e., biochemical properties, utilization of carbon sources, chemical sensibilities) differing only in a few metabolic characteristics (Supplementary Table S2). For instance, strain PIC25 was able to utilize sodium butyrate, α-keto-butyric acid, D-frutose-6-PO4, D-serine, whereas strain PIC105 was unable to utilize any of these substrates but could use quinic acid. PIC25 and PIC105 were able to grow in the presence of 2.5% NaCl, D-mannitol, L-arginine, L-histidine, L-malic acid, butyric acid, sucrose, D-fructose, and glycerol. Compared to the other strains, strain PICF141 clearly differed in its ability to use carbon sources as well as in its sensitivity to chemicals. In fact, PICF141 shared less than 70% of properties tested with all strains studied (Supplementary Table S2). Moreover, PICF141 was the only strain able to grow in D-maltosa and D-turanose. Finally, strain PICF141 was able to assimilate D-trealose, sucrose, α-D-glucose, D-fructose, D-galactose, myo inositol, glycerol, and L-serine.

An in-depth knowledge on the ability to use different carbon sources and chemical sensitivities, and optimum culturing medium among others, provides relevant information for the future formulation, production, storage, application, and commercialization of BCAs. The carbon substrate has a dual role in biosynthesis and energy generation, carbohydrates being the usual carbon source for microbial fermentation processes (Costa et al., 2002). Competition for carbon sources can be one of the main factors determining biocontrol efficacy when bioformulations are based on consortia of bacteria. This fact must therefore be thoroughly considered when developing multi-strain combinations (Sun et al., 2015).

Phylogenetic Analyses of Newly-Isolated Olive Rhizobacteria

Preliminary molecular identification (partial sequencing and comparison of *16s rRNA* and *gyrB* genes) (Ruano-Rosa et al., 2017) of the three selected strains enabled their assignment to

the highly-ubiquitous and metabolically-versatile *Pseudomonas* genus. This genus comprises 238 species and 18 subspecies according to the List of Prokaryotic Names (http://www.bacterio.net/pseudomonas.html) (June, 2017). The sequence of the *gyrB* gene suggested *P. indica* as the closest species to strains PIC25 and PIC105 (e-values 0.0, and 93 and 98% identity, respectively), while strain PICF141 showed as closely related to *P. fluorescens* (e-value 0.0, and 95% identity). *Pseudomonas indica* was proposed as novel species by Pandey et al. (2002). The first strain of this species was isolated from an oilfield in Gujarat (India).

To more accurately identify the three novel *Pseudomonas* spp. strains a MLSA (7610 nucleotide positions from partial sequences of the housekeeping genes *atpA*, *dnaJ*, *gyrB*, *nusA*, *recA,* and *16S rRNA*) was carried out. The sequences of these housekeeping genes were obtained from their genomes (see below). Phylogenetic trees were generated including 21 *Pseudomonas* spp. type strains belonging to 13 different species and strains PIC25, PIC105, and PICF141 strains (Supplementary Figure S1). *Pseudomonas* is a rather complex genus subjected to continuous taxonomic revisions (Gomila et al., 2015), its heterogeneity being significantly resolved by polyphasic taxonomic studies (Achouak et al., 2000), *16S rRNA* gene sequence identity (Anzai et al., 2000), DNA-DNA hybridization relatedness (Palleroni, 1984), and MLSA (Mulet et al., 2010). According to the latter study, the genus was divided into two main intrageneric groups (IG), namely IG *P. aeruginosa* and IG *P. fluorescens*. The first IG includes three main groups represented by the species *P. aeruginosa, P. stutzeri,* and *P. oleovorans*, while the other IG comprises six main groups represented by the species *P. fluorescens, P. syringae, P. lutea*, *P. putida P. anguilliseptica,* and *P. straminea*, amongst which the *P. fluorescens* group is the most complex (Mulet et al., 2010). According to this classification the novel olive rhizobacteria strains were assigned to the *P. mandelii* subgroup within the *P. fluorescens* group (strain PICF141) and to the *P. aeruginosa* group (strains PIC25 and PIC105, that showed 96.47% identity between them) (Supplementary Figure S1). These results were consistent with our preliminary molecular identification only based on the comparison of the *16S RNA* and *gyrase B* genes (Ruano-Rosa et al., 2017). The closest neighbor of strains PIC25 y PIC105 was *P. resinovorans* type strain (89 and 89.59% identity, respectively). Strain PICF141 clustered within the *P. fluorescens* group, *P. mandelii* JR-1 strain being the closest species (94.47% identity).

For a better fine tuning taxonomic identification of the novel BCAs, additional MLSA (1096 positions of partial sequences of the housekeeping genes *gyrB* and *rpoD* gene) was conducted by comparing representative strains of the *P. fluorescens, P. mandelii*, *P. aeruginosa*, *P. oleovorans,* and *P. stutzeri* subgroups (33 *Pseudomonas* type strains belonging to 24 different species) (**Figure 1**). Results were congruent with the previous analysis, and strains PIC25 and PIC105 clustered within the *P. aeruginosa* group while strain PICF141 did so within the *P. mandelii* subgroup. This analysis indicated that the closest species to strains PIC25 and PIC105 was *P. indica* (Pandey et al., 2002), showing 94.24 and 98.63% identities, respectively. Moreover, the evolutionary distances separated *P. indica* type strain from either PIC25 (0.060) or PIC105 (0.014). Considering these results we assign strain PIC105 to the *P. indica* species. Despite the fact that PIC25 and PIC105 showed 96.47% identity and an evolutionary distance of 0.061, strain PIC25 was kept as *incertae sedis* within the *P. aeruginosa* group until further evidence, although showing close relatedness with *P. indica*. Indeed, PIC25 and PIC105 were able to grow in the presence of 2.5% NaCl, D-mannitol, L-arginine, L-histidine, L-malic acid, and butyric acid (Supplementary Table S2), as previously reported for *P. indica* IMT37 and IMT70 (Pandey et al., 2002). In addition, PIC25 and PIC105 strains shared with IMT40 strain the ability to grow in sucrose, D-fructose and glycerol.

Finally, strain PICF141 was also kept as *incertae sedis* within the *P. mandelii* subgroup, *P. lini* (Delorme et al., 2002) being the closest relative (95.49% identity and 0.041 evolutionary distance) (**Figure 1**). Interestingly, strain PICF141 and *P. lini* CFBP 5737T shared the ability to metabolize D-trealose, sucrose, α-D-glucose, D-fructose, D-galactose, myo inositol, glycerol, and L-serine (Supplementary Table S2; Delorme et al., 2002). In contrast, PICF141 was able to grow in D-maltosa and D-turanose (Supplementary Table S2) while its closest species was unable to use these two compounds as carbon source (Delorme et al., 2002).

General Characteristics of the PIC25, PIC105, and PICF141 Genomes

A summary of the genome sequencing projects for strains PIC25, PIC105, and PICF141 is shown in Supplementary Table S3. The draft genomes of PIC25 (predicted size 6,053,123 bp; GC content 63.6%), PIC105 (5,806,705 bp; 64.2%), and PICF141 (6,008,661 bp; 58.8%) included 79, 69, and 57 large mapped contigs (largest contig sizes were 169,273, 252,661, and 300,602 nt, respectively). Non-mapped contigs were 36 (for PIC25), 33 (PIC105), and 14 (PICF141). Additional genome characteristics such as predicted number of coding sequences, number of rRNA operons, etc, are summarized in **Table 4**. The classification of coding DNA sequences into functional categories according to the COG (Clusters of Orthologous Groups) is shown in Supplementary Table S4.

Comparative Analysis of the PIC25 and PIC105 Genomes with *P. indica* Species

Since strains PIC25 and PIC105 displayed considerable phylogenetic, phenotypic, and metabolic similarities between them, and that their closest species was *P. indica,* their genomes were compared with the draft genomes of two recently-sequenced *P. indica* strains, JCM21544 and NBRC 103045, available in the databases (https://www.ncbi.nlm.nih.gov/genome/?term=Pseudomonas+indica). The bioinformatics analysis enabled the identification of a putative *P. indica* core genome consisting of 4398 predicted protein coding genes (**Figure 2**). This core genome represented 79.3% (PIC25) and 77.3% (PIC105) of the predicted proteome of the olive rhizobacteria under study. A total of 662 genes were specific for PIC25 and 768 for PIC105. When analyzed PIC25 and PIC105 strains together, the core genome for the olive rhizobacteria

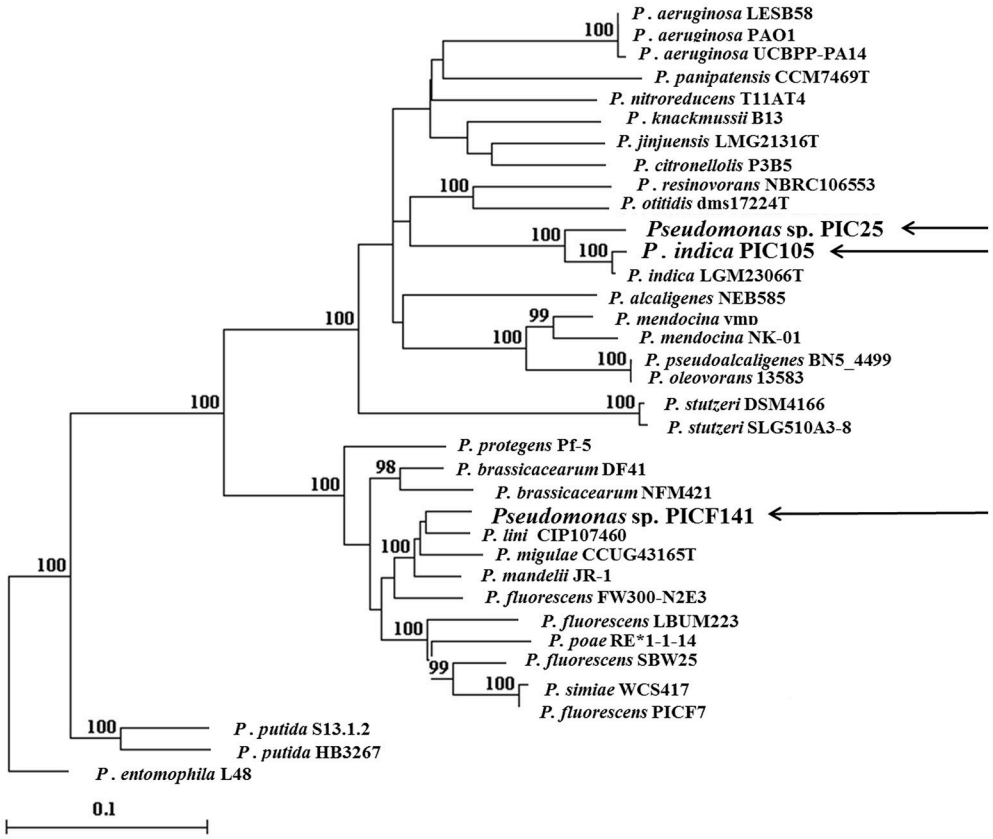


FIGURE 1 | Phylogenetic tree showing the taxonomic position of the three new *Pseudomonas* spp. strains (arrowed) isolated from the olive rhizobacteria. The tree was inferred by the Neighbor-Joining method, based on the alignment of concatenated partial sequences of *gyrB* and *rpoD* genes (see text for details). Bar indicates sequence divergence. Bootstrap values (>95%) based on 1,000 re-sampled datasets are shown at branch nodes. *Pseudomonas entomophila* L48 was used as out-group.

consisted of 4871 putative protein-coding genes, while 677 and 819 predicted protein coding genes were unique for PIC25 and PIC105, respectively. Whilst both strains share a large number of genes with the two *P. indica* strains so far sequenced, the analysis carried out was not conclusive enough to claim that all strains can be accurately assigned to the same species; that is, to *P. indica*. Additionally, a functional comparison of PIC25 and PIC105 strains was made using the Gene Ontology terms associated to the strain-exclusive genes. No significant functional deviation was found. The unique GO term showing significant difference was the GO:0000746 P:conjugation (Biological Process category): 3.44% (PIC105) vs. 0% (PIC25). Another term (Biological Process category too) showing differences was the GO:0009405 P:pathogenesis, that was slightly more present in PIC25 (3.67 vs. 2.21%) (Supplementary Tables S5–S8).

Ruling Out the Presence of Potential Pathogenicity/Virulence Factors in Strains PIC25 and PIC105

Considering that strains PIC25 and PIC105 belong to the IG *P. aeruginosa,* and that some representatives of the *P. aeruginosa* species can behave as opportunistic pathogens in immuno-compromised patients (Stover et al., 2000), it is essential to discard that the new olive rhizobacteria harbor pathogenic traits and/or virulence factors. To this end, the genomes of strains PIC25 and PIC105 were compared with the genomes of two *P. aeruginosa* strains, one non-pathogenic (M18, Wu et al., 2011) and another pathogenic (LESB58, Winstanley et al., 2009). Even though strains PIC25 and PIC105 clustered within the *P. aeruginosa* group, the analysis revealed that they only shared 40 genes with strain LESB58 and 43 genes strain M18 (at >90% identity) (data not shown). Besides, none of the virulence-related genes (e.g., *flgL*, *fliC*, *flaG*, *fliD*, *fliS*, *fliT,* etc.,) against mammals present in LESB58 or M18 strains according to Wu et al. (2011) were found in the genomes of PIC25 and PIC105. Another characteristic feature of *P. aeruginosa* species is the production of the blue pigment pyocyanin (Wilson et al., 1988). Pyocianin is a phenazine exerting toxic effects on eukaryotic cells through reactive oxygen species (Mahajan-Miklos et al., 1999). The presence of pyocyanin biosynthesis genes was also checked in PIC25 and PIC105 genomes. Indeed, *PhzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2* gene clusters, involved in the final steps of the pyocyanin biosynthetic cascade (Mavrodi et al., 2001), were not found in PIC25 and PIC105

TABLE 4 | General parameters of the three olive rhizosphere *Pseudomonas* spp. genomes.

Attribute	Genome (total)					
	PIC25		PIC105		PICF141	
	Value	% of total	Value	% of total	Value	% of total
Genome size (bp)	6,053,123	100	5,806,705	100	6,008,661	100
DNA coding region (bp)	5,295,441	87.5	5,076,306	87.4	5,216,964	86.8
DNA G+C content (bp)	3,849,786	63.6	3,726,163	64.2	3,531,891	58.8
Total genes	5,779	100	5,326	100	5,462	100
Protein-coding genes	5,585	96.6	5,120	96.1	5,245	96.0
RNA genes	194	3.4	206	3.9	217	4.0
Genes in internal clusters	NA	–	NA	–	NA	–
Protein-coding genes with function prediction	3,369	58.3	3,572	67.1	3,872	70.9
Protein-coding genes assigned to COGs	2,737	47.4	2,744	51.5	2,907	53.2
Proteins with signal peptides	NA	–	NA	–	NA	–
Proteins with transmembrane helices	NA	–	NA	–	NA	–
CRISPR repeats	3	0.0018	5	0.0022	0	0.0000

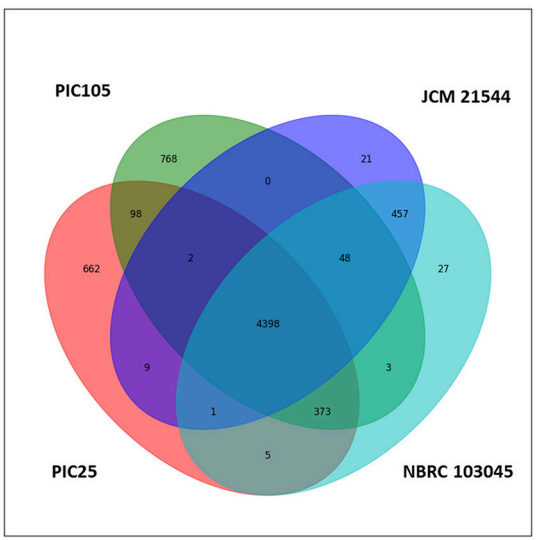


FIGURE 2 | Comparison between the genomes of *Pseudomonas* sp. PIC25 and *Pseudomonas indica* PIC105, JCM21544, and NBRC 103045. The Venn diagram shows the number of orthologous coding sequences shared by the four strains (4398 genes; core genome) and those ones that are specific for PIC25 (662) and PIC105 (768).

genomes. The enzyme anthranilate synthase participates in the synthesis of pyocyanin. *Pseudomonas aeruginosa* possesses two functional anthranilate synthases, each comprised of large and small subunits encoded by the products of the *trpE* and *trpG* and *phnA* and *phnB* genes, respectively. These enzymes are not functionally redundant (Essar et al., 1990a,b). Only *TrpE* and *trpG* genes were identified in PIC25 and PIC105 genomes. Besides, pyocyanin production was tested at both 30 and 37°C. Results showed that after 30 days of culturing in liquid SSM, PIC25, and PC105 did not produce pyocyanin, in contrast to the reference strain *P. aeruginosa* BIRD-69 (data not shown). Altogether, these results indicate that strains PIC25 and PIC105 do not seem to carry undesirable traits present in some pathogenic representatives of the *P. aeruginosa* group, ruling out potential risks as for human toxicology concerns (Kamilova et al., 2015).

In Silico Identification of Secretion Systems T3SS, T4SS, and T6SS

Bacterial secretion systems (TSSs) play relevant roles throughout the whole range of plant-bacteria interactions (i.e., pathogenic, endophytic, or mutualistic symbioses) (Costa et al., 2015; Green and Mecsas, 2016). The presence of TSSs in the genomes of strains PIC25, PIC105, and PICF141 was confirmed by using the "T346Hunter" web-based tool (Martínez-García et al., 2015a). "T346hunter" allowed the identification of flagellar and non-flagellar type III (T3SS), type IV (T4SS), and type VI (T6SS) secretion systems, although differences were found among the olive rhizobacteria under study. Moreover, presence of some of these TSSs in the reference strain *P. fluorescens* PICF7 was confirmed, corroborating our previous results for this BCA (Martínez-García et al., 2015b). The three TSSs were identified in the genomes of PIC25 and PIC105, while in PICF141 and PICF7 only T3SS and T6SS were detected. Finally, flagellar T3SS2 and 3, and T6SS1 and 2 were present in all strains (Supplementary Table S9). Overall, strains PICF7 and PICF141 showed a very similar TSSs profile, only differing in that PICF141 harbors flagellar T3SS1 while strain PICF7 has the non-flagellar T3SS1 (Supplementary Table S9).

Non-flagellar T3SS (NF-T3SS) and T6SS are complex molecular machineries that deliver effector proteins from bacterial cells into the environment or into other eukaryotic or prokaryotic cells, with significant implications for the strains encoding them. T3SSs have been detected in both beneficial and pathogenic bacteria, and have been related to plant root colonization, rhizosphere competence, environmental

competition, defense against amoebas, or oomycete suppression (Rezzonico et al., 2005; Matz et al., 2008; Mavrodi et al., 2011). For instance, T3SSs have been identified in the PGPR *P. fluorescens* SBW25 (Preston et al., 2001) and BBc6R8 (Cusano et al., 2011), and in *P. brassicacearum* Q8r1-96 (Mavrodi et al., 2011). Strain Q8r1-96, as well as *P. fluorescens* A506, Q2-87, SS101, SBW25, and *Pseudomonas* sp. BG33R, harbor gene clusters encoding for secretion protein T3SS (*rsp/rsc*). In the pathogenic species complex *P. syringae,* T3SS is responsible of the transport of type III effector proteins (T3Es) into plant cells (Cornelis, 2010), involved in the suppression of plant defense responses and alteration of the eukaryotic cell physiology (Chang et al., 2004; Lindeberg et al., 2008). T6SSs, also present in the three *Pseudomonas* spp. here selected as BCAs, were originally thought to be exclusively implicated in the delivery of virulence effectors to eukaryotic hosts. However, other reports have indicated that this TSS play a key role in the interaction among bacteria, being well-distributed among environmental bacteria including plant-associated *Pseudomonas* spp. (Russell et al., 2011; Loper et al., 2012). Thus, one to three gene clusters coding for T6SS are present in the genomes of *P. fluorescens* group strains analyzed by Loper et al. (2012). Other beneficial pseudomonads like *P. putida* W619 also harbors, among other TSS, several T6SSs (Reinhold-Hurek and Hurek, 2011).

T4SS is a more functionally-diverse TSS system not only involved in effector translocation but also in conjugation and DNA uptake/release. There are three functional types of T4SSs (Alvarez-Martinez and Christie, 2009; Wallden et al., 2010). One of them is used for the conjugation process, which is the major mechanism to spread antibiotic resistance genes among pathogenic bacteria. Others T4SSs are involved in DNA uptake (transformation) and release from the extracellular milieu. Finally, there are T4SSs that are used to transfer proteins. Most of the T4SSs in this category are found in pathogenic bacteria, playing important roles in virulence such as establishing pathogen-host interaction and/or transferring toxic effector proteins or protein complexes into the cytoplasm of the host cell. While in strain PIC105 only T4SS1 was found, the three T4SS types were detected in the PIC25 genome. Their functionality and roles remain to be elucidated.

In Silico Detection of Genetic Traits Involved in Bacteria-Plant Interaction

The "PIFAR" open-access web-based tool (Martínez-García et al., 2016) allowed the identification of several genetic factors involved in plant-bacteria interactions. A summary of the genes identified in the three newly-identified strains, as well as in *P. fluorescens* PICF7, is provided in Supplementary Table S10. Specific information about genetic factors can be found in **Table 5**. The number of annotated genes involved in plant-bacteria interaction present in strains PIC25, PIC105, and PICF141 was very similar, ranging from 108 to 116 (Supplementary Table S10). However, this number was lower than that detected in *P. fluorescens* PICF7 (>150 genes). The same gene clusters were identified in the genomes of strains PIC25 and PIC105 for factors such as proteases, detoxification, EPSs, LPSs, MDRs, volatiles, and MAMPs, confirming the close

TABLE 5 | Genes identified in the genomes of *Pseudomonas* spp. strains PIC25, PIC105, PICF141, and PICF7 involved in plant-bacteria interaction according to the web-based tool PIFAR (Martínez-García et al., 2016).

Genetic factors involved in plant-bacteria interaction		*Pseudomonas* spp. strains			
		PIC25	PIC105	PICF141	PICF7
Proteases	*HtrA*	+	+	+	+
	Serralysin protease C	+	+	+	+
PCWDEs	*LipA*	+	+	+	+
	Pec lyase C	+	+	–	–
	Pectate lyase	+	+	–	–
	Cellulase		+	+	+
Adhesion	*AttC*	+	+	+	+
	AttG	–	–	+	+
	XadM	–	–	+	+
	Haemagg act	+	+	+	+
	Usher	–	–	+	+
	Pilin	+	–	+	+
	Fimbrial	–	–	+	+
	Cellulose synthase	–	–	+	–
Deto+ification	*SapABCDF*	+	+	+	+
	KatB	+	+	+	+
	KatE	+	+	+	+
	KatG	–	–	+	–
	Pip	+	+	+	+
	Dps	+	+	–	+
	Cbb	+	+	+	+
	Copper resistance cueAR	+	+	+	+
	Copper resistance ABCDRS	–	–	+	+
	Polymixin resistance	–	–	–	+
EPSs	Alginate	+	+	+	+
	GalU	+	+	+	+
	GpsX	–	–	+	+
Metabolism	*TrpGC*	+	+	+	+
	Glutamate synthase gltBD	+	+	+	+
	AroC	+	+	+	+
	AroK	+	–	+	+
	AroQ	+	+	+	+
	AcnB	+	+	+	+
	AsnB	+	+	+	+
	Mqo	+	+	+	+
	Purine biosynthesis *purD*	+	+	+	+
	Purine biosynthesis *purC*	+	+	–	–
	Citrate transporter	–	–	–	+
LPSs	Wzt	+	+	+	+
	Rfb303	+	+	+	+
	Glicosyl tranferase WxocB	+	+	–	–
MDRs	ACR tran	+	+	+	+
	Multi Drug Res	+	+	+	+
	MatE	+	+	+	+
	OEP	+	+	+	+

(Continued)

TABLE 5 | Continued

Genetic factors involved in plant-bacteria interaction		*Pseudomonas* spp. strains			
		PIC25	PIC105	PICF141	PICF7
Volatiles	*BudB*	+	+	+	+
	2,3-butanediol	+	+	+	+
Antibiotics	Fusaricidin	+	+	+	+
	Amphisin	+	+	+	−
	Hydrogen cyanide	−	−	+	−
	Insecticidal fit toxin	−	−	+	−
	Viscosin	−	−	−	+
	Gluconic acid	−	−	−	+
MAMPs	Chemotaxis protein cheA	+	+	+	+
	Chemotaxis protein cheW	+	+	+	+
	Chemotaxis protein cheY	+	+	+	+
	Chemotaxis response regulator protein-glutamate cheB	+	+	−	+
Type III effectors	*XopAE*	+	+	−	−
	XopK	+	+	−	−
	Xop+	+	+	−	−
	XopAL2	+	+	−	−
	HopG1	+	−	−	−
	HopN1	+	+	−	−
	HopV1	+	+	−	−
	HopS1	+	−	−	−
	HopO1	+	−	−	−
	HopT1	+	−	−	−
	HopB1	−	−	+	+
	RipQ	+	+	−	−
	RipTPS	+	+	−	−
	AvrE1	−	+	−	+
Siderophores	Pyochelin	−	−	+	+
	Pyoverdine*	−	−	−	+
	Pseudobactin*	−	−	−	+
	Hemophore	−	−	−	+
	Arthrobactin	−	−	−	+
Phytohormones	IAA2	−	−	+	+
	Salicylic acid	−	−	−	+
	Salicylic hydroxylase	−	−	−	+
Biofilm	Phosphoglucomutase protein yhxB	−	−	+	+

*+, The complete gene cluster for the genetic factor is present in the genome; −, The gene cluster for the genetic factor was not found or incomplete. *According to PIFAR nomenclature pyoverdine and pseudobactin are considered different siderophores.*

relatedness of these two bacterial strains. As expected, the profile of genetic factors in strain PICF141 differed significantly from that in PIC25 and PIC105, showing more similarities with strain PICF7 and in agreement with their assignment to the *P. fluorescens* group (**Table 5** and Supplementary Table S10). For instance, less adhesion and EPS factors were found in strains PIC25 and PIC105 compared to strains PICF7 and PICF141. In contrast, more PCWDE and LPS factors, and particularly T3Es, were identified in strains PIC25 and PIC105 (**Table 5** and Supplementary Table S10). Production of 2,3-butanediol was not detected in any of the studied strains. Biosynthesis of 2,3-butanediol from pyruvate requires three key enzymes, α-acetolactate synthase (ALS, EC 4.1.3.18), α-acetolactate decarboxylase (ALDC, EC 4.1.1.5), and 2,3-butanediol dehydrogenase (BDH, EC 1.1.1.76; also called acetoin reductase, EC 1.1.1.4) (Ji et al., 2011). Only the gene putatively coding for α-acetolactate synthase was found, what could explain the absence of 2,3-butanediol production. The *in silico* analysis also confirmed that the genomes of the three newly-identified strains harbor the genes encoding for the biosynthesis of several antibiotics (e.g., fusaricidin and amphisin). In contrast, genes coding for the insecticidal fit toxin and for HCN production were only identified in strain PICF141. *In vivo* production of HCN by this strain was previously confirmed, as well as the presence of *hcnBC* genes by PCR analysis (see above).

"PIFAR" also searches for putative T3Es by using three databases of well-known bacterial pathogens like *P. syringae* (http://pseudomonas-syringae.org/), *Ralstonia solanacearum* (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi) and *Xanthomonas* spp. (http://www.xanthomonas.org/t3e.html). The first T3E was identified because its presence led to a hypersensitive response in resistant plants (Staskawicz et al., 1984). Many relevant plant diseases are caused by bacterial pathogens that deliver effector proteins into the eukaryotic host cell by using the T3SS (e.g., Lindeberg et al., 2012), thereby suppressing the plant defense response (Boch, 2009). Twelve and nine T3Es previously described in the pathogenic bacteria *P. syringae* (*Hop* or *AvrE1*; (Baltrus et al., 2011)), *R. solanacearum* (*Rip*; Poueymiro and Genin, 2009; Mukaihara et al., 2010) and *Xanthomonas* (*Xop*; White et al., 2009) that modulate host responses, enabling successful infection and multiplication in plants (Zhou et al., 2008; Guo et al., 2009) were identified in PIC25 and PIC105, respectively (**Table 5**). In contrast, only one to two T3Es were predicted for PICF141 (*HopB*) and PICF7 (*HopB* and *AvrE1*). While T3Es have been reported mostly in pathogenic bacteria, they have been described in beneficial bacteria as well. For instance, two T3Es identified in *P. fluorescens* Q8r1-96, a strain responsible for the suppressiveness of agricultural soils to take-all disease of wheat, are encoded by the ortholog genes *hopAA1-1* and *hopM1* present in the pathogenic bacterium *P. syringae* (Mavrodi et al., 2011).

Differences were also found for gene clusters putatively encoding for biofilm, siderophores and hormones production (**Table 5** and Supplementary Table S10). Complete gene clusters for these traits were only identified in strains of the *P. fluorescens* group that were the only ones to show positive for pyoverdine production (data not shown). Nevertheless, genes coding for siderophore biosynthesis, regulation, and transport were annotated in the genomes of PIC25 and PIC105. While both strains were positive for siderophore activity (**Table 3**),

production of the major siderophore pyoverdine was not detected (data not shown) in agreement with data from "PIFAR" analysis. Therefore, siderophore(s) other than pyoverdine must be produced by these strains. It should be mentioned that "T346hunter" (for secretion systems) and "PIFAR" (for plant-microbe genetic factors) web-based tools only report positive matches for (nearly) complete gene clusters, and that factors defined by several genes are reported only if at least 90% of such genes are found within a given bacterial genome (Martínez-García et al., 2015a, 2016).

Root Colonization Ability of Novel BCAs from the Olive Rhizosphere

An indispensable prerequisite for a BCA to succeed in biocontrol is the efficient colonization of the specific niche where it will be applied. In the particular case of beneficial *Pseudomonas* spp. traits involved in rhizosphere and/or root colonization have been studied and reviewed in detail (Mercado-Blanco, 2015; Pizarro-Tobías et al., 2015). For instance, *P. chlororaphis* PCL1391 mutants impaired in root colonization have been shown to lose their biocontrol effectiveness against *Fusarium*

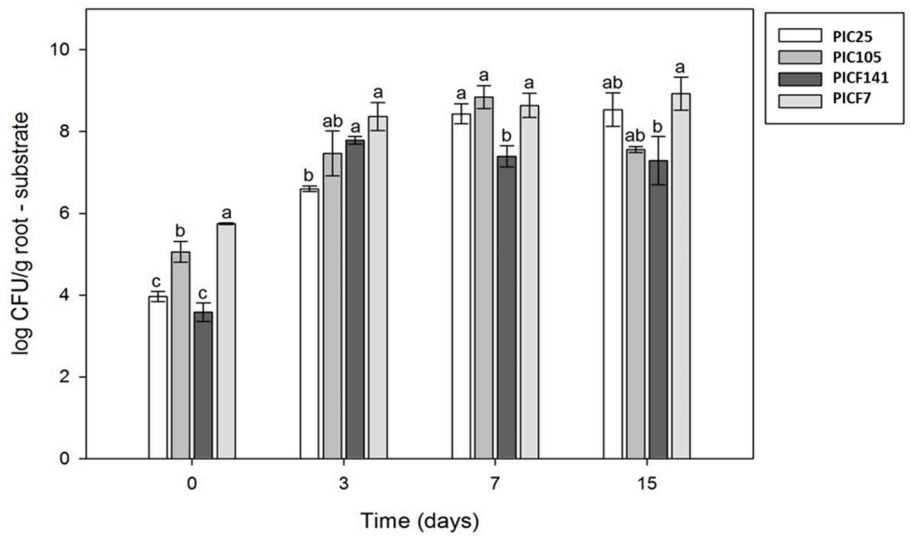


FIGURE 3 | Maize roots colonization ability of the olive rhizobacteria strains *Pseudomonas* sp. PIC25, *Pseudomonas indica* PIC105 and *Pseudomonas* sp. PICF141. Mean number of bacterial cells strongly adhered to maize roots (1 g) are expressed as log CFU/seed at 0, 3, 7, and 15 days after inoculation. Error bars represent the standard deviations ($n = 3$). Different letters indicate significant ($P < 0.05$) differences between treatments in the same sampling day. *Pseudomonas fluorescens* PICF7 was used as reference.

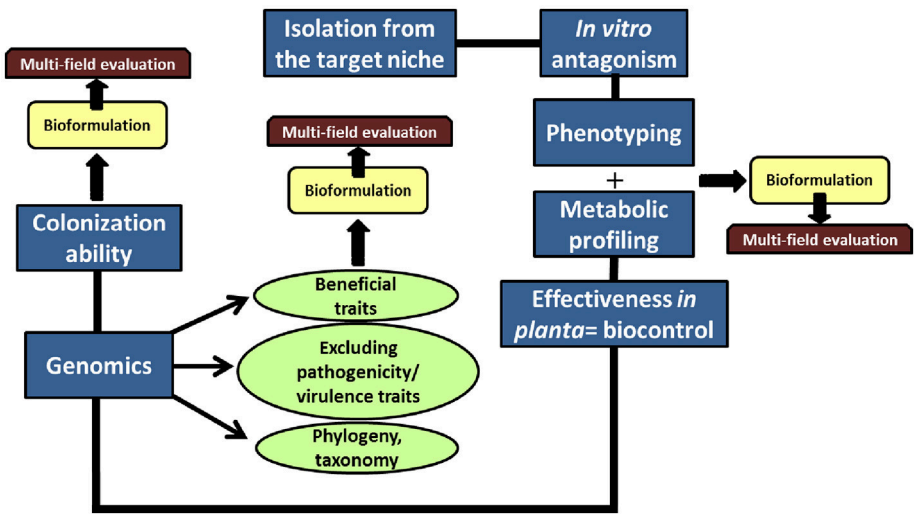


FIGURE 4 | Flowchart of actions to isolate, identify and characterize novel and effective BCAs. This was the strategy followed in this study using the olive root/rhizosphere as target, but it is proposed to be implemented in similar ecological niches.

oxysporum f. sp. *radicis-lycopersici* in tomato plants (Chin-A-Woeng et al., 2000). Since evaluation of colonization ability of the olive rhizobacteria here studied pose some difficulties due to intrinsic characteristics of woody plant roots (e.g., their large biomass, complicated anatomy, longevity), and experiments with them are usually time consuming (Cazorla and Mercado-Blanco, 2016), tests were carried out using maize as model plant. By doing so, homogeneity of the plant material, effective seeds sterilization, rapid plant growth, and score of reliable results in a short period of time (15 days) were obtained (Roca et al., 2013).

Results from maize root colonization assays showed an increase in the number of bacterial cells attached to the roots for all strains at 3 days after inoculation (DAI), although differences were found (**Figure 3** and Supplementary Figure S2). Indeed, significantly lower colonizing efficiency at this time point was evident for strain PIC25, compared to that showed by strains PICF141 and PICF7. Strain PIC105 displayed intermediate root colonization efficiency (**Figure 3**). However, at 7 DAI, strain PICF141 was the least effective in maize root colonization (around 10^7 CFU/g root), in contrast to strains PIC25, PIC105, and PICF7 (above 10^8 CFU/g root). At the end of the assay (15 DAI), the latter three olive rhizobacteria did not show significant differences in their colonization abilities, even though relevant differences on the presence of adhesion factors in their genomes were found (**Table 5** and Supplementary Table S10). Moreover, despite the fact that strains PICF141 and PICF7 showed very similar adhesion factors profiles, strain PICF7 colonized significantly better maize roots than strain PICF141 at 7 and 15 DAI (**Figure 3**). This suggests that other factors involved in adhesion and colonization must be involved to explain the observed differences. Overall, these results indicate that all strains studied present good colonization ability of roots, and that the slight differences found along time do not seem to be crucial to compromise their biocontrol effectiveness, particularly in the case of strain PICF141 that displayed the best biocontrol performance.

CONCLUSIONS

This study provides a fairly comprehensive approach to identify, characterize, and evaluate new BCAs (**Figure 4**). By implementing this set of sequential actions we succeeded in identifying three *Pseudomonas* spp. strains, indigenous from the olive rhizobacteria and effective against the D pathotype of *V. dahliae* under non-gnotobitic conditions. Among them, strain PICF141 was the most promising BCA. Therefore, we demonstrated that young plants propagated in nurseries are already an important source of beneficial microorganisms that can be used as biocontrol tools within an integrated management strategy of VWO.

Identification of biocontrol bacteria should not be exclusively based on their performance *in vitro*. Repeated *in planta* experiments conducted under non-gnotobiotic conditions must always be performed to overcome the potential biases usually obtained from antagonism tests carried out under very specific (and artificial) growing conditions. Indeed, *in vitro* experiments showed that strain PIC105 was the most effective to antagonize different olive pathogens. However, *in planta* assays demonstrated strain PICF141 as the most effective against VWO, with a comparable performance to that observed for the well-known BCA *P. fluorescens* PICF7.

The MLSA here performed allowed to assign strains PIC25 and PIC105 to the *P. aeruginosa* group and strain PICF141 to the *P. mandelii* subgroup within the *P. fluorescens* group. Moreover, based on our data we identified strain PIC105 as *P. indica*. It is the first time that a *P. indica* representative is described as a BCA. Full genome sequencing enabled us to acquire valuable information regarding to beneficial traits involved in key aspects like colonization, plant growth promotion and biocontrol abilities of these strains. Besides, *in silico* analyses were useful to confirm the absence of undesirable traits that could compromise the future use of formulations based on these bacterial strains.

Further in-depth insights on specific metabolic and phenotypic abilities of these strains will be valuable for the future development of effective formulations, which can be based on a single strain or tailored consortia. Additionally, it remains to be assessed whether these newly-identified strains are able to endure and protect olive plants under field conditions.

AUTHOR CONTRIBUTIONS

JM-B conceived the study. All authors participated in the experimental design. CG-LC, DR-R, PP-T, AV-C, JN, AR, and JM-B performed in planta bioassays and conducted experiments aimed to identify and characterize bacterial strains. PP-T, JN and AR conducted root colonization experiments. DR-R and AV-C carried out *in vitro* antagonism tests. GL and JT performed bioinformatics analyses. CG-L and JM-B wrote the article. AR, PP-T, and GL made direct contribution to the final manuscript. All authors have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00277/full#supplementary-material

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REVIEW
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Bacillus velezensis FZB42 in 2018: The Gram-Positive Model Strain for Plant Growth Promotion and Biocontrol

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Bacillus velezensis FZB42, the model strain for Gram-positive plant-growth-promoting and biocontrol rhizobacteria, has been isolated in 1998 and sequenced in 2007. In order to celebrate these anniversaries, we summarize here the recent knowledge about FZB42. In last 20 years, more than 140 articles devoted to FZB42 have been published. At first, research was mainly focused on antimicrobial compounds, apparently responsible for biocontrol effects against plant pathogens, recent research is increasingly directed to expression of genes involved in bacteria–plant interaction, regulatory small RNAs (sRNAs), and on modification of enzymes involved in synthesis of antimicrobial compounds by processes such as acetylation and malonylation. Till now, 13 gene clusters involved in non-ribosomal and ribosomal synthesis of secondary metabolites with putative antimicrobial action have been identified within the genome of FZB42. These gene clusters cover around 10% of the whole genome. Antimicrobial compounds suppress not only growth of plant pathogenic bacteria and fungi, but could also stimulate induced systemic resistance (ISR) in plants. It has been found that besides secondary metabolites also volatile organic compounds are involved in the biocontrol effect exerted by FZB42 under biotic (plant pathogens) and abiotic stress conditions. In order to facilitate easy access to the genomic data, we have established an integrating data bank 'AmyloWiki' containing accumulated information about the genes present in FZB42, available mutant strains, and other aspects of FZB42 research, which is structured similar as the famous SubtiWiki data bank.

Keywords: *Bacillus velezensis*, FZB42, AmyloWiki, induced systemic resistance (ISR), non-ribosomal synthesized lipopeptides (NRPS), non-ribosomal synthesized polyketides (PKS), volatiles, plant growth promoting bacteria (PGPR)

INTRODUCTION AND SHORT HISTORY OF GRAM-POSITIVE PGPR RESEARCH

Bacteria that are associated with plant roots and exert beneficial effects on plant development are referred to as plant-growth-promoting rhizobacteria (PGPR; Kloepper et al., 1980). It is well accepted today, that numerous PGPR are also enabled to control plant diseases.

Main subject of present and past research about microbial inoculants with beneficial action on plant health and growth are plant-associated representatives of the bacterial genus *Pseudomonas*, known as strong and persistent colonizer of plant roots (Burr et al., 1978). However, its commercial use is limited by difficulties in preparing stable and long-living bioformulations. As early as at the end of the 19th century a bacterial soil-fertilizing preparation Alinit® consisting of spores of the soil bacterium *Bacillus ellenbachensis*, later reclassified as *Bacillus subtilis*, was introduced by the German landowner Albert Caron (1853–1933) on his estate in Ellenbach (Caron, 1897). Alinit was marketed as "bacteriological fertilizer for the inoculation of cereals" by "*Farbenfabriken* former Friedrich Bayer," the later Bayer AG, in Elberfeld, Germany. The history of these early attempts in using bacterial inoculants is comprehensively described by Kolbe (1993). After a long period of silence, the plant-growth-promoting effect of *Bacillus* spp. was rediscovered in Broadbent et al. (1977). Today, formulations based on plant-beneficial endospore-forming Bacilli are by far the most widely used agents on the biopesticide market (Borriss, 2011). Especially, members of the *B. subtilis* species complex (rRNA group 1) which includes at present more than 20 closely related species (Fan et al., 2017a), and, to a minor extent, of the genus *Paenibacillus* spp., are able to suppress efficiently plant pathogens, such as viruses, bacteria, fungi and nematodes in vicinity of plant roots. This review describes the current 'state of the art' of the model strain for PGPR – and biocontrol, *Bacillus velezensis* FZB42, and the integrative data bank 'AmyloWiki,' recently established for this bacterium.

FZB42 (=BGSC 10A6, DSM23117), the prototype of gram-positive bacteria with phytostimulatory and biocontrol action, has been genome sequenced in Chen et al. (2007) and is subject of intensive research. Since its isolation from beet rhizosphere (Krebs et al., 1998) more than 140 articles about FZB42 have been published[1]. FZB42 and its closely related 'cousin' FZB24, are successfully used as biofertilizer and biocontrol bacteria in agriculture being especially efficient against fungal and bacterial pathogens[2]. Beneficial effects of FZB42/FZB24 on plant growth and disease suppression in field trials were reported for potato (Schmiedeknecht et al., 1998), cotton (Yao et al., 2006), strawberry (Sylla et al., 2013), wheat (Talboys et al., 2014), lettuce (Chowdhury et al., 2013), and tomato (Elanchezhiyan et al., 2018), for example.

In past, FZB42 and related phytostimulatory Bacilli were subjects of intensive efforts to clarify their taxonomic position. The group of plant-associated, endo-spore forming rhizobacteria (Reva et al., 2004) is known as member of the *B. subtilis* species complex (Fritze, 2004), which included originally *B. subtilis*, *B. licheniformis,* and *B. pumilus* (Gordon et al., 1973). In 1987, the species *B. amyloliquefaciens* (Priest et al., 1987) was added, and FZB42 and some other biocontrol bacteria were found as belong to this species (Idriss et al., 2002). By taking advantage of availability of an increasing number of genome sequences, we distinguished two subspecies: *B. amyloliquefaciens* subsp. *amyloliquefaciens* (type strain DSM7^T) and *B. amyloliquefaciens* subsp. *plantarum* (type strain FZB42^T) (Borriss et al., 2011). According to extended phylogenomic analysis *B. amyloliquefaciens* subsp. *plantarum* was shown as a later heterotypic synonym of *B. velezensis* (Dunlap et al., 2016), Recently, we proposed to establish an "operational group *B. amyloliquefaciens,*" which includes *B. amyloliquefaciens*, known for its ability to produce industrial enzymes (amylases, glucanases and proteases), *B. siamensis,* mainly occurring in Asian food, and PGPR *B. velezensis,* the main source for bioformulations increasingly used in agriculture for protecting plant health and to stimulate plant growth (Fan et al., 2017a, **Figure 1**).

FZB42, THE GRAM-POSITIVE PROTOTYPE FOR BIOCONTROL OF PLANT PATHOGENS

Biocontrol effects exerted by *B. velezensis* FZB42 and other antagonistic acting Bacilli are due to different mechanisms: besides direct antibiosis and competition by secretion of a spectrum of secondary metabolites in the rhizosphere (Borriss, 2011), the beneficial action on the host-plant microbiome (Erlacher et al., 2014), and stimulation of plant induced systemic resistance (ISR, Kloepper et al., 2004; Chowdhury et al., 2015a) are of similar importance.

Remarkably, in contrast to Gram-negative biocontrol bacteria and fungal plant pathogens, application of FZB42 did not lead to durable changes in composition of rhizosphere microbial community (Chowdhury et al., 2013; Kröber et al., 2014). Moreover, application of FZB42 was shown to compensate negative changes within composition of the root microbiome caused by plant pathogens (Erlacher et al., 2014).

Induced systemic resistance is triggered by a range of secondary metabolites, which are called 'elicitors.' Different signaling pathways, such as jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) are activated to induce plant resistance. Mutant strains of FZB42, devoid in synthesis of surfactin (srf), were found impaired in triggering of JA/ET dependent ISR in lettuce plants, when challenged with plant pathogen *Rhizoctonia solani* (Chowdhury et al., 2015b). The lower expression of the JA/ET-inducible plant defensin factor (PDF1.2) in a *sfp* mutant strain, completely devoid in non-ribosomal synthesis of lipopeptides and polyketides, compared to the *srf* mutant strain, only impaired in surfactin synthesis, suggests that secondary metabolites other than surfactin might also trigger plant response.

[1] http://amylowiki.top/reference.php

[2] https://www.abitep.de/index.php/de/agrar.html

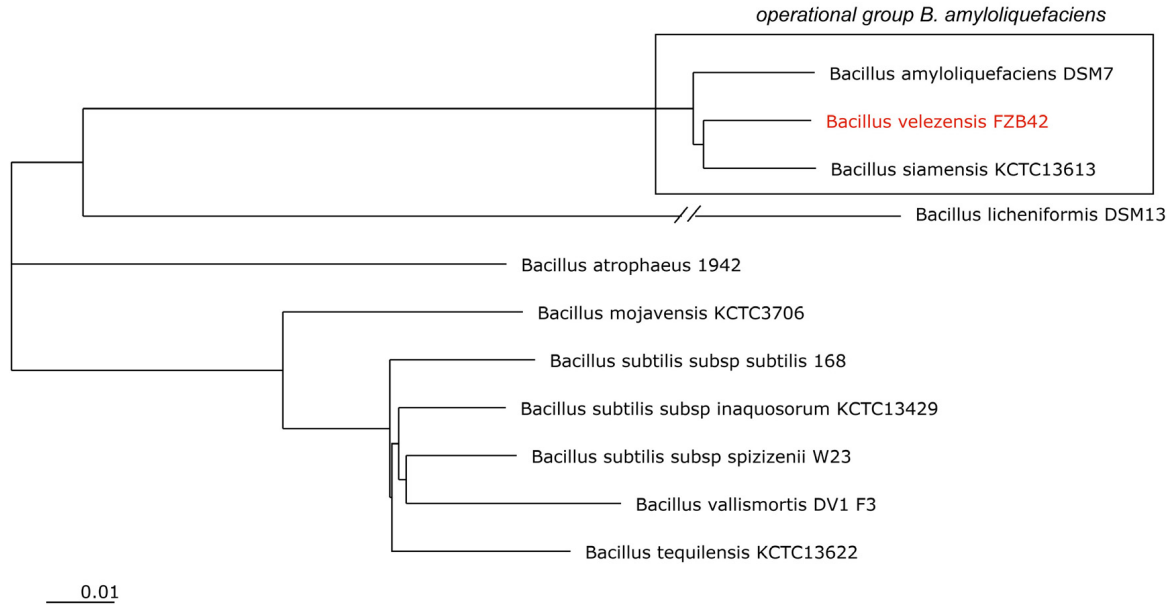


FIGURE 1 | NJ phylogenomic tree, constructed from 11 type strain genomes with highest similarity to B. subtilis 168^T. The genome of B. licheniformis DSM13 was used as outgroup. The tree was build out of a core of 1946 genes per genome, 21406 in total. The core has 586283 AA-residues/bp per genome, 6449113 in total. B. velezensis FZB42 (labeled in red) is a member of the operational group B. amyloliquefaciens (boxed). The scale bar corresponds to 0.01 substitutions per site.

Gray leaf spot disease caused by *Magnaporthe oryzae* is a serious disease in perennial ryegrass (*Lolium perenne*). A mutant strain of FZB42 (AK3) only able to produce surfactin but no other lipopeptides such as bacillomycin D, and fengycin was shown to induce systemic resistance (ISR). Similarly, treatment with crude surfactin suppressed the disease in perennial ryegrass. ISR defense response was found connected with enhanced hydrogen peroxide (H_2O_2) development, elevated cell wall/apoplastic peroxidase activity, and deposition of callose and phenolic/polyphenolic compounds. Moreover, a hypersensitive response reaction and enhanced expression of different defense factors, such as peroxidase, oxalate oxidase, phenylalanine ammonia lyase, lipoxygenase, and defensins were caused by surfactin and also the surfactin producing mutant strain (Rahman et al., 2015).

Recent studies performed with mutant strains of *B. velezensis* SQR9, which is closely related with FZB42, revealed that non-ribosomal synthesized lipopeptides fengycin and bacillomycinD, the non-ribosomal synthesized polyketides macrolactin, difficidin, and bacillaene, the dipeptide bacilysin, exopolysaccharides, and volatile organic compounds (VOCs) contribute to ISR response in *Arabidopsis* plantlets after infection with plant pathogens *Pseudomonas syringae* pv. tomato and *Botrytis cinerea* (Wu G. et al., 2018).

Volatile organic compounds produced by *B. velezensis* GB03 have been reported to trigger synthesis of ET/JA-responsive plant defense gene PDF1.2 (Ryu et al., 2004; Sharifi and Ryu, 2016). Thirteen VOCs produced by FZB42 were identified using gas chromatography-mass spectrometry analysis. A direct effect against plant pathogens was registered: benzaldehyde, 1,2-benzisothiazol-3(2 H)-one and 1,3-butadiene significantly inhibited the colony size, cell viability, and motility of *Ralstonia solanacearum*, the causative agent of bacterial wilt in a wide variety of potential host plants (Tahir et al., 2017). Furthermore, transcription of type III (T3SS) and type IV secretion (T4SS) systems were down regulated. In addition, synthesis of other genes contributing to pathogenicity, such as *eps*-genes responsible for extracellular polysaccharides, and genes involved in chemotaxis (*motA, fliT*) were found repressed. Simultaneously, the VOCs significantly up-regulated the expression of plant genes related to wilt resistance and pathogen defense. Over-expression of plant defense genes *EDS1* and *NPR1* suggested that the SA pathway is involved in the ISR response elicited by surfactin (Tahir et al., 2017).

A recent analysis performed with FZB42 VOCs confirmed that signal pathways involved in plant systemic resistance were positively affected. JA response (VSP1 and PDF1.2) and SA response genes (PR1 and FMO1) were triggered in *Arabidopsis* plantlets after incubation with the volatiles. Noteworthy, defense against nematodes were elicited by volatiles in *Arabidopsis* roots (Hao et al., 2016).

An interesting mechanism of FZB42 to avoid leaf pathogen infection has been recently described. The foliar pathogen *Phytophthora nicotianae* is able to penetrate inside of plant tissues by using natural entry sites, such as stomata. Recently it was shown that colonizing of plant roots by FZB42 restricted entry of the pathogen into leave tissues of *Nicotiana benthamiana*. It was found that FZB52 turned on the abscisic acid (ABA) and SA-regulated pathways to induce stomatal closure after pathogen infection. In addition, it was shown, that several SA- and JA/ET-responsive genes in the leaves became activated in presence of FZB42, suggesting that these signaling pathways are also

contributing to plant defenses against *P. nicotianae* (Wu L. et al., 2018).

Besides their indirect action against pathogens via triggering of ISR, polyketides and lipopeptides act directly against bacterial and fungal plant pathogens. They comprise two families of secondary metabolites non-ribosomally synthesized by multimodular enzymes, polyketide synthases (PKSs) and Peptide synthetases (NRPS), acting in assembly line arrays. The monomeric building blocks are either organic acids (polyketides) or amino acids (lipopeptides), respectively (Walsh, 2004). Their synthesis is depending on an enzyme (Sfp) that transfers 4′-phosphopantheine from coenzyme A to the carrier proteins of nascent peptide or polyketide chains. In Bacilli, e.g., FZB42, a special class of PKSs that lacks the cognate AT domain and require a discrete AT enzyme acting iteratively *in trans* (trans AT) was detected (Shen, 2003). The broadly conserved antiterminator protein LoaP (Nus G family) was identified as regulator of macrolactin and difficidin gene clusters *in B. velezensis* FZB42 on the level of transcription elongation (Goodson et al., 2017). Unfortunately, structural instability of these polyketides excluded their use as antibacterial agents.

Lipopeptides are another important class of secondary metabolites, also non-ribosomally synthesized by giant multifunctional enzymes (peptide synthetases, NRPS). Similar to PKS, three catalytic domains are involved in each elongation cycle: (1) The A-domain (adenylation domain) select its cognate amino acid; (2) The PCP domain (peptidyl-carrier domain) is equipped with a PPan prosthetic group to which the adenylated amino acid substrate is transferred and bound as thioester; (3) The condensation domain (C-domain) catalyzes formation of a new peptide bond (Duitman et al., 1999). The lipopeptide bacillomycin D is an efficient antifungal compound produced by FZB42. Its 50% effective concentration against the fungal pathogen *Fusarium graminearum* was determined to be approximately 30 μg/ml. Bacillomycin D induced morphological changes in the plasma membranes and cell walls of *F. graminearum* hyphae and conidia. Furthermore, bacillomycin D induced the accumulation of reactive oxygen species and caused cell death in *F. graminearum* hyphae and conidia. Bacillomycin D suppresses *F. graminearum* on corn silks, wheat seedlings, and wheat heads (Gu et al., 2017).

THE GENOMES OF FZB42 AND *B. subtilis* 168, A COMPARISON

Today, *B. subtilis* is considered as being a plant-associated bacterium (Wipat and Harwood, 1999; Borriss et al., 2018). A direct comparison between the genomes of *B. subtilis* 168 and *B. velezensis* FZB42 (**Table 1**) revealed that 534 FZB42 genes are not occurring in *B. subtilis* 168, but 3158 genes are shared between both species. By contrast, there are only 423 singletons defined for FZB42 *vs. Bacillus subtilis* 168. In this context one has to mention, that the singleton numbers don't correspond to the numbers in the Venn diagram. The Venn diagram (**Figure 2**) shows the numbers of reciprocal best hits between subsets of genomes. However, a gene without reciprocal best hit to another genome does not necessarily have to be a singleton. A singleton is defined as a gene without any hit against any other genome than the own one.

Many genes, essential for a plant-associated lifestyle, are shared between *B. subtilis* 168 and FZB42 as well. Relevant examples are YfmS, a chemotaxis sensory transducer, which is involved in plant root colonization (Allard-Massicotte et al., 2017), and BlrA (formerly YtvA) a blue light receptor related to plant phototropins (Borriss et al., 2018). However, due to a century of 'domestication' under laboratory conditions, the type strain *B. subtilis* 168 has lost its ability to colonize roots and to control plant diseases. Its ability to form biofilms on solid surfaces (e.g., rhizoplane) is attenuated by several mutations detected in the genes *sfp* (necessary for production of lipopeptides and polyketides), *epsC* (required for extracellular polysaccharide synthesis), *swrA* (essential for swarming differentiation on solid surfaces), and *degQ*, which stimulates phosphorylation of DegU. By contrast, the closely related wild type *B. subtilis* 3610 forms robust biofilms and is able to produce antimicrobial compounds (**Table 1**). It was shown that by introducing wild type alleles of these four genes and the spo0F phosphatase encoding *rapP* gene, residing on a large plasmid occurring in *B. subtilis* 3610 but not in *B. subtilis* 168, the laboratory strain 168 forms biofilms which are essentially the same as in 3610. This demonstrates that domestication of *B. subtilis* 168 is only due to the four gene mutations mentioned above and loss of the plasmid occurring in strain 3610 (McLoon et al., 2011). Notably, FZB42 does not harbor a *rapP* containing plasmid, but is able to produce robust biofilm similar to *B. subtilis* 3610.

FZB42 releases several cellulases and hemicellulases degrading the external cellulosic and hemicellulosic substrates present in plant cell walls. Final products of enzymatic hydrolysis are free oligosaccharides, which act as elicitors of plant defense (Ebel and Scheel, 1997). Some genes encoding extracellular hydrolases, such as *amyE* (alpha-amylase), *eglS* (endo-1,4-β-glucanase), and *xynA* (xylanase) occurred only in the plant-associated representatives of the '*B. amyloliquefaciens* operational group' but not in their soil-associated counterparts (Borriss et al., 2011; Zhang et al., 2016). Similarly, an operon involved in xylan degradation (*xylA, xynP, xynB, xylR)* is present in *B. subtilis* 168 and *B. velezensis* FZB42 but not in *B. amyloliquefaciens* DSM7^T suggesting that both strains have in common some genes involved in plant macromolecule degradation (Rückert et al., 2011).

Bacillus velezensis harbored additional genes involved in hexuronate (galacturonate and fructuronate) degradation. Three genes were found unique for *B. velezensis* FZB42 and other members of this species: *kdgK1*, (2-dehydro-3-deoxygluconokinase), *kdgA* (2-dehydro-3-deoxyphosphogluconate aldolase), and the transcription regulator *kdgR*. They are part of the six-gene *kdgKAR* operon (He et al., 2012). In addition *yjmD*, a gene with putative galacticol-1-phosphate dehydrogenase function and two further genes: *uxuA* encoding mannonate dehydratase, and *uxuB* encoding mannonate oxidoreductase are part of the six-gene transcription unit. A second operon, containing the genes *uxaC*, *uxaB*, and *uxaA* encoding enzymes for metabolizing different hexuronates to D-altronate and D-fructuronate, occurs

TABLE 1 | Comparison of the genomes of *Bacillus subtilis 168* (domesticated), *Bacillus subtilis 3610* (wild type), *Bacillus amyloliquefaciens* DSM7 (non-plant associated), and *Bacillus velezensis* FZB42 (plant associated).

	***B. subtilis* 168**	***B. subtilis* 3610**	***B. amyloliquefaciens* DSM7**	***B. velezensis* FZB42**
NCBI accession	AL009126.3	*NZ_CM000488.1*	NC_014551.1	NC_009725
Size (bp)	4,215,606	4,214,598	3,980,199	3,918,589
ANIb (AL009126.3)	*	100.00 (99.61)	76.43 (72.32)	76.35 (75.15)
ANIb (NC_009725)	76.49 (69.21)	76.47 (69.24)	93.84 (85.28)	*
Transcription units	1609	2837	2584	2506
Total genes	4381	4398	3999	3808
Protein genes	4194	4283	3870	3687
RNA genes	187	115	128	120
tRNAs	86	91	94	88
Pseudogenes	70	0	121	84
Pathways	246	184	245	238
Enzymatic reactions	1217	768	1489	1446
Transport reactions	51	80	157	150
Polypeptides	4235	4284	3870	3687
Protein features	3890	3610	3389	3305
Protein complexes	188	42	51	20
Enzymes	962	697	1114	1041
Transporters	51	134	162	175
Compounds	992	679	1257	1267
Non-ribosomal synthesized secondary metabolites				
BGC0000433	Surfactin[1] 356968 – 422359	Surfactin 356500 – 421899	Surfactin 313124 – 378534	Surfactin 322618 – 388025
BGC0000181	–	–	–	Macrolactin 1374169 – 1460068
BGC0001089	Bacillaene[1] 1768695 – 1878521	Bacillaene 1767850 – 1877685	Bacillaene 1773732 – 1876436	Bacillaene 1688756 – 1791439
BGC0001103/ BGC0001090	–	–	Iturin/Mycosubtilin 1968514 – 2008850	Bacillomycin D 1851172 – 1988997
BGC0001095	Fengycin[1] 1934525 – 2017957	Fengycin 1933702 – 2017134	Fengycin[2] 1948515 – 2058936	Fengycin 1851172 – 1988997
Triketide pyrone	T3pks 2189857 – 2191463	T3pks 2296123 – 2337238	T3pks 2170363 – 2211463	T3pks 2122078–2123684
BGC0000176	–	–	–	Difficidin 2260090 – 2360537
Nrps2	–	–	Orphan Nrps2 2500781 – 2552402	–
Nrps1	–	–	–	Orphan Nrps1 2885927 – 2868410
BGC0000309	Bacillibactin[1] 3260519 – 3310260	Bacillibactin 3259511 – 3309252	Bacillibactin 3033649 – 3100417	Bacillibactin 3001250 – 3068038
BGC0001184	Bacilysin 3850668 – 3892086	Bacilysin 3849661 – 3891079	Bacilysin 3636549 – 3677967	Bacilysin 3576267 – 3617685
Ribosomal synthesized antimicrobial compounds (RiPPs)				
BGC0000558	Sublancin 2259521 – 2279691	Sublancin 2258687 – 2278857	–	–
BGC0000602	Subtilosin_A 3826058 – 3847669	Subtilosin_A 3825052 – 3846663	–	–
BGC0000616	–	–	Amylocyclicin 3076887 – 3081038	Amylocyclicin 3044505 – 3048679
BGC0000569	–	–	–	Plantazolicin 726469 – 736360
Antibacterial peptide Lci	–	–	Lci 1296288- 1296563	Lci 310858 - 311142

*Phylogenomic relationship was determined by ANIb (average nucleotide identity, JSpeciesWS, Richter et al., 2016), either using B. subtilis 168 or B. velezensis for comparison. General data were taken from MetaCyc data base (Caspi et al., 2018). Gene clusters encoding secondary metabolites were identified by antiSMASH version 4.1.0 (Blin et al., 2017). The MIBiG accession numbers (Medema et al., 2015) are indicated. [1]Not expressed in B. subtilis 168, but expressed in its wild type counterpart B. subtilis 3610. [2]Fengycin gene cluster is only fragmentary in DSM7. Not expressed in DSM7 (Borriss et al., 2011). *Means that ANIb analyses performed with B. subtilis 168 (AL009126.3) and FZB42 (NC_009725) against itself results in 100% identity.*

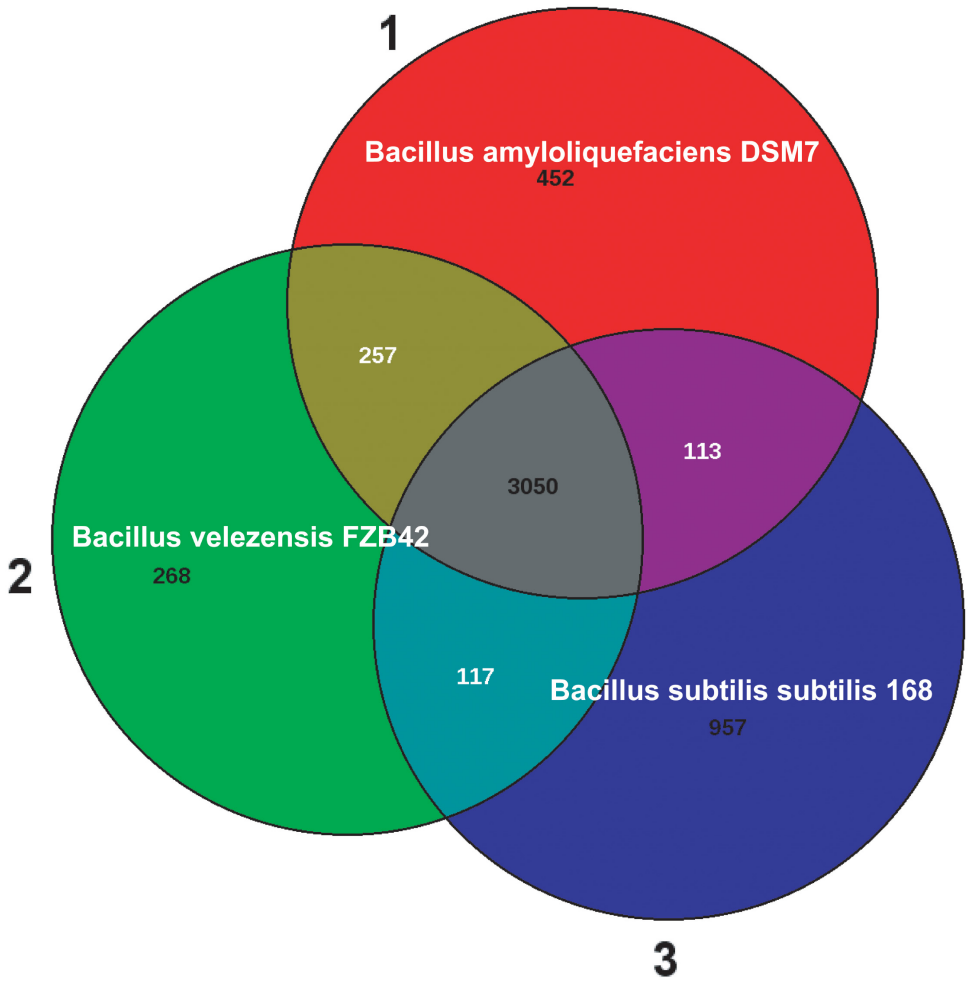


FIGURE 2 | The Venn diagram of *Bacillus amyloliquefaciens* DSM7 **(1)**, *Bacillus velezensis* FZB42 **(2)**, and *Bacillus subtilis subtilis* 168 **(3)**. The numbers of reciprocal best hits between subsets of genomes are shown. Note, 100% identical paralogous genes were not counted in the Venn diagram numbers (Blom et al., 2016). The three strains share 3050 genes according to the best hit calculation, whilst 268 genes were found unique in FZB42. A direct comparison between FZB42 and *B. subtilis* 168 revealed that they have 3122 genes in common, whilst 522 genes were found unique in FZB42.

distantly from the *kdgAR* operon. In *Escherichia coli* K12 UxuA, KdgK, and KdgA are involved in a degradative pathway of aldohexuronates (Portalier et al., 1980). Whilst the complete biochemical pathway from galacturonate to KDG is present, no gene encoding D-glucuronate isomerase was detected, suggesting that *B. velezensis* is not able to metabolize D-glucuronate (He et al., 2012).

Nearly 10% of the FZB42 genome is involved in synthesizing antimicrobial compounds, such as the polyketides bacillaene, macrolactin and difficidin (Chen et al., 2006; Schneider et al., 2007) and the lipopeptides surfactin, bacillomycin D and fengycin (Koumoutsi et al., 2004). In total, the FZB42 genome harbors 13 gene clusters devoted to non-ribosomal and ribosomal synthesis of secondary metabolites with putative antimicrobial action. In two cases, in the *nrs* gene cluster and in the type III polyketide gene cluster their products are not identified till now (**Table 1**). Similar to *B. subtilis* 168^T, the genome of the non-plant associated soil bacterium *B. amyloliquefaciens* $DSM7^T$ possesses a much lower number of gene clusters involved in synthesis of antimicrobial compounds than FZB42 (**Table 1**).

Notably, the gene clusters involved in non-ribosomal synthesis of the antifungal lipopeptides bacillomycin D and fengycin, and the polyketides difficidin and macrolactin are missing or fragmentary in $DSM7^T$ and other representatives of *B. amyloliquefaciens* suggesting that synthesis of these secondary metabolites might be important for the plant associated life style. Five out of a total of 13 gene clusters are located within variable regions of the FZB42 chromosome, suggesting that they might be acquired *via* horizontal gene transfer (Rückert et al., 2011). Most of them (bacillomycin D, macrolactin, difficidin, plantazolicin, and the orphan *nrsA-F* gene cluster) are without counterpart in $DSM7^T$ and *B. subtilis* 168^T. Moreover, it has been shown experimentally that $DSM7^T$, due to a deletion in the fengycin gene cluster, is unable to produce fengycin (Borriss et al., 2011), notably the gene cluster for synthesis of iturinA is present in the $DSM7^T$ genome (**Table 1**).

Besides type I PKS also genes encoding type III polyketide synthases are present in the genome of FZB42. By contrast to type I PKSs, type III PKSs catalyze priming, extension, and cyclization reactions iteratively to form a huge array of different polyketide products (Yu et al., 2012). In *B. subtilis* gene products of *bspA-bspB* operon were functionally characterized, and found to be involved in synthesis of triketide pyrones. The type III PKS BspA catalyzes synthesis of triketide pyrones and BspB (YpbQ) is a methyltransferase catalyzing its posttranslational modification to alkylpyrones ethers (Nakano et al., 2009). However, their biological role needs further elucidation. Orthologs of *bspA* and *bspB* are present in FZB42 and DSM7^T (**Table 1**).

Another group of secondary metabolites are bacteriocins, which represent a class of post-translationally modified peptide antibiotics (Schnell et al., 1988). Together with peptides without antibiotic activity, they are generally termed RiPPs (ribosomally synthesized and post-translationally modified peptides). RiPP precursor peptides are usually bipartite, being composed of an *N*-terminal leader and *C*-terminal core regions. RiPP precursor peptides can undergo extensive posttranslational modification, yielding structurally and functionally diverse products (Burkhart et al., 2015). In recent years, two RiPPs with antibacterial activity (bacteriocins) were identified in FZB42: plantazolicin (Scholz et al., 2011) and amylocyclicin (Scholz et al., 2014).

An antibacterial substance still produced by a FZB42 mutant strain, unable to synthesize non-ribosomally any antimicrobial compound, was identified together with the gene cluster responsible for its biosynthesis. The *pzn* genes cluster encodes a small precursor peptide PznA that is post-translationally modified to contain thiazole and oxazole heterocycles. These rings are derived from Cys and Ser/Thr residues through the action of a modifying "BCD" synthetase complex, which consists of a cyclodehydratase (C), a dehydrogenase (B), and a docking protein (D) (Scholz et al., 2011). After modification and processing of the precursor peptide plantazolicin contains an unusual number of thiazoles and oxazoles (Kalyon et al., 2011). The structure variant plantazolicin A inhibits selectively *Bacillus anthracis* (Molohon et al., 2016), and is efficient against plant pathogenic nematodes (Liu et al., 2013), whilst the precursor molecule PZNB is inactive (Kalyon et al., 2011).

The head-to-tail cyclized bacteriocin amylocyclicin was firstly described in *B. amyloliquefaciens* FZB42 (Scholz et al., 2014). Circular bacteriocins are non-lanthionine containing bacteriocins with antimicrobial activity against Gram-positive food-borne pathogens (van Belkum et al., 2011). Amylocyclicin was highly efficient against Bacilli, especially against a *sigW* mutant of *B. subtilis* (Y2) (Butcher and Helmann, 2006). An orthologous gene cluster was also detected in *B. amyloliquefaciens* DSM7^T (**Table 1**).

Lci was reported as an antimicrobial peptide synthesized by a *B. subtilis* strain with strong antimicrobial activity against plant pathogens, e.g., *Xanthomonas campestris* pv. *oryzae* and *Pseudomonas solanacearum* PE1. Its solution structure has a novel topology, containing a four-strand antiparallel β-sheet as the dominant secondary structure (Gong et al., 2011). The gene is not present in the *B. subtilis* 168 genome, but was detected in FZB42 and *B. amyloliquefaciens* DSM7^T (**Table 1**). Lci was found highly expressed in FZB42 biofilms (Kröber et al., 2016).

FZB42 GENE EXPRESSION IS AFFECTED BY PLANTS AND VICE VERSA

Nowadays, global gene expression studies were increasingly performed to enlarge our knowledge base about effect of plants on gene expression in Gram-positive plant associated bacteria (Borriss, 2015a). The first combined transcriptome- and proteome analysis in *Bacillus,* using both, DNA-microarrays and 2-D protein gel electrophoresis, was conducted with *B. subtilis* 168 (Yoshida et al., 2001). Plant-bacteria interactions were studied with *B. subtilis* OKB105 in presence of rice seedlings. Transcriptome analysis revealed that expression of 176 bacterial genes was affected by the host plant (Shanshan et al., 2015).

In this context several studies were performed with FZB42, too. Transcription of many genes involved in carbon and amino acid metabolism was turned on, when maize root exudates were added to FZB42 cells growing in planktonic culture suggesting that nutrients present in root exudates are utilized by bacteria cells (Fan et al., 2012). Dependency of FZB42 from nutrient sources present in root exudates was corroborated in a second transcriptome study performed with DNA-microarrays. In this case root exudates with different composition obtained from maize plantlets growing under stress conditions (N, P, Fe, and K limitation) were used. In case of root exudates obtained from N-deprived maize plantlets containing decreased amounts of aspartate, valine and glutamate, FZB42 cells were found to be downregulated in transcription of genes involved in protein synthesis indicating a general stress response. By contrast, P-limited root exudates led to enhanced transcription of FZB42 genes involved in motility and chemotaxis, possibly suggesting a chemotactic response toward carbohydrates in root exudates (Carvalhais et al., 2013). Transcriptional profiling via RNA-sequencing in the taxonomically related *B. velezensis* SQR9 revealed that maize root exudates stimulated at first expression of metabolism-relevant genes and then genes involved in production of the extracellular matrix (Zhang et al., 2015).

Response of FZB42 on maize root exudates during late exponential and stationary growth phase was also investigated on the level of protein synthesis applying 2-D gel electrophoresis and MALDI TOF MS for protein identification. Elicitors of plant innate immunity such as flagellins, elongation factor Tu, and cold shock proteins were detected in the extracellular fluid (Kierul et al., 2015). Corresponding to the results obtained in our transcriptome studies, we found that the expression of genes involved in utilization of nutrients and transport was enhanced in presence of root exudates. The protein with the highest secretion in presence of maize root exudates was acetolactate synthase AlsS, an enzyme involved in post-exponential phase synthesis of acetoin and 2,3 butandiol (Kierul et al., 2015).

On the other hand, plants are also affected in their gene expression, when colonized by bacteria including representatives of *the B. amyloliquefaciens* operational group. Transcript analysis of rape seedlings confronted with a root-colonizing *B. velezensis*

strain revealed that gene expression was more affected in leaves than in roots. Altogether the treatment caused a metabolic reprogramming in plant leaves (Bejai et al., 2009; Sarosh et al., 2009). Similar effects on plant gene expression were reported for root-colonizing *B. subtilis* FB17. A microarray study performed with *Arabidopsis* plantlets exposed to FB17 showed that expression of auxin-regulated genes and genes involved in metabolism, stress response and plant defense were up-regulated. Some *Arabidopsis* mutants deficient in three of the up-regulated genes, were found less colonized by FB17 (Lakshmanan et al., 2013). Further papers reporting about triggering of ISR response in plants by lipopeptides and VOCs from *B. velezensis* (Chowdhury et al., 2015b; Wu G. et al., 2018) were already discussed in a previous section.

Another study performed with FZB42 revealed that gene expression is dependent on life style. Ability to form biofilms is essential for colonizing plant root surfaces. Differential gene expression suggested that under biofilm-forming conditions transcription of 331 genes was increased and of 230 genes was decreased (Kröber et al., 2016).

The differential RNA-sequencing (dRNA-seq) technology was employed to unveil the structure of the FZB42 transcriptome (Fan et al., 2015). The unique feature of this technique is that two libraries split from the same RNA sample are compared. One library is subjected to terminator exonuclease that preferentially degrades processed RNAs with 5′-monophosphate group, thus primary transcripts with 5′-triphosphate group are enriched in relative terms (Sharma et al., 2010). Applying this method, we obtained the first global transcription start sites (TSSs) map of a PGPR *Bacillus* species. We determined a comprehensive transcriptome profile for FZB42 by identifying 4,877 TSSs for protein-coding genes. This includes >2,000 primary TSSs, >700 secondary TSSs, and nearly 200 orphan TSSs. The primary TSSs have been identified for 60% of all FZB42 genes. In addition, >1,300 internal TSSs and >1,400 antisense TSSs were also identified. A lot of coding genes were shown to be transcribed from multiple TSSs and perhaps own different UTRs. Some mRNAs contained overlapped transcripts (Fan et al., 2015). The global charting of FZB42 TSSs can favor the identification of promoter regions, *cis*-acting regulatory elements, and cognate transcriptional regulators.

By applying the dRNA-seq technique differentially expressed genes under different growth conditions were identified. For example, a large group of genes that are specifically regulated by root exudates during stationary growth were identified. The results obtained extended and corroborated our previous results obtained by using microarrays (Fan et al., 2012). Knowledge of the genes affected in their expression by plant root exudates contributes to our understanding of rhizobacterial physiology and its interaction with their host plants. They are listed as 'Interaction with plants' in AmyloWiki[3]. Moreover, this study allowed us to propose 46 previously unrecognized genes. 78 polycistronic transcripts covering 210 genes were identified and 10 previously mis-annotated genes were corrected (Fan et al., 2015).

[3]http://amylowiki.top/interaction.php

NON-CODING SMALL RNAs

Over the last decade, a growing number of non-coding regulatory small RNAs (sRNAs) have been identified in bacteria (Li et al., 2013), although the functions of most of them are still unknown. Most of sRNAs do not encode a protein, but function as an RNA regulator directly targeting multiple mRNAs. It is revealed that many sRNAs contribute to bacterial adaptation to changing environments and growth conditions (Thomason et al., 2012), therefore it is feasible to expect that sRNAs may also coordinate mutual effects of rhizobacteria on plants.

Besides graphing the profile of expressed protein-coding genes, dRNA-seq technology also offers a possibility to identify genome-wide sRNAs. We detected hundreds of non-coding RNAs in FZB42, including 136 antisense RNAs, 53 *cis*-encoded leader sequence or riboswitches, and 86 sRNA candidates (Fan et al., 2015). Among them 21 sRNAs were further validated by Northern blotting. According to their gene positions, the majority of the sRNAs perhaps act in-trans targeting the mRNAs encoded from a distant locus. Generally, sRNAs often binds to their target mRNAs, at 5′UTR in many cases, and thus modulate mRNA translation (Waters and Storz, 2009). Since the genome-wide TSS annotation of FZB42 informs about potential sRNA target sites of mRNAs, our study has provided a valuable basis for studying rhizobacterial sRNA regulation.

The function of the identified sRNAs has not been characterized in detail. However, some of the sRNAs were found related to a specific growth phase or to respond to environmental cues (soil extract or maize root exudates) (Fan et al., 2015). Furthermore, one sRNA was found to be involved in *Bacillus* sporulation and biofilm formation (data not shown). Since, sRNAs are more studied in Gram-negative than in Gram-positive bacteria, systematic detection of sRNAs in FZB42 extends our knowledge base about plant-associated Gram-positive bacteria, especially to rhizobacteria–plant interactions.

PROTEIN MODIFICATION

In recent years post-translational modifications (PTM) of proteins, such as protein phosphorylation, acetylation, methylation, and succinylation, attracted increasing attention due to their important physiological significance in organisms (Choudhary et al., 2014). Whereas most studies of PTM were performed in eukaryotic cells, nowadays the role of PTM in prokaryotes is increasingly investigated. Acetylation of lysine residues in FZB42 was studied using a combination of immune-affinity purification and high- resolution LC-MS/MS. A total of 3,268 acetylated lysine residues were detected in 1254 proteins, accounting for 32.9% of the entire proteins of FZB42. Remarkably, a high proportion (71.1 and 78.6%) of the proteins related to the synthesis of polyketides and lipopeptides were found acetylated. The finding implies an important role of lysine acetylation in the regulation of FZB42 antibiotic biosynthesis (Liu et al., 2016).

Using a similar technique, we profiled lysine malonylation of proteins in FZB42. In total, we identified 809 malonyl-lysine

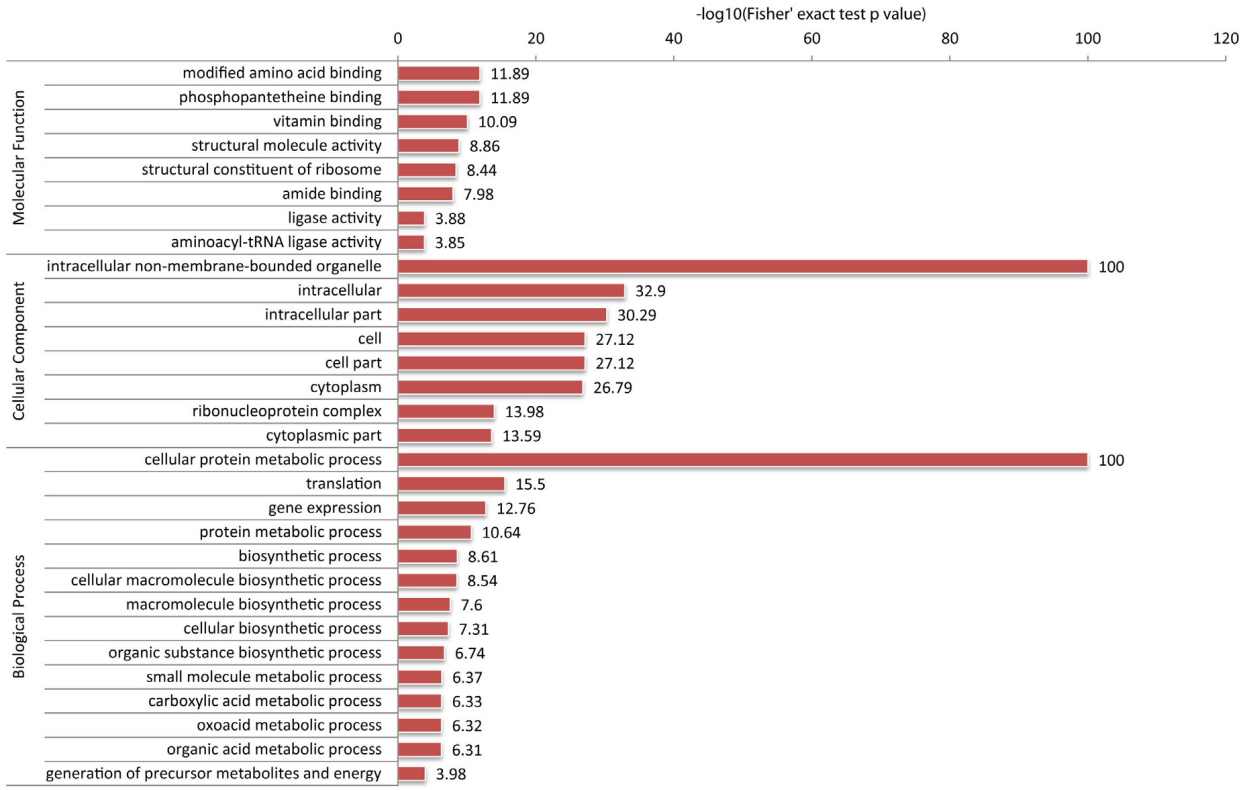


FIGURE 3 | Distribution of FZB42 malonylated proteins in various functional categories according to the GO database. The ratio of Kmal sites located in the protein to all Kmal sites was compared with the ratio of malonylated proteins to all proteins in the database. The one-tailed Fisher's exact test was used to test the enrichment and the result with p-value < 0.05 is considered significant.

sites in 382 proteins (**Figure 3**). Lysine malonylation targets the proteins implicated in a wide range of biological functions, such as fatty acid biosynthesis and metabolism, central carbon metabolism, translation processes, and NAD(P) binding. A group of proteins involved in bacterium-plant interaction was also malonylated. Moreover, malonylation seems to occur on proteins with higher surface accessibility, although the significance of the site preference remains unclear. Similar to lysine acetylation, 33 polyketide synthases (PKS) and polypeptide synthetases (NRPS) involved in non-ribosomal synthesis of bacillaene, difficidin, macrolactin, and bacillomycinD, fengycin and surfactin, were found highly malonylated. They account for 8.6% of all malonylated proteins. The PKSs and NRPSs possessed 128 malonylation sites, averagely 3.8 sites per protein, which is significantly higher than the mean of 2.1 malonylation sites per protein. The polyketide synthases, BmyA, BaeM, BaeN, and BaeR contain more than 10 malonylation sites. BaeR is the most highly malonylated protein carrying 17 malonylation sites (Fan et al., 2017b,c).

Together with the data obtained for acetylation, the high malonylation rate of PKSs and NRPSs indicates a potential effect of protein modification on biosynthesis of antibiotics in FZB42. Better understanding of the underlying mechanism of how PTM affects PKSs and NRPSs may facilitate the development of FZB42 antibiotic production and application.

AmyloWiki, AN INTEGRATING DATA BASE FOR FZB42

With the increasing reception of FZB42 as a model organism for Gram-positive PGPR, and in order to celebrate its whole genome sequencing around 10 years ago (Chen et al., 2007), we have established an integrated database 'AmyloWiki'[4] for collecting and gathering all the information known to date about this bacterium (**Figure 4**). More than 140 articles about FZB42 can be found in AmyloWiki[5] and are in part assigned to the corresponding genes/proteins. AmyloWiki centers the achievement of FZB42 studies till now including diverse information such as its 3979 genes, its transcriptome structure, protein regulators and their targets. 595 genes of FZB42 involved in plant-bacteria interactions were listed[6]. It informs also about recently identified sRNA genes and post-translational modification sites (see previous sections). A growing list of FZB42-site directed mutant strains, available for scientific community, is also presented. AmyloWiki shares some features with SubtiWiki, the popular database for *B. subtilis* 168 (Zhu and Stülke, 2018); however, specific features of FZB42 such as genes

[4]http://amylowiki.top/

[5]http://amylowiki.top/reference.php

[6]http://amylowiki.top/interaction.php

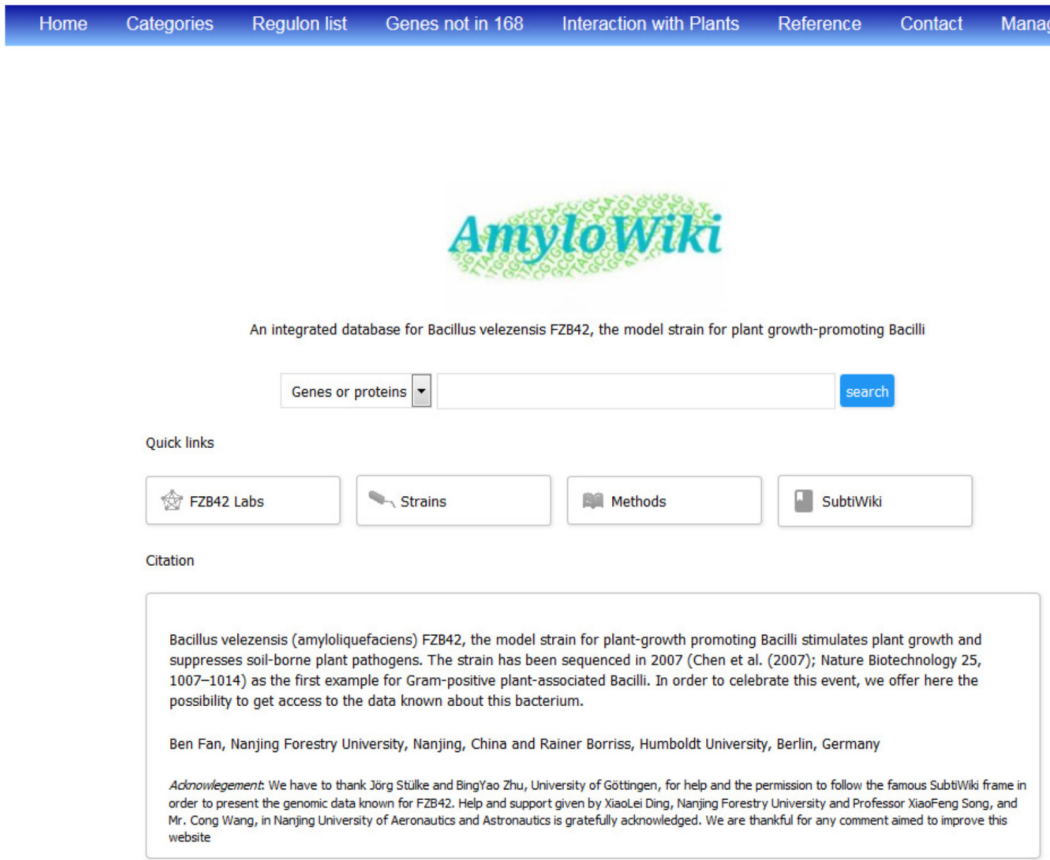


FIGURE 4 | Home page of AmyloWiki, an integrated data base of FZB42.

not occurring *in B. subtilis* 168, and genes involved in antagonism against plant pathogens and plant-microbe interaction, are highlighted in AmyloWiki. To facilitate communication and information exchange, a growing list of groups studying FZB42 is available, and many possibilities for interactive data exchange and feedback with the users are given.

AmyloWiki is configured to be a comprehensive and user-friendly database, built upon typical XAMPP (X-Windows, Linux or Mac OS + Apache + MySQL + PHP + Perl) environment. Apache 2.4.23 was used to construct a webserver. All data sets were processed and stored in MySQL (5.0.11). PHP language (version 5.6.28) was used to built database management system and interface. Webpages were designed with HTML5, CSS3 and JavaScript techniques. AmyloWiki provides a series of functions such as data submission, resource downloading, searching, advanced retrieval, and feedback.

Briefly, most information of user's interest can be returned by performing a searching. User can search with different of the query strings, such as gene name, gene locus, and PubMed ID. The items that matched the query string will be returned in the result page. This can be exemplified by searching a gene, as happens most often. The basic information of the gene such as its product, locus, synonyms, homolog in *B. subtilis*, position, length and others, will be provided on the top of the result page. The genomic context of the gene can be viewed in a visualized window with scrollable function to check its neighbor genes. The organization of the gene, if it is present in an operon, the functions the gene involved, and its functional categories/subcategories are offered next. Other associated information includes the phenotypes of the mutant, its transcriptional start sites, protein/non-coding RNA regulators, sigma factors, PTM sites and so on. The references concerning the gene are listed at the bottom of the retrieval page.

For the convenience of the user, all datasets of AmyloWiki can be downloaded at the "Download" page. The data can be downloaded in an Excel-compatible format for their specific analysis. AmyloWiki will be maintained by us with a frequent update to improve its configuration and to keep the information comprehensive. For example, it is planned to add in future experimental protocols specifically worked out and used for FZB42, like transformation and bioassay. Here, support given by experienced groups dealing with FZB42 is highly welcomed. The pages for data submission and correction are designed for authorized users in order to update relevant information. Unauthorized users are encouraged to submit their latest data *via* E-mail to the authors of the website. Then their information will be verified and included in AmyloWiki.

CONCLUSION AND OUTLOOK

In order to improve consistency in performance of bioinoculants in a sustainable agriculture we have to integrate them as part of modern crop management programs allowing to decrease the amount of agrochemicals, including harmful chemopesticides. A full understanding of the complex relationship between plant, soil, climate, microbiota, and the microbial inoculant is a necessary precondition for application success of biologicals. Basic research which has been restricted in past to selected representatives of taxonomic groups ('model organisms') such as *B. subtilis* 168, *E. coli* K12, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* has considerably deepen our understanding of those groups in general. We recommend using FZB42 as a model for research on Gram-positive rhizobacteria. This will greatly enhance scientific progress in the field and might contribute to a better consistency in application of environmental friendly beneficial Bacilli in modern agriculture. After 20 years of basic and applied research FZB42 has been proven as suitable for selecting to this task. The following features favor use of FZB42 as model organism:

(1) Apathogenicity: Concerning biosafety issues, no representatives of the *B. subtilis* species complex including *B. velezensis* have been listed as risk group in 'The Approved List of biological agents' (Advisory Committee on Dangerous Pathogens, 2013). However, *B. cereus* and *B. anthracis* were listed in human pathogen hazard group 3, excluding their use as biocontrol agents in agriculture.
(2) long term successful application of a commercialized product based on FZB42 in agriculture
(3) genetic amenable and a large collection of defined mutant is available for interested scientists
(4) large body of scientific knowledge (>140 articles) about FZB42 is already available
(5) an integrative data base 'AmyloWiki' has been established about FZB42 aimed to enhance collaboration of several groups dealing with Gram-positive PGPR and biocontrol bacteria

Most of the biocontrol agents currently in use are based on living microbes. Representatives of the *B. subtilis* species complex, including *B. velezensis*, *B. subtilis,* and *B. pumilus* are increasingly used for commercial production of biofungicides (Borriss, 2016). Most of them are stabilized liquid suspensions or dried formulations prepared from durable endo-spores. They are developed for seed coating, soil or leave application. Unfortunately, it is very unlikely that concentration of *Bacillus* synthesized cyclic lipopeptides in their natural environment is sufficient for antibiosis (Debois et al., 2014). A possibility for circumventing this problem are combined bioformulations consisting of both, *Bacillus* spores and antagonistic acting metabolites. However, only a few bioformulations currently on the market, such as SERENADE® prepared from *B. subtilis* QST713 and Double Nickel 55 prepared from *B. amyloliquefaciens* D747 (both strain names need to be corrected as *B. velezensis*, Fan et al., 2017a), contain together with living spores antimicrobial compounds, such as cyclic lipopeptides (iturins, fengycin). Unfortunately, also in these products only the number of spores is declared as active ingredient of the biofungicide, but concentration of the metabolites is not indicated, excluding an exact treatment of pathogen infected plant parts. Labeling a fixed concentration of the active principle for suppressing the target would allow a better comparison of chemical and biological pesticides (Borriss, 2015b). To the best of our knowledge, no bioformulations containing exclusively antimicrobial metabolites are commercially available, although companies like ABiTEP performed extended large scale trials with concentrated and stabilized *Bacillus* supernatants in order to suppress plant pathogens.

AUTHOR CONTRIBUTIONS

BF and RB outlined and wrote the manuscript. BF, CW, XLD, XFS, and RB developed the integrated data base AmyloWiki. LW, HW, and XG contributed essential scientific results reported in this review. All authors have approved and corrected the final version of the manuscript.

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Corrigendum: *Bacillus velezensis* FZB42 in 2018: The Gram-Positive Model Strain for Plant Growth Promotion and Biocontrol

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Keywords: *Bacillus velezensis*, FZB42, AmyloWiki, induced systemic resistance (ISR), non-ribosomal synthesized lipopeptides (NRPS), non-ribosomal synthesized polyketides (PKS), volatiles, plant growth promoting bacteria (PGPR)

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A Corrigendum on

***Bacillus velezensis* FZB42 in 2018: The Gram-Positive Model Strain for Plant Growth Promotion and Biocontrol**

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In the original article, there was an error in referring to the approved group of *Bacillus cereus*.

A correction has been made to the **Conclusion and Outlook** section, "(1) Apathogenicity":

"(1) Apathogenicity: Concerning biosafety issues, no representatives of the *B. subtilis* species complex including *B. velezensis* have been listed as risk group in 'The Approved List of biological agents' (Advisory Committee on Dangerous Pathogens, 2013). By contrast, use of strains of *B. cereus* needs special attention, since they are a member of risk group 2 and closely related to *B. anthracis*, a human pathogen and the member of risk group 3."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

REFERENCES

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Chemosensitization of *Fusarium graminearum* to Chemical Fungicides Using Cyclic Lipopeptides Produced by *Bacillus amyloliquefaciens* Strain JCK-12

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Fusarium head blight (FHB) caused by infection with *Fusarium graminearum* leads to enormous losses to crop growers, and may contaminate grains with a number of Fusarium mycotoxins that pose serious risks to human and animal health. Antagonistic bacteria that are used to prevent FHB offer attractive alternatives or supplements to synthetic fungicides for controlling FHB without the negative effects of chemical management. Out of 500 bacterial strains isolated from soil, *Bacillus amyloliquefaciens* JCK-12 showed strong antifungal activity and was considered a potential source for control strategies to reduce FHB. *B. amyloliquefaciens* JCK-12 produces several cyclic lipopeptides (CLPs) including iturin A, fengycin, and surfactin. Iturin A inhibits spore germination of *F. graminearum.* Fengycin or surfactin alone did not display any inhibitory activity against spore germination at concentrations less than 30 μg/ml, but a mixture of iturin A, fengycin, and surfactin showed a remarkable synergistic inhibitory effect on *F. graminearum* spore germination. The fermentation broth and formulation of *B. amyloliquefaciens* JCK-12 strain reduced the disease incidence of FHB in wheat. Furthermore, co-application of *B. amyloliquefaciens* JCK-12 and chemical fungicides resulted in synergistic *in vitro* antifungal effects and significant disease control efficacy against FHB under greenhouse and field conditions, suggesting that *B. amyloliquefaciens* JCK-12 has a strong chemosensitizing effect. The synergistic antifungal effect of *B. amyloliquefaciens* JCK-12 and chemical fungicides in combination may result from the cell wall damage and altered cell membrane permeability in the phytopathogenic fungi caused by the CLP mixtures and subsequent increased sensitivity of *F. graminearum* to fungicides. In addition, *B. amyloliquefaciens* JCK-12 showed the potential to reduce trichothecenes mycotoxin production. The results of

this study indicate that *B. amyloliquefaciens* JCK-12 could be used as an available biocontrol agent or as a chemosensitizer to chemical fungicides for controlling FHB disease and as a strategy for preventing the contamination of harvested crops with mycotoxins.

Keywords: *Bacillus amyloliquefaciens* JCK-12, Fusarium head blight, cyclic lipopeptides, synergistic antifungal effect, chemosensitization

INTRODUCTION

Fusarium graminearum causes Fusarium head blight (FHB), a globally devastating fungal disease of small grain cereals, especially on wheat and barley (McMullen et al., 1997). FHB results in extensive yield loss of grains (Goswami and Kistler, 2004). Over the past several decades, severe FHB outbreaks have occurred in wheat growing areas across the world, and an estimated US$ 8 billion loss was incurred from FHB in the United States between 1993 and 2001 (Nganje et al., 2004). Moreover, due to climate change, it is expected that FHB epidemics will become more severe and further losses in crop yield will occur (Madgwick et al., 2011). FHB also reduces the quality and feeding value of crops by producing various toxic metabolites, including trichothecenes and zearalenone mycotoxins. Deoxynivalenol (DON), the most important trichothecene, poses a significant threat to animal health and food safety (Marasas et al., 1984; Joffe, 1986; Snijders, 1990; McMullen et al., 2012) and facilitates disease development by acting as a virulence factor (Desjardins et al., 1996).

Fusarium head blight disease can be prevented or controlled through several strategies including crop rotation, tillage practices, fungicides application, and planting less susceptible cultivars. Among these, chemical control applied during the anthesis period is the most effective strategy (Homdork et al., 2000; Mesterházy et al., 2011). However, because of the rising cost of chemical pesticides, growing perception about their negative effects such as accumulation of toxic residues in crops and development of resistance in pathogens in recent years, it is urgently required to develop safer control agents fulfilling the consumer demand of pesticide-free production. Moreover, FHB management using chemical fungicides has a major problem, in that *F. graminearum* infections can occur beyond the 30-day preharvest interval when chemical fungicides cannot be legally applied (Francl et al., 1999; McMullen et al., 2012). These late-season infections lead to significant accumulation of the *Fusarium*-produced mycotoxin DON. Thus, biological control using antagonistic microorganisms has been suggested as an alternative strategy and can be used as part of an integrated management for FHB disease (Dal Bello et al., 2002; Kim et al., 2003).

Several bacterial strains have been reported as antagonistic microorganisms against *F. graminearum* (Zhao et al., 2014). *Bacillus* strains have especially been spotlighted as effective biological control agents due to their strong antimicrobial activities as well as their resistance to environmental stress conditions (Nagórska et al., 2007). The biocontrol mechanisms of *Bacillus* strains are related to antagonism, competition, systemic resistance induction, root colonization, and promotion of plant growth (Ongena and Jacques, 2008). In particular, some *Bacillus* strains produce a variety of lipopeptides with considerable structural diversity, which are key players in their antagonistic activities toward viruses, mycoplasmas, bacteria, yeast, fungi, and nematodes (Zeriouh et al., 2011). *B. subtilis* or related species have been known to produce three cyclic lipopeptides (CLPs), iturin A, fengycin, and/or surfactin. The iturin family shows a strong antifungal activity against both yeast and fungi, (Moyne et al., 2001; Yu et al., 2002), whereas the fengycin family has a broad range of fungitoxic activity, mainly against filamentous fungi, and are also known to elicit induced systemic resistance (ISR) (Vanittanakom et al., 1986). The surfactin family also induces ISR in plants and functions as strong biosurfactants (Ongena and Jacques, 2008). To date, the effectiveness of *Bacillus* strains for the control of *F. graminearum* has been mainly evaluated using bacterial culture supernatants, whereas few studies have been performed using a formulation based on *Bacillus* strains in field trials (Khan et al., 2004; Palazzini et al., 2007; Khan and Doohan, 2009). Moreover, no study has yet been conducted to investigate the synergistic effects of biological control agents and chemical fungicide mixtures under the field condition.

In the last decade, crop protection using chemical fungicides has been an influential tool for the control of various plant pathogenic fungi. However, by the extensive use of chemical fungicides, there is now a significant increase in the incidence of resistance in fungal pathogens to agricultural fungicides. Therefore, chemical fungicides undermine the reliability of their effectiveness, which is relatively short-lived and ultimately uneconomical. Chemosensitization is one of the strategies to resolve this problem. It is based on the enhanced sensitivity of pathogen to fungicides by co-application with a non- or slightly active fungicidal substance and a commercial fungicide at levels where, neither compound alone would be effective. Since antifungal chemosensitization is considered a novel antifungal intervention strategy, the model fungal system has been used for the development of chemosensitizers to improve the efficacy of conventional agents against fungal pathogens (Niimi et al., 2004; Kim et al., 2008).

In this study, we isolated *B. amyloliquefaciens* strain JCK-12 possessing strong antifungal activity against *F. graminearum* and observed that *B. amyloliquefaciens* JCK-12 and several chemical fungicides in combination have synergistic antifungal interactions. Our study shows the possibility of *B. amyloliquefaciens* JCK-12 as an available biocontrol agent for controlling FHB or as a chemosensitizer to improve the sensitivity of chemical fungicides. Therefore, the objectives of this study were (1) to identify useful bacterial strains for FHB

control, (2) to determine the antagonistic efficiency of the JCK-12 strain for FHB control, and (3) to increase the utilization value of JCK-12 by characterizing its biocontrol mechanism.

MATERIALS AND METHODS

Strains, Culture, and Growth Conditions

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie, 1999), hH1-GFP (Hong et al., 2010), and HK12, a constitutive GFP expresser (Son et al., 2011b), was grown on potato dextrose agar (PDA) at 25°C for propagation of mycelium and in carboxyl methyl cellulose (CMC) medium for asexual sporulation (Cappellini and Peterson, 1965). For evaluating antifungal activity, *Botrytis cinerea*, *Colletotrichum coccodes*, *Fusarium oxysporum* f. sp. *niveum*, *Phytophthora capsici*, and *Rhizoctonia solani* were received from Dr. GJ Choi, Korea Research Institute of Chemical Technology, Daejeon, Korea. *Fusarium oxysporum* f. sp. *lycopersici* (KACC40043), *Fusarium verticillioides* (KACC45825), and *Magnaporthe oryzae* (KACC46522) were received from the Korean Agricultural Culture Collection (Wanju, Korea). We also received *Raffaelea quercus-mongolicae* from the National Institute of Forest Research (Seoul, South Korea). Each plant pathogenic fungus was incubated on PDA except for *P. capsici*, for which V8 agar medium was used (Ristaino, 1990). Bacterial strains were inoculated in tryptic soy broth (TSB, Difco, Detroit, MI, United States) for 3 days at 30°C with agitation (200 rpm). All strains were stored in 20% glycerol at −70°C.

Isolation of Rhizospheric Bacteria

Soil samples were taken from the fields grown tomatoes, peppers, or onions of four regions in Korea (Daejeon, Gwangju, Jeongeup, and Geoje). Samples were collected from soil which around plants roots between 10 and 20 cm depth, placed in a cool box for transport, and stored at 4°C. Each 1 g of soil sample was suspended in 10 ml of sterile distilled water and shaken vigorously for 2 min. The supernatant was serially diluted in sterile distilled water (10^{-1} to 10^{-7}), and plated on tryptic soy agar medium (TSA, Difco). After incubation at 30°C for 1–2 days, isolated colonies were purified on TSA, and stored cryogenically at −70°C.

Screening of Antifungal Bacteria by a Dual Culture Bioassay

The antagonistic activity of bacterial strains isolated from rhizospheres against *F. graminearum* was evaluated by a dual culture bioassay (Lam et al., 2000). A mycelial plug (5 mm diameter) of *F. graminearum* was placed at the center of the PDA plate and the bacterial strains were inoculated at four cardinal points 25 mm from the center of the plate. As control, only *F. graminearum* was inoculated. After 4 days of incubation at 25°C, the inhibition zone was measured. Inhibition was graded by relating the inhibited growth area per inoculation streak to the total area of the Petri dish. The following scale was used: -, no visible inhibition; *, no fungal growth on 0.1–3% of plate area/bacterial streak; **, no fungal growth on 3–8% of plate area/bacterial streak; ***, no fungal growth on >8% of the plate area/bacterial streak. All data were obtained from three replicates.

Inhibition of Spore Germination

The fermentation broth of each bacterial strain was centrifuged at 7,000 rpm for 20 min and the supernatant was filtered through a membrane filter (0.2 μm, Advantec, Tokyo, Japan). The axenic fermentation broth was tested for inhibitory activity against conidial germination of *F. graminearum*. Conidia were obtained from *F. graminearum* cultures incubated in CMC for 4–7 days. The spore suspension was filtered through four layers of sterile cheesecloth to remove mycelia and then adjusted to 1.0×10^6 conidia/ml using a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany). The axenic fermentation broth was applied at a concentration of 20, 10, 5, and 2.5%, and autoclaved TSB was used as a negative control. The final volume of the spore suspension was adjusted to 200 μl in each well of a 48-well plate and cultured at 200 rpm and 25°C. A spore was considered to be germinated if the germ tube was longer than or equal to the greatest dimension of the swollen spore (Dantigny et al., 2006). Three replicates of 100 spores for each experiment were observed using a Zeiss Axio Imager A2 Microscope (Carl Zeiss, Oberkochen, Germany).

Molecular Identification of JCK-12

Identification of the bacterial strain was performed by evaluating the 16S rRNA, gyrase subunit A (*gyrA*), and bacterial RecA (*recA*) gene sequences. The genomic DNA of the bacterial strain was purified using the Bacterial genomic DNA purification kit (ELPIS-Biotech, Daejeon, South Korea) according to the manufacturer's recommendations. Next, 16S rRNA, *gyrA*, and *recA* genes were amplified by PCR using specific primer pair sets (Supplementary Table 1) and sequenced (Genotech Co., Daejeon, South Korea). The gene sequences of some related species were downloaded from the GenBank database and aligned using BioEdit version 5.0.9.1 (Hall, 1999). The phylogenetic trees were constructed by the neighbor-joining (NJ) method in MEGA version 6.0 with 1,000 bootstrap replicates (Tamura et al., 2013).

Antifungal Activity of JCK-12 against Various Plant Pathogenic Fungi

The antifungal potential of strain JCK-12 was examined by a dual culture assay using several plant pathogenic fungi (Fokkema, 1978). A 5 mm agar plug of each plant pathogenic fungus was placed on one side of a Petri dish containing PDA medium for most of the test fungi except for *P. capsici*, for which V8 agar medium was used. JCK-12 was then streaked at a distance of 35 mm from the fungal pathogen agar plug on the same dish. These paired cultures were incubated at 25°C. Plates inoculated only with test pathogens served as controls. The mycelial growth of each fungus was measured for 3–13 days until the mycelia of the corresponding control fungus grew to 35 mm. Antagonistic activities were evaluated by subtracting the distance of the fungal growth radius of the control culture. The experiments were repeated three times with three replicates.

Extraction and Purification of Antifungal Cyclic Lipopeptides (CLPs)

After incubation of JCK-12 at 30°C for 3 days with agitation (150 rpm), the fermentation broth (3 l) was centrifuged at 8,874 × *g* for 20 min at 4°C to remove the bacterial cells. The bacterial cell-free supernatant of JCK-12 was partitioned twice with equal volumes of *n*-butanol. The butanol layer was concentrated to dryness on a rotary evaporator and then tested for inhibitory activity against *F. graminearum* spore germination. The butanol extract highly inhibited spore germination. The butanol extract (10 g) was separated by silica gel column chromatography (70–230 mesh, 300 g, 4.2 cm i.d × 60 cm; Merck, Darmstadt, Germany) using chloroform-methanol-distilled water-acetic acid (14:6:1:0.021, v/v/v/v) to yield three fractions F1–F3. Among these three fractions, only F2 was active. The bioautographic method revealed that CLP is responsible for the inhibitory activity of F2 against *F. graminearum* spore germination. To refine the active cyclic lipopeptides (CLPs), F2 (2.72 g) was dissolved in water and precipitated by adjusting the pH to 2.0 with 6 mol/l HCl. The solution was then stored overnight at 4°C, the resulting precipitates were collected by centrifugation at 8,874 × *g* for 20 min, and then extracted twice with methanol. The refined F2 was further purified on an ODS column (200–400 mesh, 55 g, 3.2 cm i.d. × 20 cm, Sigma–Aldrich, St. Louis, MO, United States) using methanol-distilled water (3:7), methanol-distilled water (5:5), methanol-distilled water (7:3), and methanol (100%) as mobile phases, and the antifungal active fraction was collected. The antifungal active fraction (148.3 mg) was next separated through a Sephadex LH-20 column (50 g, 1 cm i.d. × 40 cm; Sigma–Aldrich) using methanol (100%) and the separated antifungal compound was finally purified by preparative TLC (Prep TLC; 20 × 20 cm, 0.5 mm, Merck). The prep TLC was developed in chloroform-methanol-distilled water-acetic acid (14:6:1:0.021, v/v/v/v) as a mobile phase. The antifungal compound was then scraped from the developed Prep TLC and eluted with methanol, yielding the final compound (25.2 mg).

To confirm the production of other CLPs produced by JCK-12, the butanol extract of the JCK-12 culture supernatant was separated by prep TLC using chloroform-methanol-distilled water-acetic acid (14:6:1:0.021, v/v/v/v). After developing and drying the prep TLC plate, it was sprayed with water. Two white colored regions (fraction 1 and fraction 2) were separately scraped from the prep TLC and then extracted with methanol.

Identification of Compounds Isolated from JCK-12 by HPLC and ESI-MS/MS Analyses

The antifungal active compound and the two CLP fractions, fraction 1 and fraction 2, isolated from JCK-12 were analyzed by HPLC using the following conditions. A C_{18} column (Atlantis T3, 5 μm, OBD 19 × 250 mm; Waters, Wexford, Ireland) was used, and the antifungal compound and fraction 1 were detected at 230 nm, whereas fraction 2 was detected at 215 nm. The mobile phases were as follows: solvent A, water with 0.1% trifluoroacetic acid (TFA); and solvent B, acetonitrile with 0.1% TFA. The compound was eluted with a linear gradient of solvent A increasing from 10 to 100% at a flow rate of 1 ml/min for 60 min. Iturin A (Sigma–Aldrich), fengycin (Sigma–Aldrich), and surfactin (Sigma–Aldrich) were used as standard chemicals. The retention times and UV spectra of the three CLPs were compared with those of the standard chemicals. To clearly confirm the identities of the CLPs isolated by prep TLC, electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis was performed on a SYNAPT G2 time-of-flight (TOF) mass spectrometry system (Waters, Manchester, United Kingdom) at a mass range of *m/z* 100–2,000.

Inhibitory Activity of Commercial CLPs against *F. graminearum* Spore Germination

In order to examine the inhibitory activity of three CLPs, iturin A, fengycin, and surfactin, which were produced by JCK-12, against conidial germination of *F. graminearum*, the three standard chemicals were treated alone or in combination at a concentration range of 2.5–60 μg/ml. Commercial surfactin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and then diluted with methanol at a concentration of 1 mg/ml. Commercial fengycin and iturin A were dissolved in methanol and ethanol, respectively, at a concentration of 1 mg/ml. Mixtures of the three CLPs (iturin A+fengycin+surfactin = I+F+S) were prepared at ratios of 1:1:1, 1:1:0, 0:1:1, and 1:0:1 (w/w/w) in order to examine the synergistic effects between the CLPs. The mixtures were applied at a concentration range of 5–60 μg/ml. Ethanol and methanol were used as negative controls at 6% and DMSO at 0.6%, the concentration of which was determined through a preliminary experiment. The solvents at the given conditions did not affect the *F. graminearum* spore germination. The experiment was performed twice with three replicates and one hundred spores for each experiment were observed under the microscopy.

Antifungal Activity of JCK-12

The antifungal activities of the JCK-12 culture supernatant and its butanol extract including three CLPs on the radial growth of *F. graminearum* were tested by a dual culture assay and pour plate method. For the dual culture assay, agar plugs (5 mm diameter) of actively growing mycelia were inoculated near the edge of a 50 mm-diameter Petri plate containing complete medium (CM) and incubated at 25°C. After 24 h, sterile paper disks with 10 μl of Luria-Bertani (LB) medium containing JCK-12 (1×10^7 cfu/ml) or 10 μl of the butanol extract (60 μg/ml) were placed on the opposite edges of the Petri dishes and incubated at 25°C for 4 days. For pour plating, CM supplemented with the butanol extract (60 μg/ml) was used. These experiments were performed twice with three replicates.

To investigate the effect of the butanol extract on the mycelial morphology of *F. graminearum*, the fungus was cultured in liquid CM containing 0, 30, or 60 μg/ml of the butanol extract for 24 h at 25°C on a rotary shaker (200 rpm). Changes in hyphal morphology were observed with differential interference contrast

(DIC) microscopy. DIC images were visualized using a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany). To visualize green fluorescent protein (GFP) expression in cells, the 38H (excitation 470/40; emission 525/50) filter set was used.

To verify whether the effect of the butanol extract on mycelial morphology was derived from the antifungal action of the three CLPs produced by JCK-12, the fungus was cultured in liquid CM containing 15 μg/ml of commercial iturin A, or 1.25 μg/ml of a mixture of the three commercial CLPs for 24 h at 25°C on a rotary shaker (200 rpm). Changes in hyphal morphology were observed by DIC microscopy.

Detection of Cell Membrane Permeability

The effect of butanol extract on cell membrane permeability was examined by propidium iodide (PI) staining as described in a previous study with slight modifications (Gao et al., 2016). Briefly, fungal strains grown for 24 h in 50 ml of liquid CM were incubated for an additional 24 h in CM supplemented with 30 μg/ml of the butanol extract. Mycelia were then harvested and incubated in 2 μM of PI for 20 min. To visualize the red fluorescent dye, a microscope using the filter set 15 (excitation 546/12; emission 590) was used. The experiments were repeated at least twice independently.

Synergistic Effects of JCK-12 with Other Antifungal Compounds

To visualize the synergistic antifungal effect of the butanol extract including three CLPs and other antifungal compounds on the hyphal growth of *F. graminearum*, the fungus was grown on CM supplemented CLPs alone (10 or 30 μg/ml) and in combination with other antifungal agents including Congo Red (60 μg/ml), Calcofluor White (0.3 mg/ml), iprodione (8.6 μg/ml), fludioxonil (0.023 μg/ml), benomyl (0.65 μg/ml), difenoconazole (0.025 μg/ml), and tebuconazole (0.0125 μg/ml). The plates were incubated at 25°C for 4 days.

Trichothecene Analysis

The total trichothecene produced (deoxynivalenol and 15-acetyl-deoxynivalenol) by *F. graminearum* was measured as described previously (Son et al., 2011a). Fungal strains were grown in minimal media containing 5 mM agmatine (MMA) supplemented with the butanol extract (0, 15, or 30 μg/ml). Seven days later, the cultures were filtered through cheesecloth, and the filtrates were extracted with an ethyl acetate–methanol solution (4:1, v/v). The dehydrated extracts were derivatized with a trimethylsilylating reagent (BSA + TMCS + TMSI, 3:2:3; Supelco, Bellefonte, PA, United States) and analyzed on a Shimadzu QP-5000 gas chromatography mass spectrometer (GC-MS; Shimadzu, Kyoto, Japan). Total trichothecene production was quantified based on the biomass produced by each strain in MMA. The experiment was repeated five times.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (iNtRON Biotech, Seongnam, South Korea) and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, United States). qRT-PCRs were performed using SYBR Green Super Mix (Bio-Rad, Hercules, CA, United States) and a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, United States) using the primer pairs for *TRI5* and *TRI6* (Supplementary Table 1). The cyclophilin gene (*CYP1*; FGSG_07439) was used as the reference gene. qRT-PCRs were performed three times with two replicates per run, and the transcript level of each target gene was calculated as described previously (Livak and Schmittgen, 2001).

Disease Control Efficacy of the JCK-12 Fermentation Broth against FHB on Wheat

Five seeds of 'Eunpamil' wheat (*Triticum aestivum* L.) were sown in the nursery soil in vinyl pots (5 cm diameter) and then stored at 4°C for 3 weeks. The seedlings were then grown in the greenhouse for 1 week and followed by transplantation into single plastic pots (20 cm diameter). The plants were grown for roughly 8 weeks in the greenhouse at 25 ± 10°C. Ten spikes were chosen per pot and used for the *in vivo* bioassay. The fermentation broth of JCK-12 was diluted with distilled water by 5- and 20-fold, and Tween-20 was then added to each solution at a concentration of 250 μg/ml as a wetter. Tween-20 solution was used as a negative control. Almuri (Syngenta, Korea), which contains 13% of difenoconazole and 13% of propiconazoles as active ingredients, was used as a positive control at a 2,000-fold dilution. A spore suspension of *F. graminearum* (2×10^5 spores/ml) was sprayed at 1 day after fungicide treatment. The aerial part of each pot was covered with a plastic bag for moisture maintenance. After 3 days, the plastic bag was removed from each pot. After 2 weeks incubation in a greenhouse, both disease incidence (DI, percentage of infected spikes) and severity (DS, percentage of infected spikes among the diseased spikes) of 10 spikes per plot were estimated to yield plot disease severity (FHB index = incidence × severity/100).

A field experiment was conducted using 'Eunpamil' at an experimental farm at Chonnam National University (Gwangju, South Korea). Each treatment plot consisted of three 2 m rows with a row spacing of 50 cm. The compound treatments and infection process, same as the greenhouse experiment, were performed, and Almuri (2,000-fold dilution) and Tween-20 solutions were applied as positive and negative controls, respectively. *F. graminearum* spores of were inoculated at 2×10^5 spores/ml using a compressed air sprayer at approximately 2 h before sunset. Both DI and DS for 30 spikes per plot were estimated at 2 weeks after inoculation. Plots were arranged as a randomized complete block design with three replicates per treatment.

Formulation of a Wettable Powder and Its Disease Control Efficacy against FHB on Wheat

To assess the potential of JCK-12 formulation as the biocontrol agent against FHB, the JCK-12 fermentation broth was dried using a pilot spray dryer (Yoojin Tech. Co., Ltd., South Korea). The dried powder (20 g) was mixed with 15 g of synthetic hydrated silicon dioxide (Rhodia Asia Pvt. Ltd., Singapore), 5 g of sodium dodecyl sulfate (Yoosung Chemical R&T Co., Ltd., South Korea) as a surfactant or wetting agent, 5 g of sodium polynaphthalene formaldehyde (Yoosung Chemical R&T Co., Ltd., South Korea) as a dispersal agent, and 55 g of kaolin. The JCK-12 formulation (JCK-12 WP20) was milled in a blender.

The JCK-12 WP20 was diluted 500-fold with tap water. Tap water was used as a negative control. Almuri (Syngenta), at a 2,000-fold dilution was used as a positive control. The synergistic effect of the JCK-12 formulation and the synthetic antifungal agents was tested using the susceptible wheat 'Eunpamil' in the same manner as described in the disease control efficacy experiment with the fermentation broth of JCK-12 against FHB (Lee et al., 2009). Both DI and DS for 30 spikes per plot were estimated at 2 weeks after inoculation. Plots were arranged as a randomized complete block design with three replicates per treatment.

To visualize the effects of JCK-12 WP20 (500-fold dilution), Almuri (4000-fold dilution) and their mixture (500-fold dilution of JCK-12 WP20 with 4000-fold dilution of Almuri) during the infection process of *F. graminearum* in wheat spikes, spore suspensions (10 μl of 1×10^5 spores/ml) harvested from carboxymethyl cellulose (CMC) inoculated with GFP-tagged strains were injected into the center spikelets of the wheat heads at the mid-anthesis after pretreatment of wheat with the JCK-12 formulation and Almuri, each alone and in combination. Six days after inoculation, the wheat spikes infected with GFP-tagged strains were evaluated. Free-hand longitudinal sections of spikes were cut using a clean scalpel and the sectioned spikes were viewed under reflected light and fluorescent light (excitation 470 and emission 525) using a SteREO Lumar V12 microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analyses

The inhibitory activity of spore germination and FHB index were subjected to analysis of variance. Means were separated at $P \leq 0.05$ using a Duncan test (SPSS Statistics, ver. 21, IBM).

RESULTS

Screening and Identification of Antifungal Bacteria

Five hundred bacterial strains were isolated from the rhizosphere of four different regions in Korea (Daejeon, Gwangju, Jeongeup, and Geoje). Of these 500 rhizospheric bacteria, five strains (JCK-7, JCK-8, JCK-9, JCK-12, and JCK-16) exhibited distinct inhibitory effects on the mycelial growth of *F. graminearum* in a dual culture assay (Supplementary Table 2). Among these, the fermentation culture filtrate of strain JCK-12 showed the strongest inhibition activity against *F. graminearum* spore germination, yielding the minimum inhibitory concentration (MIC) values of 5% (**Figure 1**). Strain JCK-12 was identified as *Bacillus amyloliquefaciens* based on BLASTn analysis and phylogenetic analyses of the amplified 16S rRNA, *gyrA*, and *recA* gene sequences (**Figure 2** and Supplementary Figures 1A,B). The nucleotide sequences of 16S rRNA, *gyrA* and *recA* of strain JCK-12 were deposited in GenBank under accession numbers KT964221, KU963797 and KU963798, respectively.

Inhibitory Activity of *B. amyloliquefaciens* JCK-12 against Mycelial Growth of Various Plant Pathogenic Fungi

In addition to *F. graminearum*, *B. amyloliquefaciens* JCK-12 inhibited the mycelial growth of all tested plant pathogenic fungi in the dual culture assay (**Figure 3** and Supplementary Table 3). The mycelial growth of *Magnaporthe oryzae* and *Botrytis cinerea* was highly inhibited with distinct inhibition zones. *Colletotrichum coccodes*, *Raffaelea quercus-mongolicae*, *Rhizoctonia solani*, and four species of *Fusarium* (*F. graminearum, F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *lycopersici*, and *F. verticillioides*) were also sensitive to JCK-12, whereas *Phytophthora capsici*, an oomycete fungus, was relatively insensitive to JCK-12. The results demonstrate that *B. amyloliquefaciens* JCK-12 has a broad spectrum of antifungal activity and could also be used as a biocontrol agent for various plant fungal pathogens used in this analysis.

Purification of Antifungal Cyclic Lipopeptides

The antifungal active compounds produced by *B. amyloliquefaciens* JCK-12 were purified using a chromatography and bioassay series. The isolated antifungal

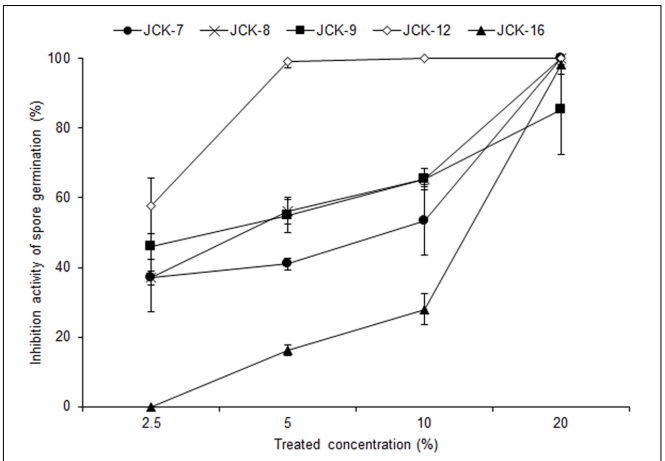


FIGURE 1 | Inhibitory activity of bacterial culture supernatants against *F. graminearum* spore germination.

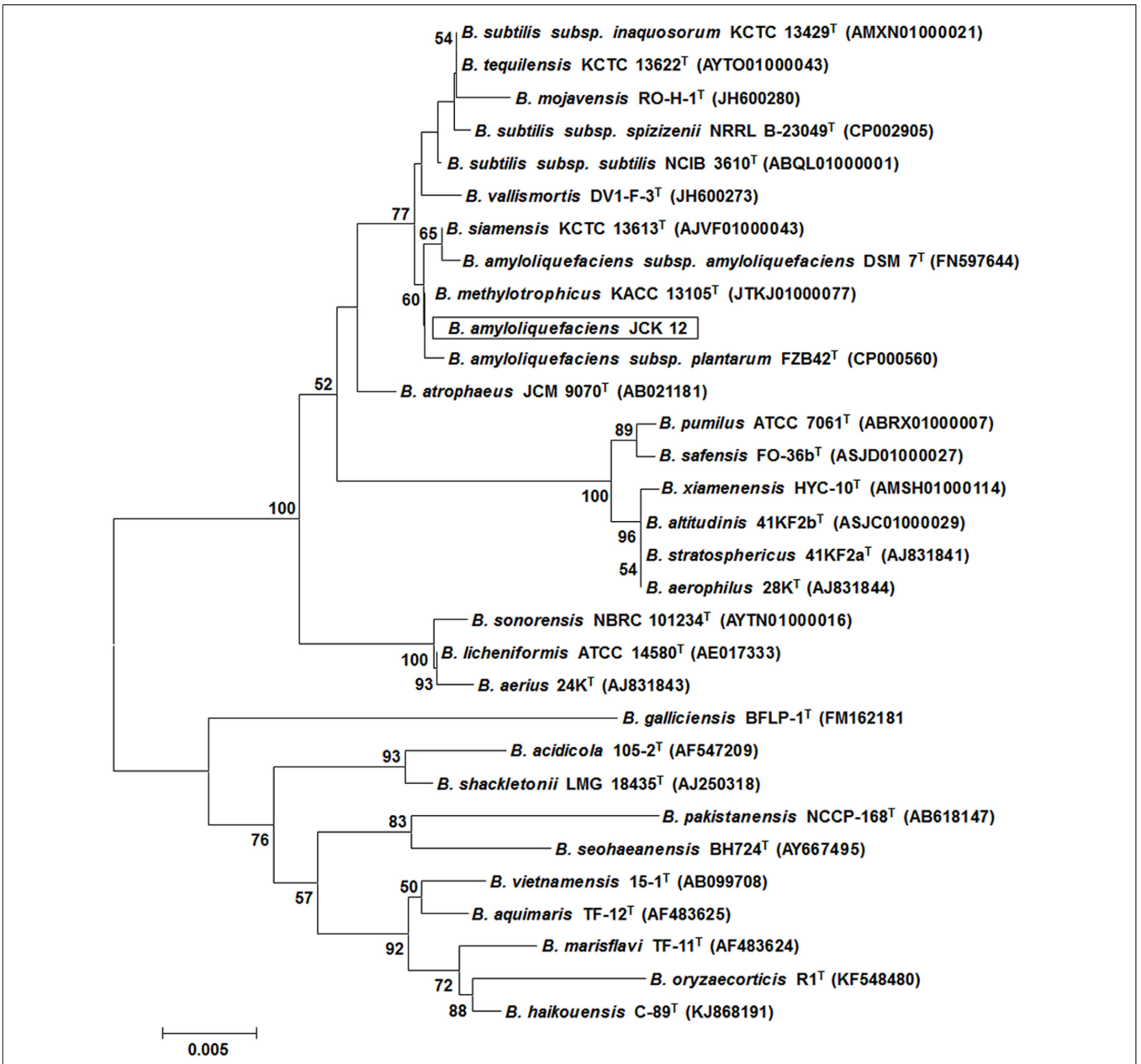


FIGURE 2 | Phylogenetic trees derived from distance analysis of 16S rRNA gene sequences of JCK-12. Sequences were aligned using MEGA 6.0 software. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with bootstrap analysis (1,000 trials). Bars indicate the percentage of sequence divergence.

fraction appeared as a single spot on the TLC plate, but three peaks were observed in the LC-MS/MS analysis (**Figure 4A**). The protonated ions ($[M + H]^+$) of the three peaks at 26.15 min (compound 1), 27.99 min (compound 2), and 28.14 min (compound 3) appeared at *m/z* 1043.9, *m/z* 1057.9, and *m/z* 1057.9, respectively. The differences of 14 Da suggest that they are homologous molecules with different lengths of fatty acid chains. In addition, the same protonated ions of compounds 2 and 3 suggested that the two compounds are isomers. LC-MS/MS analysis revealed that compound 1 was iturin A_2 and that compounds 2 and 3 were one of iturin A_3, A_4, and A_5 (**Figures 4B,C**).

Some *Bacillus* species simultaneously produce other cyclic lipopeptides (CLPs) along with iturin A (Romero et al., 2007; Kim et al., 2010). We further purified two CLPs, fraction 1 and 2, by prep-TLC using the butanol extract of the JCK-12 fermentation broth and analyzed both fractions by HPLC and ESI-MS/MS. The protonated ions ($[M + H]^+$) of fraction 1 appeared at *m/z* 1477.8. The protonated ions ($[M + Na]^+$) of fraction 2 appeared at *m/z* 1058.7 (**Figures 4D,E**). Fractions 1

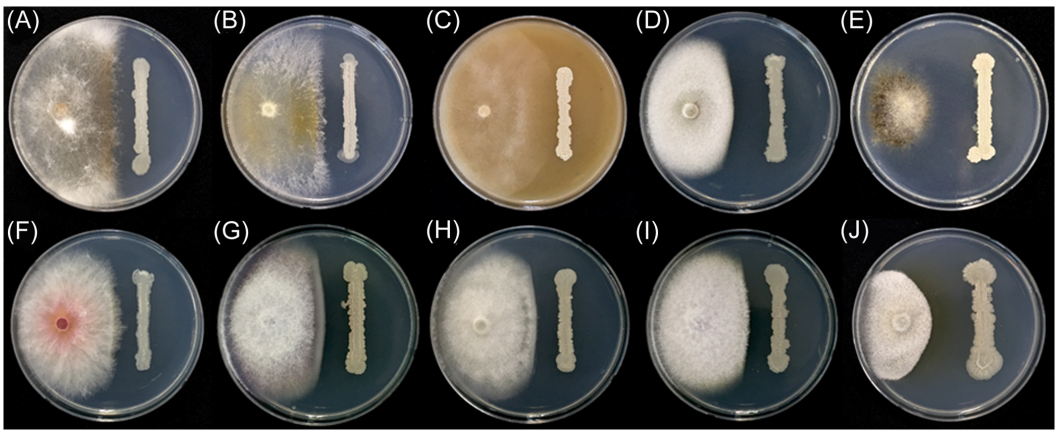


FIGURE 3 | Antifungal activity of strain JCK-12 against various plant pathogen fungi. **(A)** *Rhizoctonia solani*, **(B)** *R. quercus-mongolicae*, **(C)** *P. capsici*, **(D)** *C. coccodes*, **(E)** *B. cinerea*, **(F)** *F. graminearum*, **(G)** *F. oxysporum* f. sp. *niveum*, **(H)** *F. oxysporum* f. sp. *lycopersici*, **(I)** *F. verticillioides*, and **(J)** *M. oryzae* on plates.

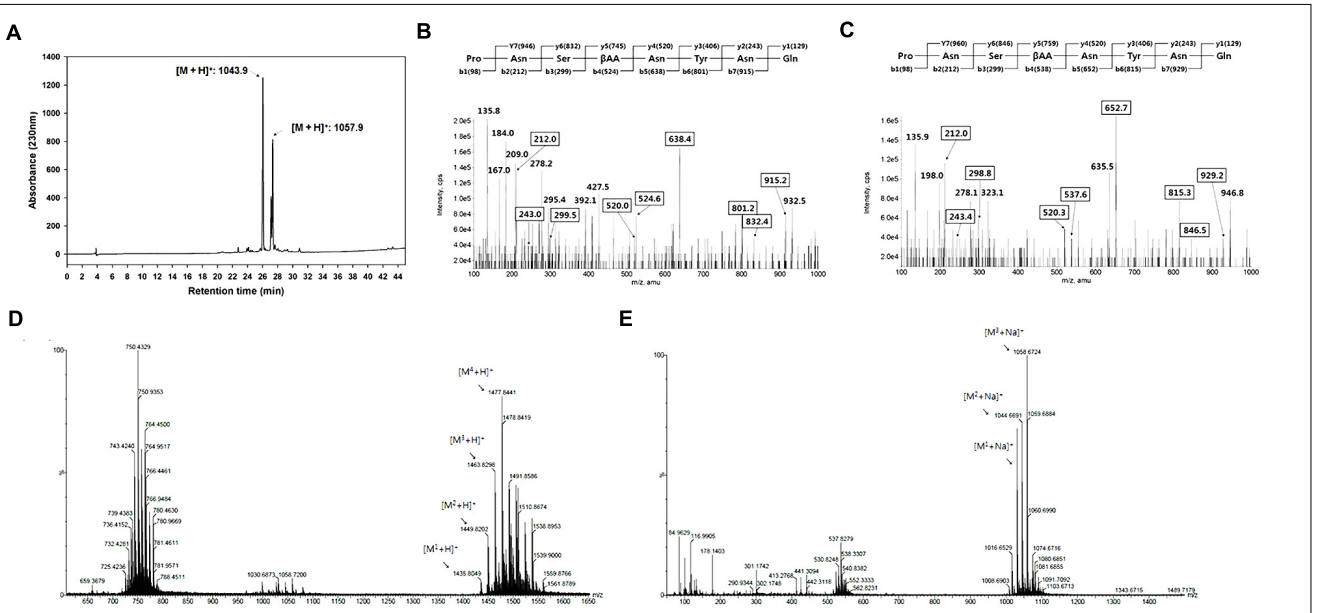


FIGURE 4 | Structural analyses of cyclic lipopeptides produced by *B. amyloliquefaciens* JCK-12. **(A)** HPLC chromatogram of antifungal metabolites, **(B)** LC-MS/MS of iturin A2 ([M + H]$^+$: 1,043.9), **(C)** LC-MS/MS of iturin A3 (or A4, A5) ([M + H]$^+$: 1,057.9), and **(D)** ESI-MS spectra of F1 and **(E)** F2 isolated from the fermentation broth culture of *B. amyloliquefaciens* JCK-12 by prep TLC. F1 and F2 represent fengycin and surfactin, respectively.

and 2 were identified as fengycin and surfactin through ESI-MS/MS analysis. The isolated CLPs were further confirmed using HPLC analysis and comparison with standard chemicals (data not shown). Taken together, these results indicated that *B. amyloliquefaciens* JCK-12 produces three CLPs, iturin A, fengycin, and surfactin.

Effects of CLPs on *F. graminearum* Conidia Germination

In order to examine the antifungal effects among the three CLPs on *F. graminearum*, we performed the conidia germination assay with three commercial CLPs in different combinations. At a concentration of 30 μg/ml, the germination inhibitory activities of iturin A, fengycin, surfactin, I + F + S (1:1:1, v/v/v), I + F (1:1, v/v), I + S (1:1, v/v), and F + S (1:1, v/v) on *F. graminearum* conidia were 88.7, 0, 0, 58.3, 78.3, 64.7, and 0%, respectively (**Figure 5A**). Of the three CLPs at 30 μg/ml, only iturin A effectively inhibited conidia germination in *F. graminearum*, whereas fengycin and surfactin had no distinct inhibitory activity at concentrations less than 30 μg/ml. Most mixtures of CLPs had an inhibitory activity on conidia germination at 30 μg/ml except in the treatment F + S (1:1, v/v). These results verified that only iturin A among the three CLPs had effective antifungal activity against *F. graminearum*.

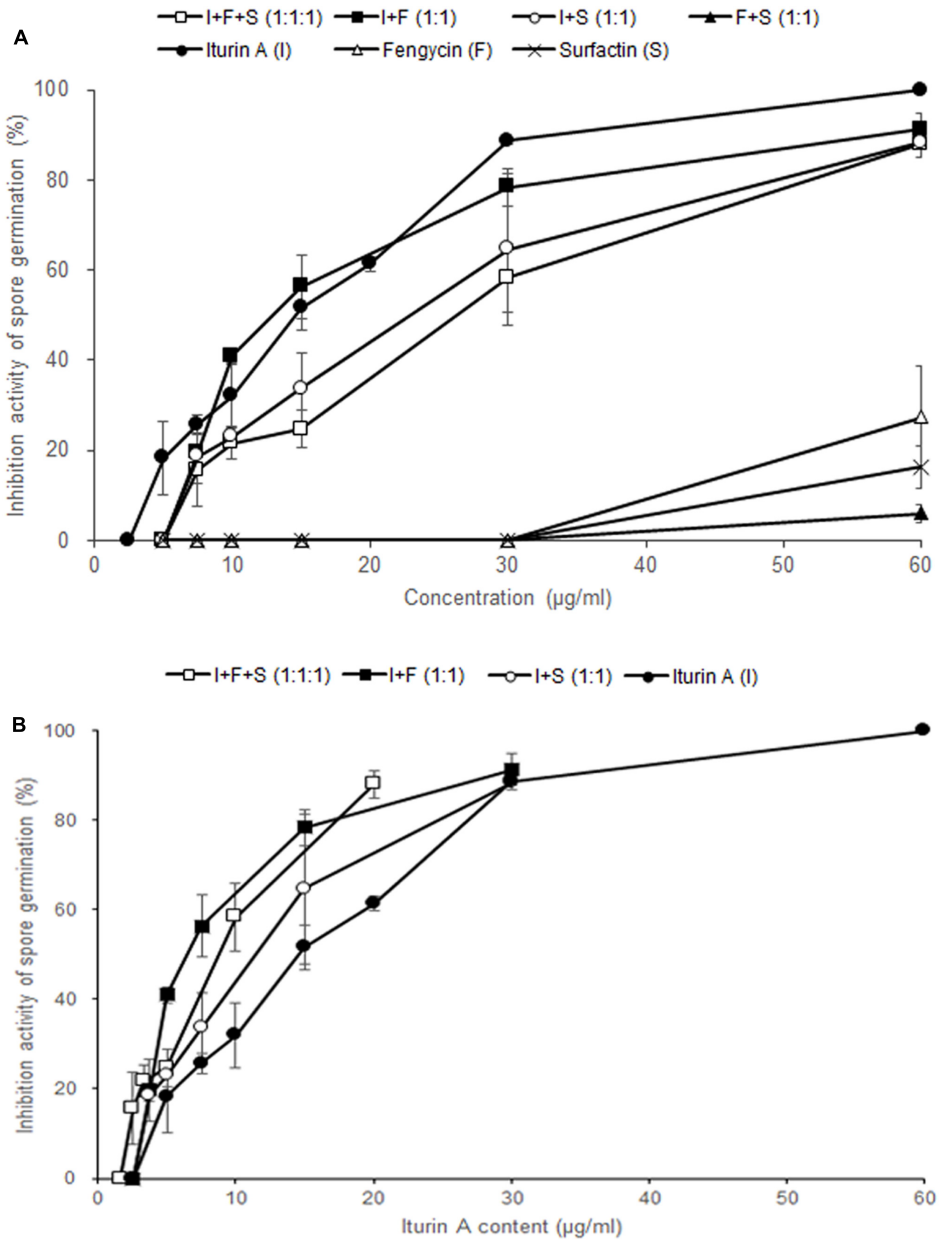


FIGURE 5 | Effect of commercial CLPs alone or in mixture on *F. graminearum* macroconidia germination. **(A)** Based on the total concentration of the three CLPs in solutions, and **(B)** based on the concentration of iturin A alone in the solutions. Error bars represent standard deviation from three replicates.

To investigate the synergistic effects of iturin A and other CLPs, we analyzed the inhibitory activity of each treatment according to the content of iturin A (**Figure 5B**). In other words, in I + F (1:1) and I + S (1:1) at 30 μg/ml, the content of iturin A is 15 μg/ml whereas the content of iturin A is 10 μg/ml in I + F + S (1:1:1) at 30 μg/ml. The mixture treatments of I + F (1:1, 78.3%) and I + S (1:1, 64.7%) containing 15 μg/ml of iturin A showed higher inhibitory activity compared to the sole treatment with iturin A (51.7%). Moreover, treatment with the I + F + S (1:1:1, 58.3%) mixture containing 10 μg/ml of iturin A exhibited approximately two-fold increase in the germination inhibitory activity compared with that upon the sole treatment with iturin A (32.0%). These results suggest that co-application of fengycin and surfactin with iturin A enhances the effectiveness of iturin A against *F. graminearum*.

Effect of the Butanol Extract on Hyphae of *F. graminearum*

Through the analysis of the mycelial growth inhibitory effects of JCK-12 and its butanol extract using the paper disk agar diffusion and pour plate methods, we confirmed that the butanol extract of *B. amyloliquefaciens* JCK-12 containing three CLPs

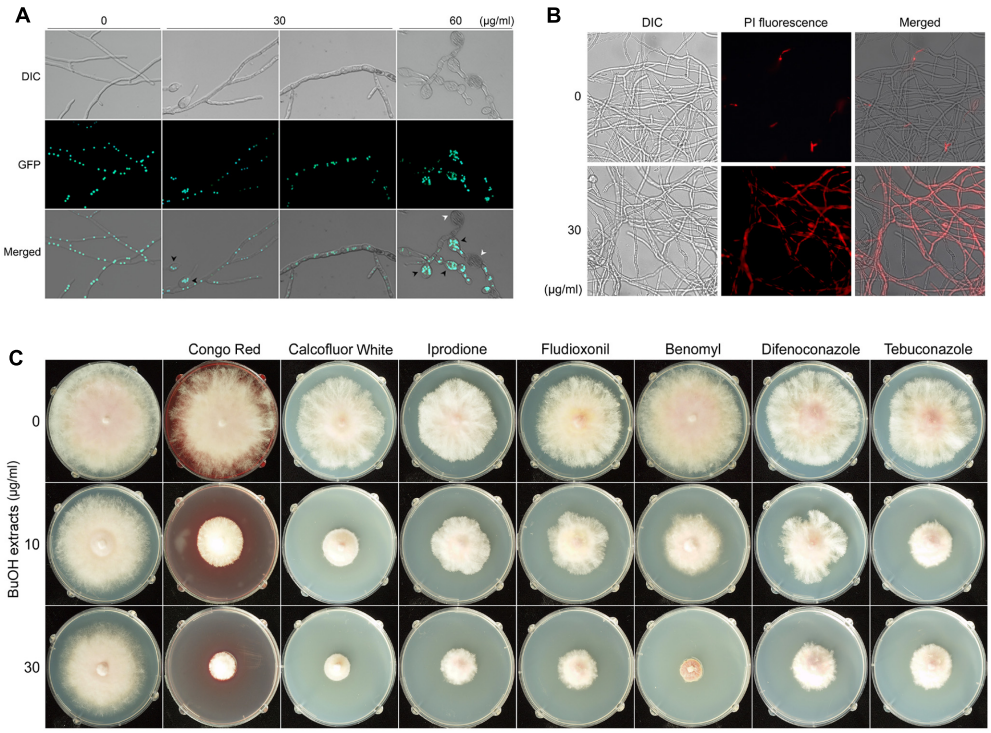


FIGURE 6 | Effects of *B. amyloliquefaciens* JCK-12 on *F. graminearum*. Effects of BuOH extracts on hyphal morphology **(A)**. Green fluorescent dots represent histone H1-green fluorescent protein (GFP). Black arrowheads indicate the balloon-shaped cells and white arrowheads indicate the exploded cells. DIC, differential interference contrast image; GFP, fluorescence microscopy image; Merged, overlays of the DIC and fluorescence microscopy images. The effect of BuOH extracts on cell membrane permeability was examined by propidium iodide (PI) staining **(B)**. Mycelia treated with BuOH extracts (0 or 30 μg/ml) were incubated in 2 μM PI for 20 min. Red fluorescence represents PI-stained hyphae. DIC, differential interference contrast image; PI, fluorescence microscopy image; Merged, overlays of the DIC and fluorescence microscopy images. The synergistic antifungal effect of BuOH extracts and other antifungal compounds on the hyphal growth of *F. graminearum* **(C)**.

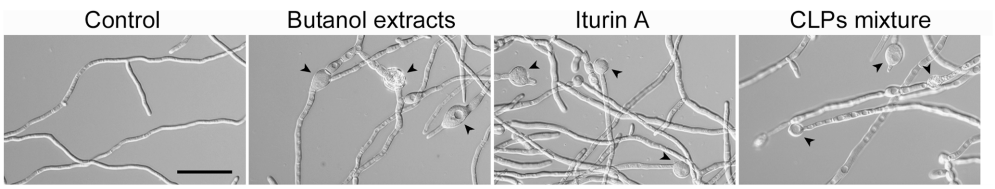


FIGURE 7 | Effect of commercial CLPs on hyphal morphology. Mycelia treated with BuOH extracts (0 or 30 μg/ml), iturin A (15 μg/ml), or the three CLPs mixture (iturin A + fengycin + surfactin = 1.25 + 1.25 + 1.25 μg/ml) were cultured at 200 rpm and 25°C. Black arrowheads indicate the balloon-shaped cells and white arrowheads indicate the exploded cells. Scale bar: 50 μm.

exhibited a strong antifungal activity against *F. graminearum* (Supplementary Figure 2). We used the butanol extract of *B. amyloliquefaciens* JCK-12 as the CLP mixture in the following experiments.

Microscopic examination revealed that treatment with the butanol extract affected the hyphal morphology of *F. graminearum* (**Figure 6A**). When the fungus was treated with 30 μg/ml of butanol extract, hyphae were swollen and a few balloon-shaped cells were observed. Upon treatment with a relatively high concentration of the butanol extract (60 μg/ml), fungal growth was severely retarded and many balloon-shaped cells were observed; some of these cells eventually exploded. To visualize the nuclei in fungal cells, a previously generated strain hH1-GFP (Hong et al., 2010) was used. We found that multiple nuclei were present in the balloon-shaped cells compared to those in the untreated control (**Figure 6A**). Similar phenotypic defect was previously reported in deletion mutants of chitin synthase genes in *F. graminearum* (Kim et al., 2009). Moreover, a recent study suggested that iturin A and plipastatin cause damages to the cell walls and plasma membranes of this fungus (Gong et al., 2015).

Based on the previous and current results on hyphal morphology, we hypothesized that CLPs produced by *B. amyloliquefaciens* JCK-12 would damage the cell walls and

membrane structures in *F. graminearum*. Since cell membrane damages could increase the cell membrane permeability (Lee and Kim, 2016), we performed propidium iodide (PI) staining to detect the effects of the butanol extract on the fungal cell membrane permeability of *F. graminearum* (**Figure 6B**). PI is a fluorescent probe that is unable to penetrate the cell membrane of healthy cells. However, it can enter the membrane-compromised cells (Cox et al., 2001). We found that the red fluorescence of hyphae treated with the butanol extract was significantly stronger than that of the untreated control. Incubation with 60 μg/ml of the butanol extract showed collapsed hyphae upon visualization of PI staining, indicating extensive cell death (data not shown). Taken together, these results indicated that the CLPs included in the butanol extract markedly increased the cell membrane permeability of *F. graminearum*.

The Synergistic Antifungal Effect of the Butanol Extract with Antifungal Agents

To determine whether changes in *F. graminearum* cell membrane permeability enhance the antifungal effects of other chemical agents such as Congo Red, Calcoflour White, iprodione, fludioxonil, benomyl, difenoconazole, and tebuconazole, we evaluated the individual and the synergistic antifungal activities of the butanol extract and other antifungal agents on the hyphal growth of *F. graminearum*. Congo Red and Calcofluor White affect fungal cell wall morphogenesis (Roncero and Duran, 1985). Iprodione, fludioxonil, benomyl, difenoconazole, and tebuconazole are used as fungicides that inhibit the germination of fungal spores and the hyphal growth of fungi (Edwards et al., 2001; Gupta et al., 2004; Kojima et al., 2006; Miñambres et al., 2010; Dzhavakhiya et al., 2012). We found a distinct synergistic interaction between the butanol extract and other antifungal agents (**Figure 6C**). Growth inhibition was significantly increased when these antifungal agents were combined with the butanol extract. These results suggest that co-application of the CLPs included in the butanol extract and other antifungal chemicals can inhibit the fungal growth more effectively because of changes in cell membrane permeability. Thus, CLPs produced by *B. amyloliquefaciens* JCK-12 may enhance pathogen sensitivity to fungicides.

Effects of CLPs on Hyphal Morphology

To confirm whether the activity of the butanol extract on *F. graminearum* results from the iturin A. fengycin, and surfactin produced by *B. amyloliquefaciens* JCK-12, we compared the effects of commercial CLPs and the butanol extract on the hyphal morphology of *F. graminearum* (**Figure 7**). When the fungus was treated with 30 μg/ml of the butanol extract, 1.25 μg/ml of the three commercial CLPs, or 15 μg/ml of iturin A, the hyphae were swollen and a few balloon-shaped cells were observed. The effect of the butanol extract on *F. graminearum* hyphal morphology was similar to that of commercial iturin A alone or the mixture of three CLPs. Moreover, the synergistic interaction of the three CLPs mixture enhanced the morphological changes at a low concentration of iturin A, which correspond to the single application of iturin A at a high concentration. Based on these results, we supposed that the CLPs included in the butanol extract could damage the cell wall and alter the membrane structure of *F. graminearum* hyphae, which may subsequently affect membrane permeability.

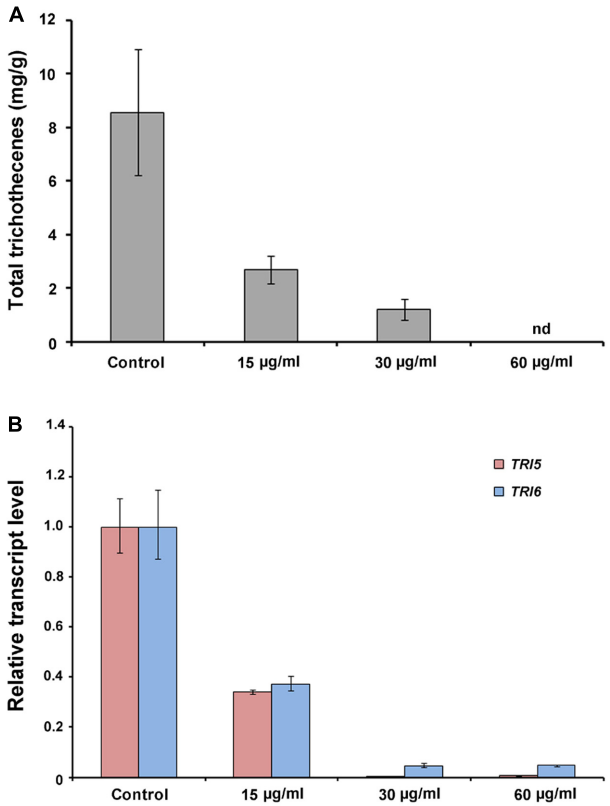


FIGURE 8 | Trichothecene production of *F. graminearum* under CLPs treatment. **(A)** Total trichothecene (DON and 15-acDON) production by *F. graminearum* strains. Fungal strains were grown in liquid minimal media containing 5 mM agmatine (MMA) supplemented with CLPs (0, 15, or 30 μg/ml) for 7 days. Trichothecenes were analyzed by gas chromatography mass spectrometry and were quantified based on the biomass of each strain. **(B)** Transcript levels of *TRI5* and *TRI6* in fungal strains treated with CLPs.

In Vitro Effect of Butanol Extract on Total Trichothecene Production

We analyzed the total trichothecene production (deoxynivalenol and 15-acetyl-deoxynivalenol) by *F. graminearum* after treatment with the butanol extract (0, 15, or 30 μg/ml). The accumulation of trichothecenes in the butanol extract-treated cultures was significantly decreased compared with that in the untreated controls (**Figure 8A**). The qRT-PCR results demonstrated that the expression of the trichothecene biosynthetic genes *TRI5* and *TRI6* was also significantly reduced by the butanol extract treatment compared with that in the untreated controls (**Figure 8B**). These results indicate that *B. amyloliquefaciens* JCK-12 plays a crucial role in disrupting trichothecene biosynthesis in *F. graminearum*.

Effect of JCK-12 Strain on FHB Disease of Wheat

To investigate the efficacy of strain JCK-12 in controlling FHB disease, we estimated the disease incidence, disease severity, and the control value of FHB on wheat under both greenhouse and field conditions after 2 weeks of treatment with the fermentation broth of strain JCK-12 (**Table 1**). In the greenhouse experiment, the fermentation broth of strain JCK-12 showed a dose dependent effect on FHB control with control values of 20.1 and 43.0% at 20- and 5-fold dilution treatments, respectively. The disease incidence and disease severity of the five-fold dilution JCK-12 treatment were comparable to those observed with Almuri. The fermentation broth of JCK-12 also suppressed the development of FHB in the field experiment with control values of 43.8 and 56.3% at 20- and 5-fold dilution treatments, respectively. However, the disease control efficacy of the fermentation broth of JCK-12 at the five-fold dilution was lower than that of Almuri.

A wettable powder type formulation of JCK-12 (JCK-12 WP20) was prepared and its disease control efficacy against FHB was then tested under greenhouse and field conditions. In addition, to examine the feasibility of the JCK-12 WP20 formulation as a chemosensitizer to increase the sensitivity of the chemical fungicides used for FHB control in cereals, JCK-12 WP20 was applied in a mixture with the synthetic fungicide, Almuri. Treatment with JCK-12 WP20 was found to reduce both the disease incidence and severity of FHB compared to the untreated control under greenhouse conditions (**Table 2**). However, it did not reduce the development of FHB under field conditions. The mixture of JCK-12 (500-fold dilution) and Almuri (4000-fold dilution) showed the strongest disease control efficacy in both greenhouse and field experiments, followed by 2000- and 4000-fold dilutions of Almuri alone.

To visualize the spread of mycelia on wheat spikes during infection, the *F. graminearum* strain HK12 constitutively expressing cytosolic GFP was used (Son et al., 2011b). In the wheat spike treated with JCK-12 WP20, Almuri, and their mixture, the fungal hyphae were seen throughout the rachis, but failed to spread to the adjacent spikelet, whereas the fungal hyphae spread through the rachis from the infected to the adjacent spikelet in the untreated wheat spike (**Figure 9**). Especially, the mycelial spread of *F. graminearum* in wheat treated with the combination of JCK-12 WP20 and Almuri was inhibited more than that in any other treatment. These results indicate that JCK-12 WP20 can markedly increase the disease control efficacy of synthetic fungicides when they are applied in a mixture.

DISCUSSION

Due to the negative effects of agricultural chemicals including environmental pollution, residual chemicals, occurrence of resistant pathogens, and limit period of usage, many studies have been performed to explore a non-hazardous alternative for controlling plant pathogenic fungi and biocontrol agents using various microorganisms (Elkahoui et al., 2012; Ruangwong et al., 2012; Ji et al., 2013). Among them, *Bacillus* species have been regarded as promising candidates for controlling plant pathogens as they have various antagonistic activities and are safe to use (Leifert et al., 1995; Siddiqui et al., 2005; Nagórska et al., 2007; Ji et al., 2013; Zhang et al., 2013; Zhao et al., 2014; Cawoy et al., 2015). In this study, we report that *B. amyloliquefaciens* JCK-12 could be used as a competent biocontrol agent against the cereal head blight fungus, *F. graminearum*. JCK-12 reduced FHB severity in both greenhouse and field conditions. Three CLPs (iturin A, fengycin, and surfactin) produced by JCK-12 cooperatively induced a strong antifungal activity by altering the cell membrane permeability of *F. graminearum*. For practical usage of JCK-12, we verified that the wettable powder type formulation of JCK-12 is an effective biocontrol agent and could be utilized as a chemosensitizer to improve the efficacy of other conventional antifungal chemicals.

Bacillus amyloliquefaciens JCK-12 strain produced iturin A, fengycin, and surfactin, which are some of the representative metabolites produced by *Bacillus* species (Ongena and Jacques, 2008). These cyclic lipopeptides mixture exhibited synergistic inhibitory effects on *F. graminearum* spore germination. Among them, it is known that iturin A and fengycin separately have antifungal activity and inhibit the growth of a wide range of plant pathogens, whereas surfactin and iturin A display a synergistic antifungal effect (Romero et al., 2007; Ji et al., 2013; Cawoy et al., 2015). However, our results showed that fengycin alone does not have a powerful antifungal activity against *F. graminearum*, whereas fengycin as well as surfactin increase the antifungal activity of iturin A as synergistic

TABLE 1 | Disease control efficacy of the JCK-12 fermentation broth culture against fusarium head blight caused by *F. graminearum* in wheat under greenhouse and field conditions.

Treatment	Dilution	Greenhouse				Field			
		DI (%)[a]	DS (%)[b]	FHB index[c]	CV (%)[d]	DI (%)	DS (%)	FHB index	CV (%)
JCK-12	×20	52.3ab[e]	29.2b	15.3	20.1	18.0b	4.3c	0.8	43.8
	×5	37.3a	22.1ab	8.2	43.0	14.0b	1.3ab	0.2	56.3
Almuri[f]	×2,000	28.2a	3.4a	1.0	57.0	4.0a	0.4a	0.0	87.5
Untreated control	–	65.5b	37.7b	24.7	–	32.0c	5.0c	1.6	–

[a]*DI, disease incidence.* [b]*DS, disease severity.* [c]*FHB index = (DI × DS)/100.* [d]*CV, control value of disease incidence.* [e]*Within columns, means followed by the same lower-case letter are not significantly different (Duncan test, $P \leq 0.05$).* [f]*Almuri was used as a positive control. Dilution value (×2,000) of Almuri represents compatibilizer concentration.*

TABLE 2 | Disease control efficacy of the wettable powder type formulation of JCK-12 against fusarium head blight caused by *F. graminearum* in wheat under greenhouse and field conditions.

Treatment	Dilution	Greenhouse				Field			
		DI (%)[a]	DS (%)[b]	FHB index[c]	CV (%)[d]	DI (%)	DS (%)	FHB index	CV (%)
Almuri	×2,000	26.9a[e]	10.7a	3.3a	94.3	6.2a	39.0ab	2.4a	82.0
	×4,000	61.0bc	20.5ab	14.0ab	79.8	8.8ab	57.4bc	5.6ab	62.6
JCK-12 WP20	×500	76.5bc	54.5b	48.6b	44.0	29.0c	87.5c	25.3c	–
JCK-12 WP20 + Almuri	×500 + ×4,000	31.4ab	7.7a	3.1a	96.4	4.8a	25.1a	2.4a	91.0
Untreated control	–	94.4c	82.0c	78.0c	–	17.5c	76.9c	13.5c	–

[a]*DI, disease incidence.* [b]*DS, disease severity.* [c]*FHB index = (DI × DS)/100.* [d]*CV, control value of disease incidence.* [e]*Within columns, means followed by the same lower-case letter are not significantly different (Duncan test, $P \leq 0.05$).* [f]*Almuri was used as a positive control. Dilution value (×2,000) of Almuri represents compatibilizer concentration.*

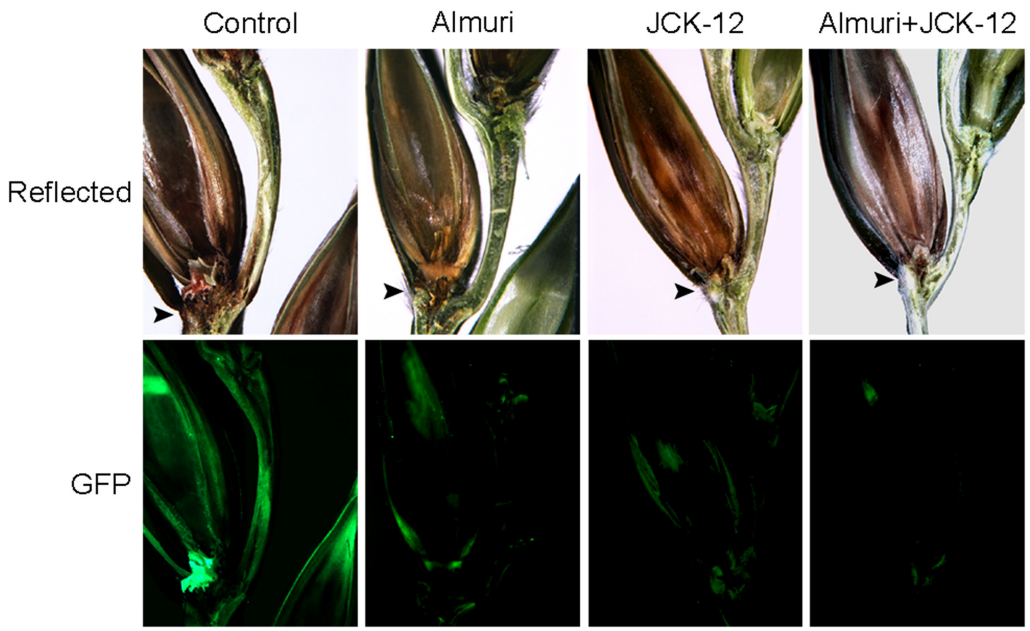


FIGURE 9 | Synergistic control effect of CLPs and the chemical fungicide Almuri. Wheat spikelets pretreated with CLPs and Almuri were inoculated with suspensions of conidia from green fluorescent protein (GFP)-expressing *F. graminearum* strains. The infected wheat heads were dissected at day 7 after inoculation and examined by fluorescence microscopy. Spreading of the GFP signal represents spreading of hyphae from the points of inoculation. Arrowheads mark the inoculated spikelets.

factors. Even the synergistic antifungal activity of fengycin and iturin A is slightly higher than that of surfactin and iturin A on *F. graminearum* spore germination. *B. amyloliquefaciens* JCK-12 strain simultaneously produces three cyclic lipopeptides showing a synergistic antifungal activity against *F. graminearum*, suggesting that strain JCK-12 has the potential to be an excellent biocontrol candidate.

Fusarium graminearum accumulates hazardous mycotoxin (DON) on infected grains. However, there is no efficient strategy to prevent DON contamination by late-season infections when chemical fungicides cannot be used (Francl et al., 1999; McMullen et al., 2012). Biological control using *Bacillus* strains has gained importance for the control of mycotoxin accumulation by *Fusarium* species in both pre- and post-harvest crops (Tsitsigiannis et al., 2012). In this study, *B. amyloliquefaciens* JCK-12 was revealed to possess the ability to decrease both fungal growth and DON production. In particular, CLPs successfully inhibited DON production by affecting DON biosynthetic gene expression (**Figure 8**). Conclusively, we demonstrate that biological control using JCK-12 is a promising strategy to prevent mycotoxins as well as FHB itself.

Previous studies have proposed a mechanism for the antifungal action of CLPs using an artificial lipid membrane, demonstrating that iturin A and surfactin penetrate the lipid bilayer of the target cell cytoplasmic membrane and form selective ion-conducting pores, resulting in increased membrane permeability (Sheppard et al., 1991; Maget-Dana and Peypoux, 1994; Carrillo et al., 2003). Furthermore, it is known that treatment with plant hydrolytic enzymes also causes cell wall thinning in the hyphal apex, and subsequently inhibits fungal growth, resulting in an imbalance of turgor pressure and balloon-like swelling of the plasma membrane in fungi

(Arlorio et al., 1992). These phenomena are similar to those shown by the cell wall of hyphae treated with Calcofluor White or Congo Red, which disturb growth in yeasts, and in chitinous and cellulosic fungi (Fèvre et al., 1990). In this study, we confirmed that CLPs lead to the formation of balloon-like plasma membrane in *F. graminearum in vivo*. In addition, our results showed that the cell membrane of *F. graminearum* is the essential site of antifungal attack by CLPs and that their antifungal activity may result from altering the fungal cell wall integrity and fungal cell membrane permeability. Moreover, the synergistic effect of the CLPs mixture can significantly enhance cell permeability in fungal hyphae, suggesting that the three cyclic lipopeptides produced by *B. amyloliquefaciens* JCK-12 strain are excellent antifungal sources of biocontrol agents. Through optimization of fermentation conditions for the production of cyclic lipopeptides and appropriate formulation for supporting their stability and control effectiveness, *B. amyloliquefaciens* JCK-12 could be used as a more effective biological agent against FHB.

The treatment of *B. amyloliquefaciens* JCK-12 extracts or formulation and chemical fungicides in combination caused cell wall damage in the hyphae of *F. graminearum* and subsequent increased sensitivity of *F. graminearum* to fungicides, indicating that *B. amyloliquefaciens* JCK-12 can be used as candidates for chemosensitizers. Antifungal chemosensitization is a novel antifungal intervention strategy to improve the efficacy of conventional agents against fungal pathogens (Niimi et al., 2004; Kim et al., 2008). Although a single application of the *B. amyloliquefaciens* JCK-12 formulation is not a complete alternative for synthetic chemicals to control FHB, *B. amyloliquefaciens* JCK-12 is a good biocontrol candidate that produces potent chemosensitizers. Therefore, our results are progressive and noteworthy in that *B. amyloliquefaciens* JCK-12 can actually increase antifungal activity under field conditions and reduce the input of chemical fungicides as supplements or chemosensitizers in a system of integrated plant disease management with no safety problems to humans or the environment.

In addition, use of this biological agent enables extension of its application period past the flowering stage when synthetic fungicides can no longer be applied. A biological control system using *B. amyloliquefaciens* JCK-12 may show great potential for reducing mycotoxin accumulation. This finding shows that *B. amyloliquefaciens* JCK-12 has promising potential for the development of effective and eco-friendly chemosensitizers that could be used in combination with chemical fungicides, to decrease the incidence of resistance development in pathogens, to reduce environmental damage by lowering the effective dosage levels of chemical fungicides, and to enhance the efficacy of antifungal agents. Our study has important practical implications in that FHB management was performed using a formulation of *B. amyloliquefaciens* JCK-12 under the field condition and we identified *B. amyloliquefaciens* JCK-12 as an potential candidate for the chemosensitizer to be used with agrochemicals based on the results of the field trials.

AUTHOR CONTRIBUTIONS

KK, YL, AP, and J-CK conceived this study. KK, YL, AH, J-IK, AP, NY, HP, CL, TL, and J-CK performed the experiments. KK, AP, HS, GC, Y-WL, and J-CK analyzed data. AP, KK, YL, HS, Y-WL, and J-CK wrote the manuscript. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02010/full#supplementary-material

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Antifungal Activity of Lipopeptides From *Bacillus* XT1 CECT 8661 Against *Botrytis cinerea*

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This work aims to explore the capacity of a *Bacillus methylotrophicus* (later heterotypic synonym of *Bacillus velezensis*) strain named XT1 CECT 8661 against the necrotrophic plant pathogen *Botrytis cinerea* and to identify the compounds responsible for its activity. Q_TOF electrospray mass spectrometry analysis allows us to detect several lipopeptides – surfactin, bacillomycin, and fengycin – in XT1 cultures. *In vitro* antibiosis studies demonstrated the efficiency of the lipopeptide fraction for the inhibition of fungal growth. In fact, microscopy studies (SEM/TEM) revealed, an alteration of the morphology of the phytopathogen in interaction with lipopeptides, with resistance structures appearing in the early stages of growth of the fungus. Our studies, carried out with tomatoes, grapes, and strawberries have demonstrated the efficiency of *Bacillus* XT1 CECT 8661 lipopeptides against *B. cinerea* infection and it capability to trigger the antioxidant activity in fruit. Overall, the results of this study highlight the potential of lipopeptides of this strain as an effective biological control agent against the colonisation of *B. cinerea*.

Keywords: *Bacillus* XT1, lipopeptides, antifungal activity, *Botrytis cinerea,* antioxidant activity

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INTRODUCTION

Botrytis cinerea [teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel] is a necrotrophic fungi known to be the cause of grey mould. These fungi can infect more than 200 plant species, including horticulturally important crops (Gao et al., 2018). Therefore, it has significant economic relevance, causing huge economic losses (Dean et al., 2012). The ability to produce conidiophores that contain conidia gives it the ability to remain quiescent until conditions are favourable enough to produce the infection (Gao et al., 2018). The production of lytic enzymes along with other phytotoxic metabolites induces cell death in plant tissues, mainly affecting those in a state of senescence or with wounds on their surface (Finiti et al., 2014; Gonzalez-Fernandez et al., 2015; Yu et al., 2015; Yahaya et al., 2016). The aforementioned ubiquity, together with the capacity to produce resistant structures and the high mutation rate of *B. cinerea,* makes the fight against this fungus a challenging task (Gonzalez-Fernandez et al., 2015; Haidar et al., 2016).

Currently, the most popular treatment to combat grey mould is the extensive use of pesticides; however, recent regulations of these products have considerably restricted the possibility of their use. These pesticides produce residual waste and contaminate ground water increasing the risks for human health and the environment (Perez-Garcia et al., 2011; Romanazzi et al., 2012; Finiti et al., 2014). As a consequence, one of the biggest challenges for sustainable agriculture

is the development of environmentally friendly alternatives such as the use of microorganisms. The biological control through bacterial strains has been an objective of particular interest due to their multiple modes of action against different plant diseases. One of these mechanisms is the production of a wide variety of biologically active compounds with great potential for biotechnological applications (Gond et al., 2015; Martinez-Hidalgo et al., 2015; Mnif et al., 2016).

Although several microorganisms have been described as potential candidates for biological control agents, numerous research studies have focused on members of the genus *Bacillus*. Species from this genus have been considered biologically safe and are commonly used in agriculture. The sporulation capacity of these microorganisms gives them a high resistance, high ubiquity in diverse habitats and stability in formulated products (Ongena and Jacques, 2008; Tanaka et al., 2015; Mnif et al., 2016). Members of the genus *Bacillus* are well known for their capacity to colonise roots, promote plant growth (PGPR) and induce systemic resistance mechanism in plants (Sicuia et al., 2015). In addition, they can produce a broad spectrum of biologically active molecules, with potential antimicrobial and antifungal properties. One of the major factors related with the antifungal activity of members of the genus *Bacillus* is due to the production of lipopeptides (Ongena and Jacques, 2008).

Lipopeptides are low-molecular-weight cyclic amphiphilic oligopeptides synthesised by multi-enzyme complexes called non-ribosomal peptide synthetases (NRPSs) (Romero et al., 2007; Gond et al., 2015; Han et al., 2015; Deng et al., 2017). Species from the genus *Bacillus* produce molecules which are mainly classified into three families depending on their amino-acid sequence: surfactin, iturin, and fengycin. These families share a cyclic β-amino or β-hydroxy fatty acid linked to a lipid tail (Tapi et al., 2010; Sumi et al., 2014; Jemil et al., 2017). Biological activities may differ from one compound to another depending on the type of amino-acid residues, the cyclisation of the peptide and the length and branching of the fatty acid chain (Ongena and Jacques, 2008; Frikha-Gargouri et al., 2017).

Different studies have demonstrated the activity of lipopeptides produced by *Bacillus subtilis* (Farace et al., 2015; Wang et al., 2015; Arroyave-Toro et al., 2017). However, the information about the antifungal activity of lipopeptides produced by *B. methylotrophicus* against *B. cinerea* is almost non-existent.

The objective of this study is to analyse the antifungal activity of *B. methylotrophicus* XT1 CECT 8661 (later reclassified as heterotypic synonym *B. velezensis*) (Ruiz-García et al., 2005) against *B. cinerea*. For this purpose, the lipopeptides produced by *B. methylothrophicus* by this strain were identified, the genes involved in their biosynthesis were detected and their antifungal activity was tested *in vitro*. Alterations of the morphology of the phytopathogen in interaction with these macromolecules was examined via microscopy studies. Furthermore, studies of antibiosis *in vivo* and determination of antioxidant compounds on grapes, tomatoes, and strawberries were carried out in order to demonstrate the ability of these compounds to protect against *B. cinerea* infection and to activate antioxidant mechanisms. To the best of our knowledge, this is the first report that describes the ability of lipopeptides to trigger the antioxidant activity in fruit, a mechanism involved in the elicitation of an induced systemic resistance phenomenon.

MATERIALS AND METHODS

Bacterial and Fungal Strains

The bacterial strain used in this study was the patented strain *Bacillus* XT1 CECT 8661, licenced to Xtrem Biotech S.L., which was isolated from a rhizospheric soil sample in the south of Spain (Béjar et al., 2014). The strain was originally classified as *B. methylotrophicus* and it was later reclassified as a heterotypic synonym of *B. velezensis*. It was routinely cultivated on a nutrient broth and nutrient agar at 28°C. The phytopathogenic fungus *B. cinerea* was kindly provided by the University of Zaragoza (Spain) and was maintained on potato dextrose agar (PDA) and potato dextrose broth (PDB) and incubated at 24°C.

Antifungal Activity of XT1 Strain

The antifungal activity of XT1 strain against *B. cinerea* was determined in both solid and liquid mediums. For the solid assay strain XT1 was spread on a 1 cm^2 area on one side of a PDA agar plate (at 1 cm from the plate wall) and an 8-mm-agar disc of the mycelium of fungi was deposited on the opposite side. The maximum and minimum values of the fungal mycelium radius obtained were measured after a 15-day incubation period at 25°C. The results were expressed as a percentage of the mycelium inhibition rate ($IR\% = A - B/A \times 100$) where A was the maximum value of the mycelium radius and B was the minimum value. For the antifungal assay in liquid medium, first, the time in which the antifungal activity was the maximum was determinate cultivating the strain XT1 in MOLP (medium optimal for lipopeptide production) (Ahimou et al., 2000) at different times (24, 48, 72, and 96 h), then the supernatant was tested against *B. cinerea* following the procedure described below. A 15-day culture of *B. cinerea* in PDB was crushed in a breaker and filtered with gauze, all under sterile conditions. The spore concentration was adjusted to 5×10^7 conidia mL^{-1} and penicillin G (2.5 mg mL^{-1}) and streptomicin (10 mg mL^{-1}) was added to the spore suspension. The experiment was carried out on multiwell culture plates (Cellstar*) with 48 wells where 900 μl of the spore solution was subjected to 300 μL of different times XT1 supernatant, obtained after centrifugation of the XT1 culture in MOLP at 10000 rpm 20 min. Inoculated medium with cycloheximide 50 μg mL^{-1} was used as a positive control for growth inhibition, whilst PDB inoculated with spore suspension without treatment was considered as the negative control. The plates were incubated at 25°C for 7 days. The results were obtained by observing the presence or absence of fungal growth (Frikha-Gargouri et al., 2017).

Lipopeptide Production

Three different liquid media have been tested for lipopeptide production: MOLP medium (Ahimou et al., 2000); SG medium (Schaeffer et al., 1965; Leighton and Doi, 1971), and a commercial

concentrated beef medium (CM) (ox concentrate 43% and yeast extract 24%). Lipopeptides were extracted according to Yazgan et al. (2001) with slight modifications. Briefly, the culture supernatant of strain XT1 was subjected to an organic extraction with one volume *n*-butanol three times using a decantation funnel. Then, the organic phase was evaporated with a vacuum concentrator.

Antifungal Activity of the Lipopeptides Produced by XT1

Lipopeptide antifungal activity was tested by preparing 20 mL of lipopeptide solution at different concentrations: 20, 10, 8, 6, 4, 2, 1, and 0.5 mg mL^{-1} (w/v in distilled water) in 50 mL tubes. Then 0.8 g PDA was added to each lipopeptide solution; the negative control was made with untreated PDA medium. The tubes were then sterilised and the medium were poured into 90 mm Petri plates. Next, a fungal plug of 15 days of mycelial growing *B. cinerea* was deposited in the middle of each plate and maintained at 25°C for 15 days. After that, the mycelial growth inhibition percentage was calculated after 15 day's incubation by the comparison between the diameter of mycelial growing in the control plates and the treatments according to the following formula: mycelial growth inhibition = 100−[(diameter of control mycelium diameter of mycelium in lipopeptide medium /diameter of control mycelium) × 100] (Borah et al., 2016).

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of the Lipopeptides

The minimal inhibitory concentration (MIC), defined as the smallest concentration of lipopetides that inhibits the fungal growth totally and the minimal fungicidal concentration (MFC), defined as the lowest concentration of lipopeptides capable of killing the fungi, was determined. The experiment was carried out in liquid medium according to the protocol described previously in the *"Antifungal Activity of XT1 Strain"* section. However, in this case 900 µl of the spore solution was exposed to 300 µL of lipopeptide dilutions 20, 10, 8, 6, 4, 2, 1, and 0.5 mg mL^{-1}. The plates were incubated at 25°C for 7 days. The results were obtained by observing the presence or absence of fungal growth. The whole content from each well, where there was no growth of *B. cinerea,* was passed to tubes with PDB medium and incubated at 25°C for 7 days for the determination of MFC (Frikha-Gargouri et al., 2017).

Stability of Lipopeptides to Heat and pH

The antifungal activity of a lipopeptide solution (10 mg mL^{-1}) from XT1 was determined after 10, 30, and 60 min of heating at 100°C and after 20 min at 121°C. The stability was also determined at different pH in the range comprised between 3 to 12 (Ghribi et al., 2012).The antifungal activity was tested in liquid medium according to the method described above (Frikha-Gargouri et al., 2017).

Genetic Characterization of Lipopeptides

Genes encoding NRPS production were amplified by polymerase reaction chain (PCR) from genomic DNA of the XT1 strain. PCR was carried out using the specific and degenerated primers described in **Table 1**. PCR amplifications were achieved in 50 µL mixtures with PCR buffer, 2 mM $MgCl_2$, 4 mM of each primer, 5U Taq polymerase, 0.2 mM of each dNTP, and 80–100 ng of genomic DNA. The amplification conditions were: 95°C for 5 min, 40 cycles of 94°C for 1 min, annealing temperature for 1 min, 72°C extension for 1 min; and a final extension at 72°C for 10 min. The annealing temperatures were 45, 43, 50, 53, and 50°C for, Af2/Tf1, As1/Ts2, BmyBF/BmyBR, ItuDF/ItuDR, and SrfA3/LicA3 primers, respectively. The amplification products were analysed by electrophoresis in a 2% (w/v) agarose gel.

Identification of Lipopeptides Using UPLC – HDMS Q-TOF

The residue obtained from lipopeptide extraccion was dissolved in 10% methanol and analysed by high-pressure liquid chromatography (UPLC) (Acquity UPLC® BEH300, Waters) coupled to a high definition mass spectrometry (SYNAPT G2 HDMS Q-TOF. Waters). Mass spectrometry was carried out by positive ionisation electrospray (ESI+). The obtained data were processed by the MassLynx™ software (Waters).

TABLE 1 | PCR primers of lipopeptides biosynthesis genes in *Bacillus* XT1.

Lipopeptide	Gene	Primers	Primer sequences (5′→3′)	PCR product size (bp)	Reference
Surfactin/Lichenicin	*srf*A3/*lic*A3	SrfA3/licA3(F)	CAAAAKCGCAKCATACCAAKTTGAG	268	Randall Simpson et al., 2011
		*Srf*A3/*lic*A3 (R)	AGCGGCAYATATTGATGCGGYTC		
Fengycin	*fen*C	Af2 (F)	GAATAYMTCGGMCGTMTKGA	443–455	Tapi et al., 2010
		Tf1 (R)	GCTTTWADKGAATSBCCGCC		
Surfactin	*srf*A-A	As1 (F)	CGCGGMTACCGVATYGAGC	419–431	Tapi et al., 2010
		Ts2 (R)	ATBCCTTTBTWDGAATGTCCGCC		
Bacillomycin	*bmy*B	BmyB (F)	GAATCCCGTTGTTCTCCAAA	370	Mora et al., 2011
		BmyB (R)	GCGGGTATTGAATGCTTGTT		
Iturin	*itu*D	ItuD (F)	TTGAAYGTCAGYGCSCCTTT	482	Chung et al., 2008
		ItuD (R)	TGCGMAAATAATGGSGTCGT		

Scanning Electron Microscopy and Transmission Electron Microscope of Mycelia Treated With Lipopeptides

Solid culture medium plates with the negative control treatments and lipopeptides 10 mg·mL^{-1} from the previously experiment described in the "*Antifungal Activity of the Lipopeptides Produced by XT1*" section, were used to observe the morphology of *B. cinerea*. The samples were fixed and observed in a FIB-FESEM (CrossBeam NVision 40®, Carl Zeiss SMT) Scanning Electron Microscope and Transmission Electron Microscope.

Bioassay Against *B. cinerea* in Grapes, Strawberries, and Tomatoes

Antifungal activity of lipopeptides produced by XT1 was tested on grapes, strawberries, and tomatoes. *B. cinerea* was grown for 15 days on a PDA medium at 25°C and conidia were collected with sterile distilled water and filtered through four layers of sterile cheesecloth. The surface of the fruit was sterilised with 5% NaOCl for 5 min and rinsed three times with plenty of sterile water. Wounds of 3 mm were performed with a sterile scalpel on the surface of the grapes, then 15 µl of a 20 mg·mL^{-1} solutions of lipopeptides were applied on the injury. One hour later, when the fruit was dried at room temperature, 15 µL of a suspension of *B. cinerea* 10^8 conidia mL^{-1} were inoculated into the wound. Strawberries and tomatoes were cut into slices and treated by spray with the same lipopeptide solution as in the previous case. Then, after 1 h, the fruit was infected by spraying with the conidia suspension of *B. cinerea*. All the treatments were incubated at 25°C, 70% humidity for 6 days. For each treatment, a total of nine fruits were used and three technical replicates were performed. Effect was measured and expressed as disease incidence (% of infected fruit).

Antioxidant Activity on Fruit Treated With Lipopeptides

The antioxidant activity was measured extracting the soluble phenols and by FRAP assay (ferric iron reducing antioxidant power assay). Total soluble phenols were extracted from 0.1 g of lyophilized fruits with 10 mL of 80% methanol and 0.1% hydrochloric acid. The mixture was placed in the dark at 4°C for 2 h. The supernatant was filtered and the extract was used for the determination of the phenol content and the FRAP assay. The amount of total phenols was determined according to the Folin-Ciocalteu's procedure described by Ribereau-Gayon (1968) with slight modifications. The phenol content was estimated from a standard curve of gallic acid (GAE) and the results expressed as mg of gallic acid 100 g^{-1} d.w (dry weight). In FRAP assay an extract of fruit (0.2 mL) (prepared as for phenol determination) was added to 2 mL of FRAP solution [0.25 mol L^{-1} acetate buffer (pH 3.6) containing 1 mmol L^{-1} 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 20 mmol L^{-1} $FeCl_3.6H_2O$] and incubated 5 min at room temperature measuring the absorbance at 593 nm. A standard of 1 mmol L^{-1} L-ascorbic acid in distilled water was prepared. Results were expressed as mmol L^{-1} of Fe^{2+} equivalents 100 g^{-1} dry weight (Benzie and Strain, 1999).

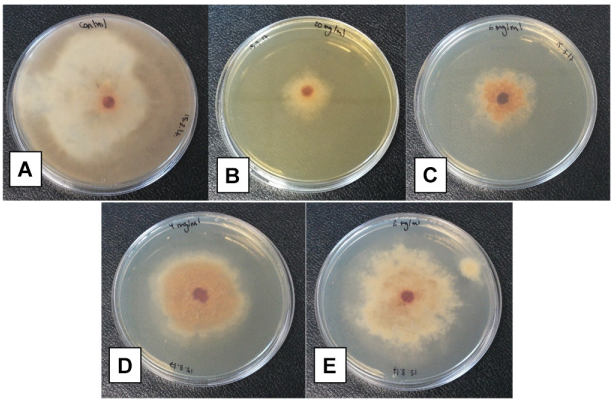


FIGURE 1 | Antifungal activity of *Bacillus* XT1 lipopeptides toward *Botrytis cinerea*. Effect of different concentrations on the antifungal potency: negative control **(A)** and in the presence of: 10 mg mL^{-1} lipopeptide **(B)**, 6 mg mL^{-1} lipopeptide **(C)**, 4 mg mL^{-1} lipopeptide **(D)**, and 2 mg mL^{-1} lipopeptide **(E)**.

Statistical Analyses

Data obtained were subjected to ANOVA and multiple pair-wise comparisons were performed by the Duncan's multiple range test.

RESULTS

Antifungal Activity of *Bacillus* XT1

Antifungal production in the supernatant was detected after 24 h of aerobic culture and reached its maximum at 72 h. The XT1 strain showed antifungal activity against *B. cinerea* in both solid and liquid media (inhibition rates of 60 and 100%, respectively).

Lipopeptide Production

The production of lipopeptides was tested in different culture media. The data showed that MOLP medium increased the lipopeptide production compared with other media. The best production yield was obtained with this medium (10 g L^{-1}); however, the production with other media such as SG and the commercial medium (CM) decreased the production of lipopeptide to 2.8 and 2.13 g L^{-1}, respectively.

Antifungal Activity of the Lipopeptides Produced by *Bacillus XT1*

The antifungal activity of *Bacillus* XT1 lipopeptides toward *B. cinerea* was also analysed. The results demonstrated that lipopeptides produced by XT1 inhibit the growth of *B. cinerea*. Inhibition rates of 72, 48, 30, and 19% of the mycelium diameter were observed for the concentrations of lipopeptides of 10, 6, 4, and 2 mg mL^{-1}, respectively, after 15 days of treatment (**Figure 1**). In general, lipopeptides from XT1 showed antagonistic activity against *B. cinerea* across a broad spectrum of concentrations in a dose response manner (**Figure 1**).

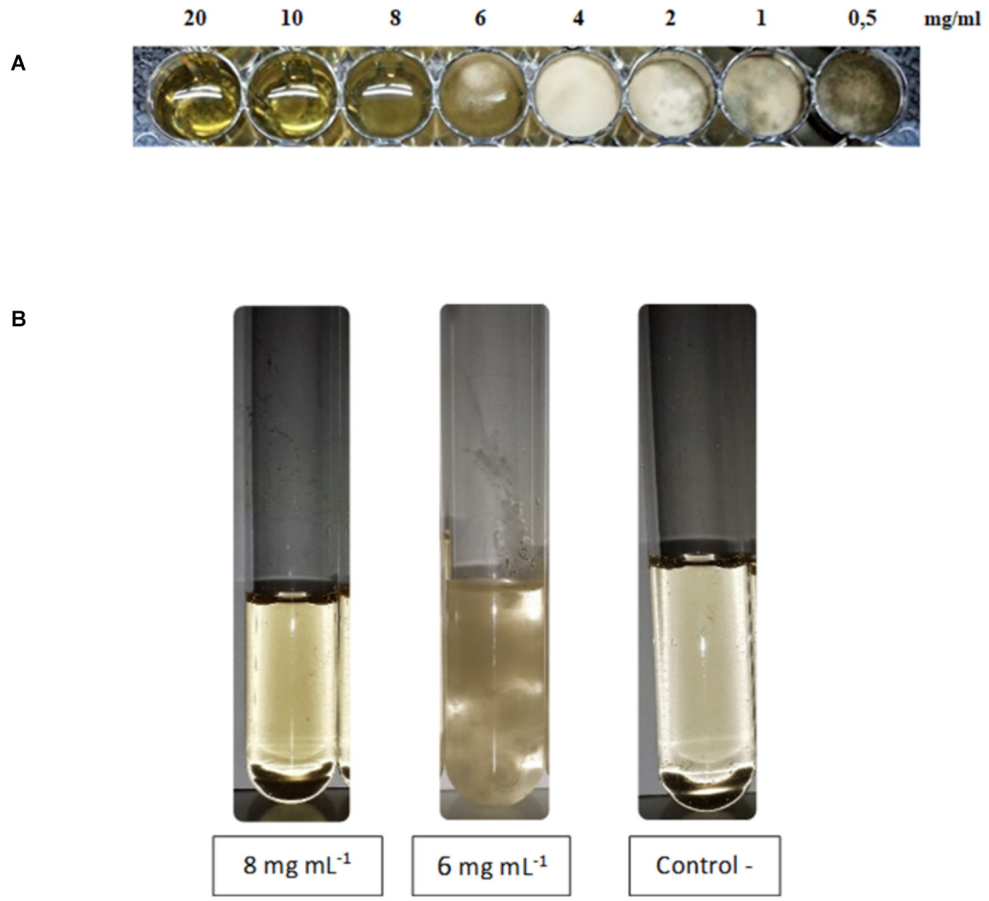


FIGURE 2 | Minimal inhibitory concentration (MIC) **(A)** and minimal fungicidal concentration (MFC) **(B)** of lipopeptides from XT1.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of the Lipopeptides

Lipopeptides from XT1 were also tested in multiwell culture plates with 48 wells to test the MIC and in culture tubes to test the MFC. As shown in **Figure 2A** lipopeptides concentrations tested ranging from 20 to 0.5 mg mL^{-1} and a significant inhibitory effect of lipopeptides was observed at concentrations as low as 8 mg mL^{-1} which corresponds to the MIC. The MFC was also 8 mg mL^{-1} (**Figure 2B**).

Stability of Lipopeptides to Heat and pH

The antifungal potency was not affected by any heat treatment. However, the antifungal potency of lipopeptides from XT1 was affected at pH 3 and pH 12. The optimum range of pH for the maximum antifungal efficacy was between 7 and 9.

Detection of Genes Involved in Lipopeptide Biosynthesis

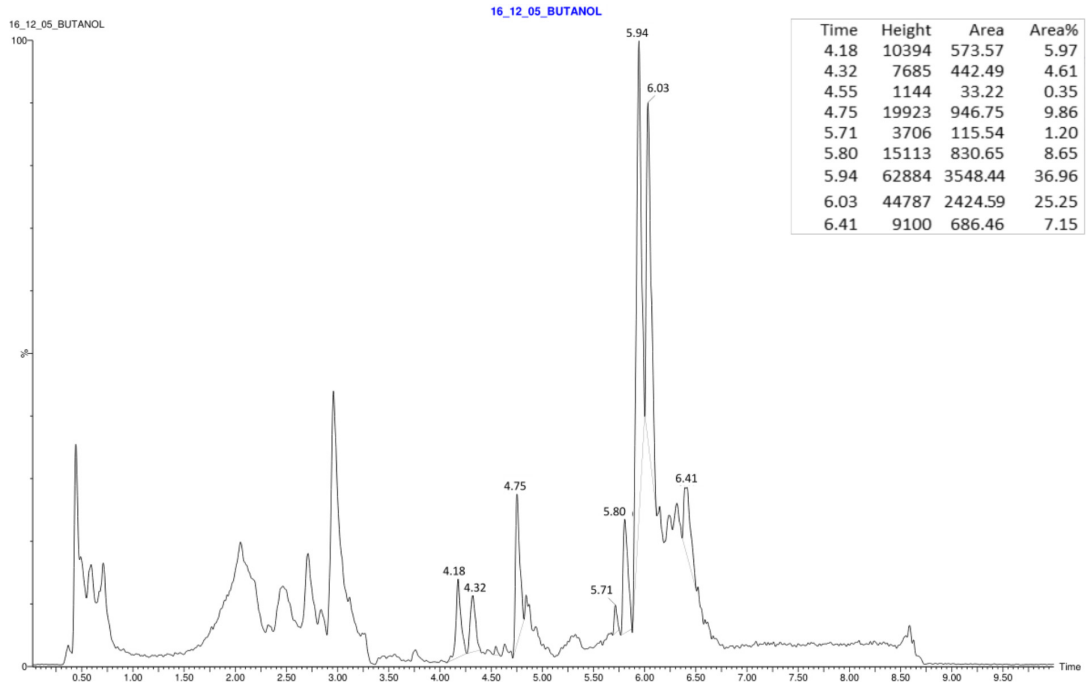
Genomic analysis of *Bacillus* XT1 indicates that it contains gene clusters for non-ribosomal lipopeptide synthetases. Amplicons of the expected sizes were obtained for fengycin, surfactin, bacillomycin, and iturin genes *srf*A-C, *fen*C, *srf*A-A, *bmy*B, and *itu*D.

Identification of Lipopeptides Using UPLC – HDMS Q-TOF

Electrospray quadrupole time-of-flight mass spectrometry (Q-TOF MS) analyses were carried out in this study to identify the metabolites produced by XT1. **Figure 3** illustrates the total ion chromatogram (TIC) spectrum of the lipopeptide extract from a XT1 culture supernatant.

These analyses showed that the strain XT1 produces several forms of different lipopeptides. Four known surfactins with an acyl chain ranging from C12 to C15 were detected, whereas three known bacillomycins D (C14, C15, and C16) were also detected. Two peaks corresponding to the fengycin A and fengycin B were also observed (**Table 2**). There were no mass signals for iturin.

Quadrupole time-of-flight mass spectrometry analysis indicated five types of lipopeptides and two predominant compounds. This analysis detected a [M+H] peak at m/z 1022.6729 and afforded the molecular formula $C_{52}H_{91}N_7O_{13}$ (i-Fit = 20.6 and DBE = 10.5) and corresponding to surfactin and [M+H] peak at m/z 1463.8038 corresponding to fengycin



Time	Height	Area	Area%
4.18	10394	573.57	5.97
4.32	7685	442.49	4.61
4.55	1144	33.22	0.35
4.75	19923	946.75	9.86
5.71	3706	115.54	1.20
5.80	15113	830.65	8.65
5.94	62884	3548.44	36.96
6.03	44787	2424.59	25.25
6.41	9100	686.46	7.15

FIGURE 3 | Total ion chromatogram (TIC) spectrum obtained from the surfactant of *Bacillus* XT1.

A with the molecular formula $C_{72}H_{110}N_{12}O_{20}$ (i-Fit = 30.2 and DBE = 23.5) (**Figures 4C** and **4A**, respectively).

Quadrupole time-of-flight mass spectrometry analysis indicated five types of lipopeptides and two predominant compounds. This analysis detected a [M+H] peak at m/z 1031.5431 (1053.5276 corresponds to the complementary sodium adduct molecular ion [M+Na]) with the molecular formula $C_{48}H_{75}N_{10}O_{15}$ (i-Fit = 101.1 and DBE = 16.5) corresponding to bacillomycin D, a [M+H] peak at m/z 1463.8038 with the molecular formula $C_{72}H_{110}N_{12}O_{20}$ (i-Fit = 130.3 and DBE = 23.5) corresponding to fengycin A and a [M+H] peak at m/z 1022.6729 and afforded the molecular formula $C_{52}H_{91}N_7O_{13}$ (i-Fit = 161.5 and DBE = 10.5) and corresponding to surfactin (**Figures 4A**, **4B**, and **4C**, respectively).

Effect of XT1 Lipopeptides on *B. cinerea* Mycelial Growth

Microscopy data of *B. cinerea* mycelial growth treated with XT1 lipopeptides are shown in **Figure 5**. Scanning electron microscopy (SEM) analyses of *B. cinerea* mycelium treated with the MIC/MFC of lipopeptides (8 mg mL^{-1}) from XT1 showed important alterations of pathogen morphology. Hyphae treated without lipopeptides grew normally with straight appearance and their surfaces were smooth (**Figure 5A**). However, after exposure to lipopeptides, one of the most striking features was the appearance of structures of resistance (**Figure 5B**).

Transmission electron microscopy (TEM) images of normal hyphae treated without lipopeptides showed smooth surfaces, intact cells, well defined enclosing cell walls and the cellular

TABLE 2 | Lipopeptide production by *Bacillus* XT1 as detected by Q-TOF MS.

Lipopeptide	Fatty chain length	$[M+H]^+$	Retention time (min)	Area (%)	Peak intensity
Bacillomycin D	C14	1031.5431	4.18	5.97	1.02e4
	C15	1045.5585	4.32	4.61	8.35e3
	C16	1059.5726	4.55	0.35	1.31e3
Fengycin A	C16	1463.8038	4.75	9.86	4.81e3
Fengycin B	C15	1477.8329	4.86	0.60	1.65e3
	C16	1491.8481	4.86	0.60	1.01e3
Surfactin	C12	994.6413	5.71	1.20	4.22e3
	C13	1008.6564	5.80	8.65	1.06e4
	C14	1022.6729	5.94	36.96	8.40e4
	C15	1036.6867	6.03	25.25	6.60e4

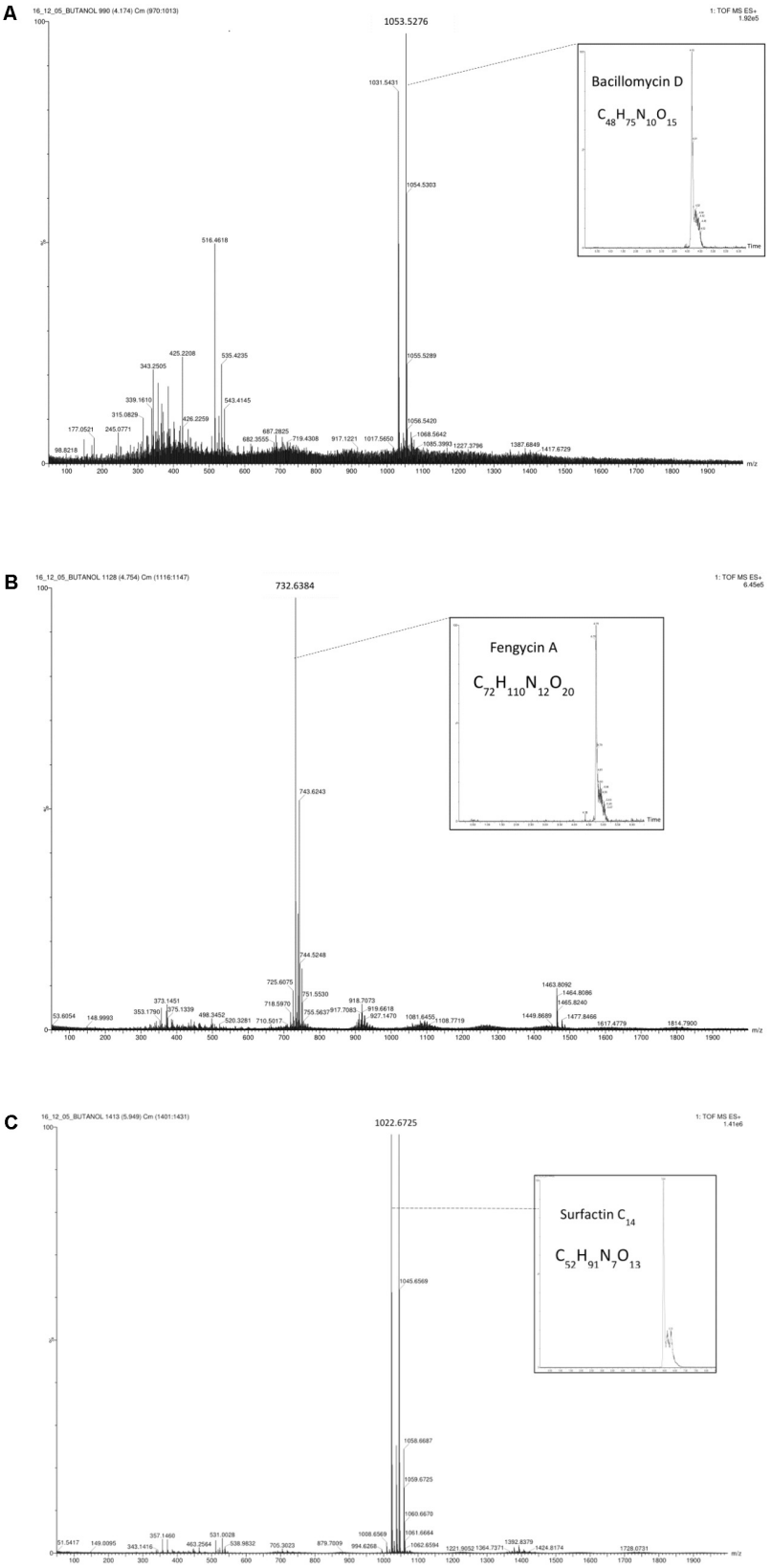


FIGURE 4 | Quadrupole time-of-flight mass spectrometry (Q-TOF MS) spectra obtained from the surfactant produced by *Bacillus* XT1: protonated linear derivatives of the [M+H] of bacillomycin D **(A)**, fengycin A **(B)**, and surfactin **(C)**.

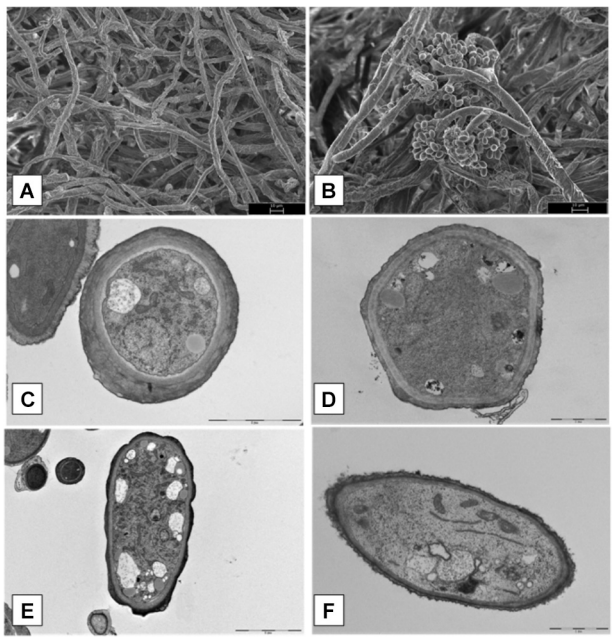


FIGURE 5 | Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) micrographs of hyphae of *Botrytis cinerea* treated with lipopeptides. Hyphae treated without and with lipopeptides (SEM) (**A,B**, respectively). Hyphae treated without and with lipopeptides (TEM) (**C,E** and **D,F**, respectively).

organelles in normal arrangements (**Figures 5C,E**). TEM images of hyphae confirmed that in the treatment of *B. cinerea* with lipopeptide organelles were degenerated and gathered in clumps (**Figures 5D,F**).

Bioassay Against *B. cinerea* in Grapes, Strawberries, and Tomatoes

The MIC/MFC of lipopeptide extract from XT1 (8 mg mL^{-1}) was applied in order to evaluate the protector effect with different fruit infected with *B. cinerea*. The lipopeptides were injected in grapes and sprayed onto strawberries and tomatoes. The level of disease infections decreased in all the fruit treated with XT1 lipopeptides.

The disease incidence in grapes, strawberries, and tomatoes treated with *B. cinerea* was 71, 100, and 100%, respectively. The disease reductions in fruit treated with XT1 lipopeptides were 100, 12, and 50%, respectively (**Table 3** and **Figures 6**, **7**). The results show that antifungal lipopeptide treated fruit reduced the disease symptoms significantly compared to non-treated fruits except in strawberries where the effect was not so evident. This reduction is higher for grapes.

Antioxidant Activity on Fruit Treated With Lipopeptides

The antioxidant activity was tested in grapes where the highest disease reduction was observed. The antioxidant activity of grape extracts, as estimated by the FRAP assay increased significantly with the inoculation of the MIC/MFC of lipopeptides produced by XT1 (**Figure 8A**). Although an increase in the antioxidant activity was also observed in grapes infected with the pathogen, the highest increases were observed in the treatment with the lipopeptides.

On the other hand, total phenol content also increased with the exposure of fruit to the lipopeptides but the increase is only significant in the uninfected grapes. In this case the increase of total phenol content is of 30% (**Figure 8B**).

DISCUSSION

The strain *Bacillus* XT1 is a gram positive, sporulated, and halotolerant rod that grows in a wide range of salt concentrations (0–12% w/v), temperature (15–40°C), and pH (5–10) (Béjar et al., 2014). The strain was originally classified as *B. methylotrophicus* XT1 CECT 8661 (deposited according to the Bucharest Treaty for patenting purposes and licenced to Xtrem Biotech S.L.). The species *B. methylotrophicus* was later reclassified as a heterotypic synonym of *B. velezensis* (Dunlap et al., 2016).

This paper describes the antifungal activity of the strain and its lipopeptides against *B. cinerea*, a filamentous fungus classified as the second most important phytopathogen worldwide. Also demonstrate the implication of XT1 lipopeptides in the *in vivo* antibiosis and in the alteration of fungus structures and in the induced systemic response of fruits affected with the fungi.

One of the major factors related with the antifungal activity of members of the genus *Bacillus* is due to the production of lipopeptides synthesised by NRPSs such as iturin, fengycin, and surfactin (Romero et al., 2007; Ongena and Jacques, 2008; Pramudito et al., 2018). Lipopeptides are produced as a mixture of macromolecules belonging to the same family or class. Genomic analysis of *Bacillus* XT1 indicated that it contains gene clusters for non-ribosomal lipopeptide synthetases related to the production of surfactin, iturin, fengycin, and bacillomycin. Detection of the

TABLE 3 | Disease incidence in grapes, strawberries, and tomatoes.

	Disease incidence (%)			
	Control	**XT1 lipopeptides**	***Botrytis***	**XT1 lipopeptides + *Botrytis***
Grapes	0 ± 0.00a	0 ± 0.00a	71 ± 0.18b	0 ± 0.00a
Strawberry	43 ± 0.25b	33 ± 0.00a	100 ± 0.00d	88 ± 0.58c
Tomato	25 ± 0.20b	0 ± 0.17a	100 ± 0.00d	50 ± 0.13c

Values followed by the same letters for each fruit did not differ significantly according to Duncan's multiple range test ($p < 0.05$).

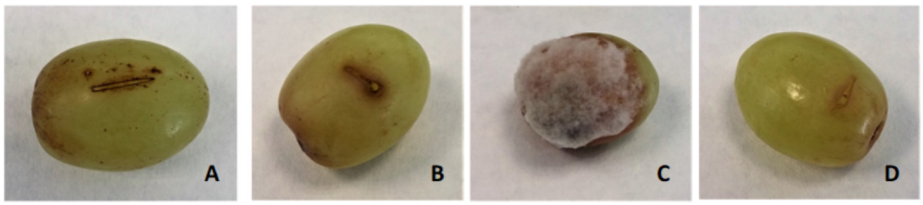


FIGURE 6 | Lipopeptides from XT1 inhibited disease severity in grapes. Disease severity of grey mould on grapes. **(A)** Grapes treated with sterile water as the negative control. **(B)** Grapes treated with lipopeptides from XT1. **(C)** Grapes infected with *Botrytis cinerea*. **(D)** Grapes infected with *B. cinerea* and treated with lipopeptides from XT1.

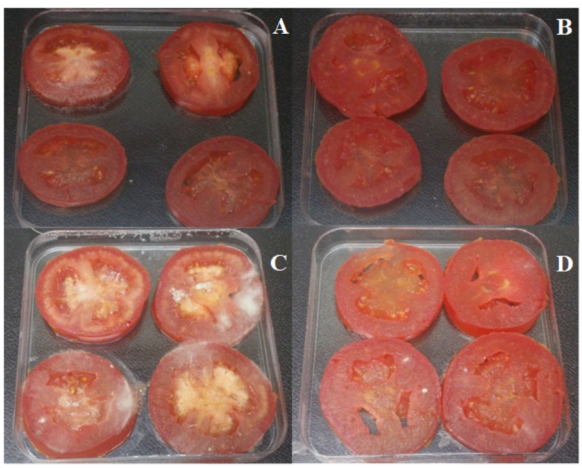


FIGURE 7 | Lipopeptides from XT1 inhibited disease severity in tomatoes. Disease severity of grey mould on tomatoes. **(A)** Tomatoes treated with sterile water as the negative control. **(B)** Tomatoes treated with lipopeptides from XT1. **(C)** Tomatoes infected with *Botrytis cinerea*. **(D)** Tomatoes infected with *B. cinerea* and treated with lipopeptides from XT1.

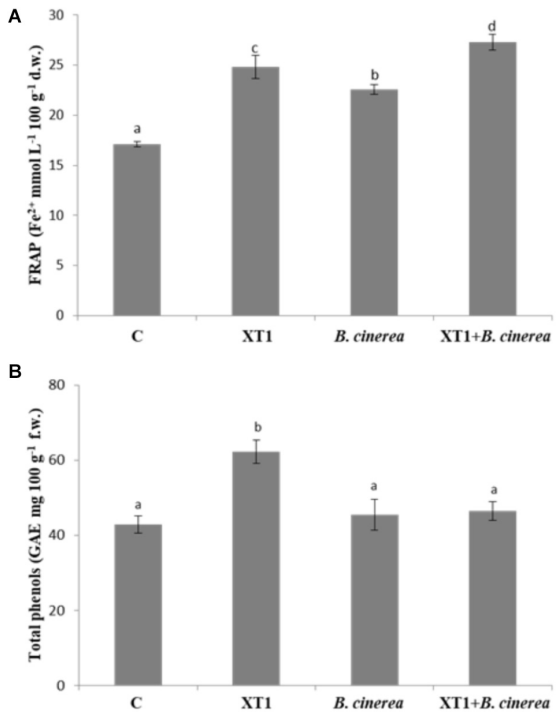


FIGURE 8 | Antioxidant activity evaluated with the FRAP assay **(A)** and total phenols **(B)** of grapes treated with lipopeptides from XT1, infected with *Botrytis cinerea* and infected with *B. cinerea* but treated with lipopeptides from XT1. Values followed by the same letters did not differ significantly according to Duncan's multiple range test ($p < 0.05$). Vertical lines represent the standard errors of the mean.

produced lipopeptides by XT1 was performed by Q-TOF MS analysis showing that strain XT1 produces all lipopeptides except iturin. Some of these macromolecules were previously shown to be produced by other *B. methylotrophicus* strains (Frikha-Gargouri et al., 2017).

Lipopeptides biosynthesis from XT1 was tested in different culture media. The data showed that the culture medium used in the growth of the microorganism could decisively influence the production of lipopeptides. Ahimou et al. (2000) described an optimum medium (named MOLP medium) for lipopeptide production by *Bacillus subtilis*. This study concluded that the medium MOLP is also the best to increase the lipopeptide production yields in *B. methylotrophicus* by *Bacillus* XT1. The influence of culture conditions on lipopeptide production was previously described for other strains of *Bacillus* genus like *B. subtilis* or *B. amyloliquefaciens* (Monteiro et al., 2005; Medeot et al., 2017). In terms of stability, the thermostable nature of XT1 lipopeptides and the fact that these antifungal compounds were affected by extremely alkaline pH were also observed in the evaluation of the activity of the *B. subtilis* biosurfactant (Ghribi et al., 2012).

Several studies have previously highlighted the antagonistic effect against different pathogens of NRP metabolites such as lipopeptides (Chowdhury et al., 2015; Mnif and Ghribi, 2015). For example, it has been demonstrated that the biocontrol of *Bacillus* strains against different pathogenic bacteria and fungi such as *Aspergillus* or *Pseudomonas syringae* is facilitated by lipopeptides such as surfactin (Bais et al., 2004). According to the literature, few studies describe the inhibitory activity of the lipopeptides produced by *Bacillus* strains against *B. cinerea.* All the studies reported the antibiosis of *B. cinerea* by the NRPs metabolites produced by *B. subtilis* but do not demonstrate directly the implications of these molecules in the antibiosis

(Tareq et al., 2014; Farace et al., 2015; Wang et al., 2015; Arroyave-Toro et al., 2017). The same occurs with *B. amyloliquefaciens* (Ji et al., 2013; Pretorius et al., 2015; Tanaka et al., 2015; Zhang et al., 2017), *B. marinus* (Gu et al., 2017), *B. atrophaeus* (Zhang et al., 2013), and *B. velezensis* (Ge et al., 2016; Romero et al., 2016; Gao et al., 2017).

With respect to the activity of lipopeptides, Romano et al. (2013) reported the non-effect of *B. amyloliquefaciens* lipopeptides against *B. cinerea* at concentrations of 0.1 mg mL^{-1}. Tareq et al. (2014) established a range of lipopeptide activity in *B. subtilis* between 0.5 to 300 μg mL^{-1} and Zhang et al. (2017) determined the maximum activity of lipopeptides of *B. amyloliquefaciens* at 30 mg mL^{-1}. Our study tested a range of concentrations from 0.5 to 20 mg mL^{-1} and confirmed the antifungal activity of XT1 lipopeptides with inhibitory and fungicidal effects of these compounds at concentrations as low as 8 mg mL^{-1} (MIC and MFC). This fact and the higher productions of lipopeptides in MOLP medium may determine the high fungicidal activity of *Bacillus* XT1 against *B. cinerea.*

Different studies show that lipopeptides from *Bacillus* sp. strains produce damage to the hyphae and survival structures of pathogenic fungi (Souto et al., 2004). Chitarra et al. (2003) suggested that lipopeptides produced by *B. subtilis* YM 10-20 may permeabilize fungal spores and inhibit their germination. Other studies show the swelling and the deformation of fungus hyphae of *Pestalotiopsis eugeniae* when treated by the lipopeptides of *B. subtilis* BS-99-H (Lin et al., 2010). The effects of the lipopeptides produced by XT1 on the morphology of *B. cinerea* were evaluated in solid media. SEM studies revealed an extensive formation of fungal spores in the intersection of the fungus-bacteria inhibition zone. The same results were observed in previous studies where the biological activity of lipopeptides from *B. amyloliquefaciens* against *Fusarium solani* was analysed (Torres et al., 2017). Gong et al. (2014) studied the effect of bacillomycin D from *B. subtilis* on *Aspergillus flavus* and concluded that due to the amphipathic nature of bacillomycin D, this compound entered the spores and the hyphae where it caused pores to be formed in the membrane, resulting in the leakage of cell contents. TEM images of hyphae confirmed that in the treatment of *B. cinerea* with XT1 lipopeptides the organelles degenerated probably due to the entry of these compounds.

In this study, the antifungal activity of lipopeptides from XT1 against grey mould disease in different fruit was also investigated. The inoculation results showed that grey mould disease on grapes and tomatoes was significantly inhibited by the lipopeptides produced by XT1. Previous studies have described the involvement of lipopeptides from *B. subtilis* in grapevine plant defence and local resistance against *B. cinerea* (Farace et al., 2015). They also showed that lipopeptides are perceived by grapevine plant cells and activate different signalling pathways. Previous studies suggest that lipopeptides act as elicitors of defence-related genes (Waewthongrak et al., 2014). However, and to the best of our knowledge, our study is the first to highlight the ability of lipopeptides to trigger the antioxidant activity of these macromolecules in fruit. The total phenol content was increased significantly with the exposure of the fruit to the lipopeptides produced by XT1. The highest increases in antioxidant activity were observed in the infected fruits and in those treated with the lipopeptides. These data may suggest that the antimicrobial effect of lipopeptides and the accumulation of antioxidant compounds are closely related with pathogen resistance.

CONCLUSION

In this study, we have investigated the high antifungal activity against *B. cinerea* of a patented strain, *Bacillus* XT1 CECT 8661. The lipopeptides produced by XT1 are involved in the biological control of *B. cinerea* and trigger the antioxidant activity in fruit. Based on the inhibitory effect on the development of grey mould on grapes and tomatoes, *Bacillus* XT1 CECT 8661 could be considered as a potential alternative for chemical fungicides in reducing the damage of grey mould disease.

AUTHOR CONTRIBUTIONS

LT carried out the experimental techniques and statistical analysis. MR collaborated in the experimental techniques related with determination of lipopeptide genes and analysis of the chromatograms. VB collaborated in the design of the techniques related with the extraction and the study of antifungal activity of lipopeptides, analysed the results, and critically read the manuscript. IS designed the experimental techniques, analysed the results, and drafted the manuscript.

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Complete Genome Sequence of Industrial Biocontrol Strain *Paenibacillus polymyxa* HY96-2 and Further Analysis of Its Biocontrol Mechanism

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Paenibacillus polymyxa (formerly known as *Bacillus polymyxa*) has been extensively studied for agricultural applications as a plant-growth-promoting rhizobacterium and is also an important biocontrol agent. Our team has developed the *P. polymyxa* strain HY96-2 from the tomato rhizosphere as the first microbial biopesticide based on *P. polymyxa* for controlling plant diseases around the world, leading to the commercialization of this microbial biopesticide in China. However, further research is essential for understanding its precise biocontrol mechanisms. In this paper, we report the complete genome sequence of HY96-2 and the results of a comparative genomic analysis between different *P. polymyxa* strains. The complete genome size of HY96-2 was found to be 5.75 Mb and 5207 coding sequences were predicted. HY96-2 was compared with seven other *P. polymyxa* strains for which complete genome sequences have been published, using phylogenetic tree, pan-genome, and nucleic acid co-linearity analysis. In addition, the genes and gene clusters involved in biofilm formation, antibiotic synthesis, and systemic resistance inducer production were compared between strain HY96-2 and two other strains, namely, SC2 and E681. The results revealed that all three of the *P. polymyxa* strains have the ability to control plant diseases via the mechanisms of colonization (biofilm formation), antagonism (antibiotic production), and induced resistance (systemic resistance inducer production). However, the variation of the corresponding genes or gene clusters between the three strains may lead to different antimicrobial spectra and biocontrol efficacies. Two possible pathways of biofilm formation in *P. polymyxa* were reported for the first time after searching the KEGG database. This study provides a scientific basis for the further optimization of the field applications and quality standards of industrial microbial biopesticides based on HY96-2. It may also serve as a reference for studying the differences in antimicrobial spectra and biocontrol capability between different biocontrol agents.

Keywords: genome sequencing, *Paenibacillus polymyxa*, comparative genomic analysis, biocontrol mechanism, biofilm, antibiotics, induced resistance

INTRODUCTION

Paenibacillus polymyxa is a multifunctional Gram-positive (G^+) bacterium that has been reported to have applications in agriculture (Hao and Chen, 2017), medicine (Galea et al., 2017; Yu et al., 2017), and industry (Lal and Tabacchioni, 2009). *P. polymyxa* has been in the list of substances reported under the Toxic Substance Control Act (TSCA) by the United States Environmental Protection Agency (EPA)[1]. It means *P. polymyxa* is a safe and commercially available microbe. The application of *P. polymyxa* in agriculture includes two main aspects: the biocontrol of plant diseases and the promotion of plant growth. *P. polymyxa* is an important biocontrol agent that has been reported to suppress a large variety of fungal and bacterial plant diseases, such as those caused by the fungi *Fusarium oxysporum* and *Botrytis cinerea* and the bacteria *Xanthomonas campestris* and *Ralstonia solanacearum* (Xu et al., 2006; Kim et al., 2010; Fan et al., 2012; Weselowski et al., 2016). *P. polymyxa* can withstand various adverse conditions during the biopesticide manufacturing process, especially drying, and exhibits a long and resilient shelf life owing to its endogenous spore formation. Therefore, *P. polymyxa* is an important strain for the production of microbial biopesticides[2,3]. In 2004, *P. polymyxa* strain HY96-2 from the tomato rhizosphere was developed as the first microbial biopesticide based on *P. polymyxa* and registered in China, for the control of soil-borne diseases caused by *R. solanacearum* on tomatoes and *F. oxysporum* on watermelons, as well as leaf diseases caused by *B. cinerea* and *Pseudomonas syringae* on cucumbers[2]. In addition, other studies have demonstrated that HY96-2 inhibits the growth of many fungal and bacterial pathogens, such as *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, and *Erwinia carotovora* (Fan et al., 2012). Although microbial biopesticides derived from *P. polymyxa* HY96-2 have been manufactured and sold in 24 provinces across China, further research into its precise biocontrol mechanism, especially at the molecular level, is still needed.

Genomes are a very useful resource for understanding the mechanism of biocontrol agents. Kim et al. (2017) determined the key genes responsible for the production of antimicrobial agents and volatile organic compounds, indoleacetic acid (IAA) synthesis, siderophore secretion, phosphate transporter, and phosphonate cluster biosynthesis in *Paenibacillus yonginensis* DCY84T by genome sequencing and confirmed the ability of this strain to induce plant resistance and protect plant growth by a combination of physiological experiments. By comparing the genomes of *Bacillus amyloliquefaciens* FZB42 with *B. subtilis*, Chen et al. (2007) discovered that over 8.5% of the genome of *B. amyloliquefaciens* FZB42 is involved in antibiotic and siderophore synthesis, whereas Stein (2005) estimated that not more than 4–5% of the average *B. subtilis* genome is devoted to antibiotic production. Therefore, this genomic comparison demonstrated that *B. amyloliquefaciens* FZB42 is capable of protecting plants from diseases at the molecular level via the production of antibiotics.

Furthermore, despite its importance as a biocontrol agent, there have only been a small number of comprehensive studies into the biocontrol mechanism of *P. polymyxa* using genomic comparisons or other molecular methods. To further understand the biocontrol mechanism of HY96-2 at the molecular level, its genome was completely sequenced and compared with those of other *P. polymyxa* strains. Thus far, the complete genomes of seven *P. polymyxa* strains have been published, including the strains SC2 (Ma et al., 2011), E681 (Kim et al., 2010), YC0136 (Liu et al., 2017a), M-1 (Niu et al., 2011), SQR-21 (Li et al., 2014), CR1 (Eastman et al., 2014b), and YC0573 (Liu et al., 2017b). The details of these strains are presented in **Table 1**. Compared with other *P. polymyxa* strains, SC2 and E681 have been studied in greater detail and found to inhibit the growth of many plant pathogens. SC2 was isolated from the rhizosphere of pepper plants in Guizhou Province, China, and demonstrated to inhibit plant pathogenic fungi, such as *F. oxysporum*, *B. cinerea*, *Pseudoperonospora cubensis*, and plant pathogenic bacteria, the species of which were not published (Zhu et al., 2008). E681, isolated from the rhizosphere of barley plants in South Korea, was reported to possess inhibitory activity against plant pathogenic fungi such as *F. oxysporum*, *B. cinerea,* and *R. solani* (Ryu et al., 2006) and the plant pathogenic bacterium *P. syringae* (Kwon et al., 2016).

Colonization (Bianciotto et al., 2001; Bais et al., 2004; Sang and Kim, 2014), antagonism (Lugtenberg and Kamilova, 2009; Sharma et al., 2009), and induced resistance (Ongena et al., 2007; Shi et al., 2017) are the three main reported mechanisms of biological control. Biofilm formation indicates that a biocontrol agent possesses good colonization ability (Ongena and Jacques, 2008; Sang and Kim, 2014). Besides the key genes involved in biofilm formation, quorum sensing also plays an important role (Miller and Bassler, 2001). The types and amounts of antibiotics generated by a biocontrol agent affect their antimicrobial spectra and biocontrol efficacies. It has been reported that *P. polymyxa* secretes antifungal and antibacterial metabolites, which mainly include fusaricidins, polymyxins, and other antibiotics (Jeong et al., 2011; Shaheen et al., 2011). Biocontrol agents can also generate and release systemic resistance inducers including volatile organic compounds (mainly consisting of 2,3-butanediol, methanethiol, isoprene, butyl acetate, *n*-hexadecane, etc.) into the surrounding environment, both of which have been shown to have a positive effect on plant protection (Ryu et al., 2004; Lee et al., 2012; Shi et al., 2017).

To the best of our knowledge, no genomic comparison has yet been applied to analyze the biocontrol mechanism of *P. polymyxa*. Thus, in this study the complete genome of *P. polymyxa* strain HY96-2 was sequenced and compared with those of seven other strains. To elucidate the differences in the biocontrol mechanisms between strain HY96-2 and other agriculturally undeveloped *P. polymyxa* strains, the main genes (or gene clusters) of HY96-2 involved in biofilm formation, antibiotic synthesis, and systemic resistance inducer production were analyzed in comparison with the strains SC2 and E681. This

[1] https://www.epa.gov/tsca-inventory/list-substances-reported-under-tsca-inventory-notification-active-inactive-rule

[2] http://www.icama.org.cn/hysj/index.jhtml

[3] http://www.ec.gc.ca/ese-ees/9F3909AA-3024-4BBD-AC9E-2EB681ED1BBD/FSAR_Paenibacillus%20Polymyxa_EN.pdf

TABLE 1 | Sources and functions of the studied *P. polymyxa* strains (the complete genomes of which have been published).

Name	Accession number(s)	Origin	Activity	Reference
HY96-2	CP025957	Tomato rhizosphere (Nanchang, Jiangxi, China)	As biocontrol agent against fungi (*F. oxysporum*, *C. gloeosporioides*, *B. cinerea*, *R. solani*, *Alternaria solani*, *Penicillium italicum*, *Curvularia lunata*, etc.) and Gram-negative (G^-) bacteria (*R. solanacearum*, *P. syringae*, *E. carotovora*, etc.).	Industrial microbial biopesticides that have been commercially registered in China; Fan et al., 2012
SC2	CP002213- CP002214	Pepper rhizosphere (Guizhou, China)	As biocontrol agent against fungi (*F. oxysporum*, *B. cinerea*, and *P. cubensis*) and bacteria (species not stated), and as plant-growth-promoting rhizobacterium (PGPR) to dissolve phosphorus and potassium.	Zhu et al., 2008; Ma et al., 2011
E681	CP000154	Winter barley rhizosphere (South Korea)	As biocontrol agent against fungi (*F. oxysporum*, *B. cinerea*, *B. allii, R. solani*, *Pythium* sp., *Aspergillus* sp., etc.), and bacteria (*P. syringae*, *E. coli*, etc.), and as PGPR.	Ryu et al., 2006; Kim et al., 2010; Kwon et al., 2016
YC0136	CP017967	Tobacco rhizosphere (Guizhou, China)	As biocontrol agent against fungus *Phytophthora parasitica* var. *nicotianae* and as PGPR.	Liu et al., 2017a
M-1	HE577054- HE577055	Wheat rhizosphere (China)	As biocontrol agent against fungus *R. solani* and as PGPR.	Niu et al., 2011
SQR-21	CP006872	Melon rhizosphere (China)	As biocontrol agent against fungus *F. oxysporum* and as PGPR.	Yaoyao et al., 2017
CR1	CP006941	Degraded corn straw (Southern Ontario, Canada)	As biocontrol agent to antagonize fungi (*R. solani*, *Cylindrocarpon destructans*, etc.) and bacteria (*P. syringae*, *X. campestris*, etc.), and as PGPR, as well as biodegradation agent.	Eastman et al., 2014b; Weselowski et al., 2016
YC0573	CP017968	Tobacco rhizosphere (Guizhou, China)	As biocontrol agent to antagonize fungus *P. parasitica* var. *nicotianae* and as PGPR.	Liu et al., 2017b

study provides a scientific basis for the further optimization of the field applications and product quality standards of the microbial biopesticide derived from *P. polymyxa* HY96-2.

MATERIALS AND METHODS

Strains and Genomic DNA Preparation

P. polymyxa strain HY96-2 was isolated from the rhizosphere of tomato plants in the suburbs of Nanchang, Jiangxi Province, China, and preserved in the China General Microbiological Culture Collection Center (CGMCC No. 0829). HY96-2 was cultured at 30°C in Luria–Bertani broth. Genomic DNA was purified from overnight liquid cultures ($OD_{600\ nm} \approx 0.7$) using the cetyltrimethylammonium bromide method (Watanabe et al., 2010). A TBS-380 fluorometer (Turner BioSystems, United States) or NanoDrop 2500 (Thermo Scientific, United States) was applied to ensure the DNA quality ($\geq$10 μg, without degradation, $OD_{260}/OD_{280} \approx 1.8–2.0$).

Sequencing and Assembly

The whole genome was sequenced using the PacBio RS II platform with a 10-kb library. Reads were assembled using HGAP (version 2.3.0, SMRT Analysis) (Chin et al., 2013). The assembly data for the complete genome have been deposited in GenBank with the accession number CP025957.

Genome Components and Genome Annotation

Coding DNA sequence (CDS) prediction was performed using Glimmer 3.02 (Delcher et al., 1999). A circular map of the genome was obtained using Circos version 0.64 (Krzywinski et al., 2009). Genomic islands (GIs) were predicted using the GI prediction method IslandViewer 4 (Dhillon et al., 2015). tRNA and rRNA were predicted using the tRNAscan-SEv1.3.1 (Lowe and Eddy, 1997) and barrnap 0.7 software[4], respectively. Clustered regularly interspaced short palindromic repeat sequences (CRISPRs) were found using CRISPRFinder (Grissa et al., 2007). Functional annotation was based on BLASTP searches (BLAST 2.2.28+) against the NCBI non-redundant (NR) database, gene database, string database, and gene ontology (GO) database. Based on the string database, the BLASTP comparison was used to perform the Clusters of Orthologous Groups of proteins (COG) annotation, according to which the protein function could be classified (Tatusov et al., 2001). The BLAST algorithm was used to compare the predicted genes with the KEGG database, and the corresponding genes involved in specific biological pathways could be obtained according to the KEGG Orthology (KO) numbers obtained from the alignment. GO was annotated with blast2go (Conesa et al., 2005).

Genome Comparison

The complete genome sequences of seven *P. polymyxa* strains (SC2, E681, YC0136, M-1, SQR-21, CR1, and YC0573) and the related species strain *B. subtilis* 168 (AL009126) and *B. amyloliquefaciens* FZB42 (CP00560) examined in this study were obtained from GenBank. Phylogenetic analysis was conducted for *P. polymyxa* strains, *B. subtilis*, and *B. amyloliquefaciens* inferred by analyzing homologous gene. The single-copy homologous genes of each strain were selected for

[4]http://www.vicbioinformatics.com/software.barrnap.shtml

multiple sequence alignment and quality control comparison. Multiple sequence alignment was conducted by MAFFT software[5]. Quality control comparison was conducted by Gblocks software[6]. Then the phylogenetic tree was constructed by RAxML software with maximum likelihood method based on single gene or multiple genes (Stamatakis, 2014). Pan-genome analysis and nucleic acid co-linearity were conducted for the seven *P. polymyxa* strains and HY96-2. The pan-genome analysis was performed using the OrthoMCL software (Chen et al., 2006). The nucleic acid co-linearity was determined using the MUMmer 3.0 software (Kurtz et al., 2004).

The genes or gene clusters involved in biofilm formation, antibiotic synthesis, and systemic resistance inducer production were compared between strain HY96-2 and strains SC2 and E681. According to the biofilm formation pathways retrieved from the KEGG database[7], the possible pathways for biofilm formation in *P. polymyxa* were analyzed. In this study, the genes analyzed for biofilm formation in *P. polymyxa* were selected according to Molinatto et al. (2017). The gene clusters for secondary metabolites (containing antibiotics) in *P. polymyxa* were annotated using the antiSMASH database version 4.0.2 and the other antibiotics were selected based on previous studies (Walsh, 2004; Ma et al., 2011). The resistance inducers of *P. polymyxa* in this study were selected according to Lee et al. (2012) and Shi et al. (2017). The key genes involved in resistance inducer synthesis were searched for in the KEGG database. BLAST was used to compare the identities in the genes or gene clusters between HY96-2, SC2, and E681.

RESULTS

Genome Features

The complete genome of *P. polymyxa* strain HY96-2 was 5.75 Mb (**Figure 1**), in which the average GC content of the chromosome was 45.61%. A total of 5207 CDSs were predicted. Furthermore, the HY96-2 genome contained 42 rRNA and 110 tRNA genes. The general features are shown in **Table 2**. However, no plasmids were found using Webcutter version 2.0 or PlasmidFinder version 1.3.

Genomic Islands and CRISPR Prediction

Genomic islands often carry genes important for genome evolution and adaptation to surrounding environment such as those involved in pathogenesis and antibiotic resistance. Therefore, GI prediction has gradually become an increasingly important aspect of microbial genome analysis (Lu and Leong, 2016). The GIs predicted in HY96-2 are listed in Supplementary Table S1; a total of 16 GIs were predicted in the chromosome. The CRISPRs containing multiple short and repeated sequences can confer resistance to exogenous genetic elements such as phages and plasmids (Didovyk et al., 2016; Ran, 2017). A putative CRISPR was detected in the HY96-2 genome by CRISPRFinder (Supplementary Table S2).

Genome Annotation

According to the results of the COG annotation, 2605 proteins were classified into 20 COG families (Supplementary Table S3). The largest group of genes was involved in carbohydrate transport and metabolism (328 genes, 6.30%) (**Figure 2**). In total, KEGG orthologs were found for 2352 proteins by BLAST. In addition, the GO annotation results revealed that the largest group of genes was involved in the biological process domain. Among these genes, the content of genes related to the metabolic process category was highest (Supplementary Figure S1).

Genome Comparison

The phylogenetic tree indicated that HY96-2 is closest to *P. polymyxa* SQR-21 (**Figure 3**). However, *P. polymyxa* strains M-1 and SC2 were found to exhibit low homologies with strain HY96-2. We compared the 16S rRNA sequence of each *P. polymyxa* strain to calculate the percentage of homology (Supplementary Table S4). The percentage of homology between strain HY96-2 to strains M-1 and SC2 were 97.87 and 97.82%. We also performed a pan-genome analysis to compare HY96-2 and the seven other *P. polymyxa* strains, namely, SC2, E681, YC0136, M-1, SQR-21, CR1, and YC0573. As shown in **Figure 4**, 3382 gene families were found to be involved in the core genome shared by all of the studied strains. In addition, the number of gene families unique to strain HY96-2 was 448, corresponding to 468 genes, which was the highest among all of the analyzed strains (Supplementary Table S5). The annotation revealed that these specific genes encoded a large number of transcriptional regulators, helicase domain proteins, hypothetical proteins, aminotransferases, transposases, drug resistance transporters, and chloramphenicol resistance proteins, etc. The nucleic acid co-linearity results showed that strain HY96-2 has high co-linearity with the seven other *P. polymyxa* strains (**Figure 5**).

Comparison of Genes Involved in Biofilm Formation

Based on the studies of Yang et al. (2014) and Molinatto et al. (2017), we searched the genomes of strains HY96-2, SC2, and E681 for genes related to biofilm formation and compared their similarities using BLAST. These genes are as follows: the key quorum-sensing gene *luxS*, which affects biofilm formation (Yang et al., 2014); the flagellar motility-related genes, *motA*, *motB*, and *flgM* (Domka et al., 2007; Hu et al., 2011); and the *Bacillus*-based biofilm formation pathway genes, *kinB*, *spo0A*, *spo0F*, *degU*, and *degS*, etc. (Grossman et al., 1992). The results showed that 14 genes involved in biofilm formation were certainly found in the genomes of these three strains. The identities of these genes between HY96-2 and SC2 exceeded 93%, which were higher than those between HY96-2 and E681. In particular, the identities of the genes *motA*, *sfp*, and *kinB* between strain HY96-2 and E681 were below 90% (**Table 3**).

[5]http://mafft.cbrc.jp/alignment/software/

[6]http://molevol.cmima.csic.es/castresana/Gblocks.html

[7]http://www.genome.jp/kegg/pathway.html

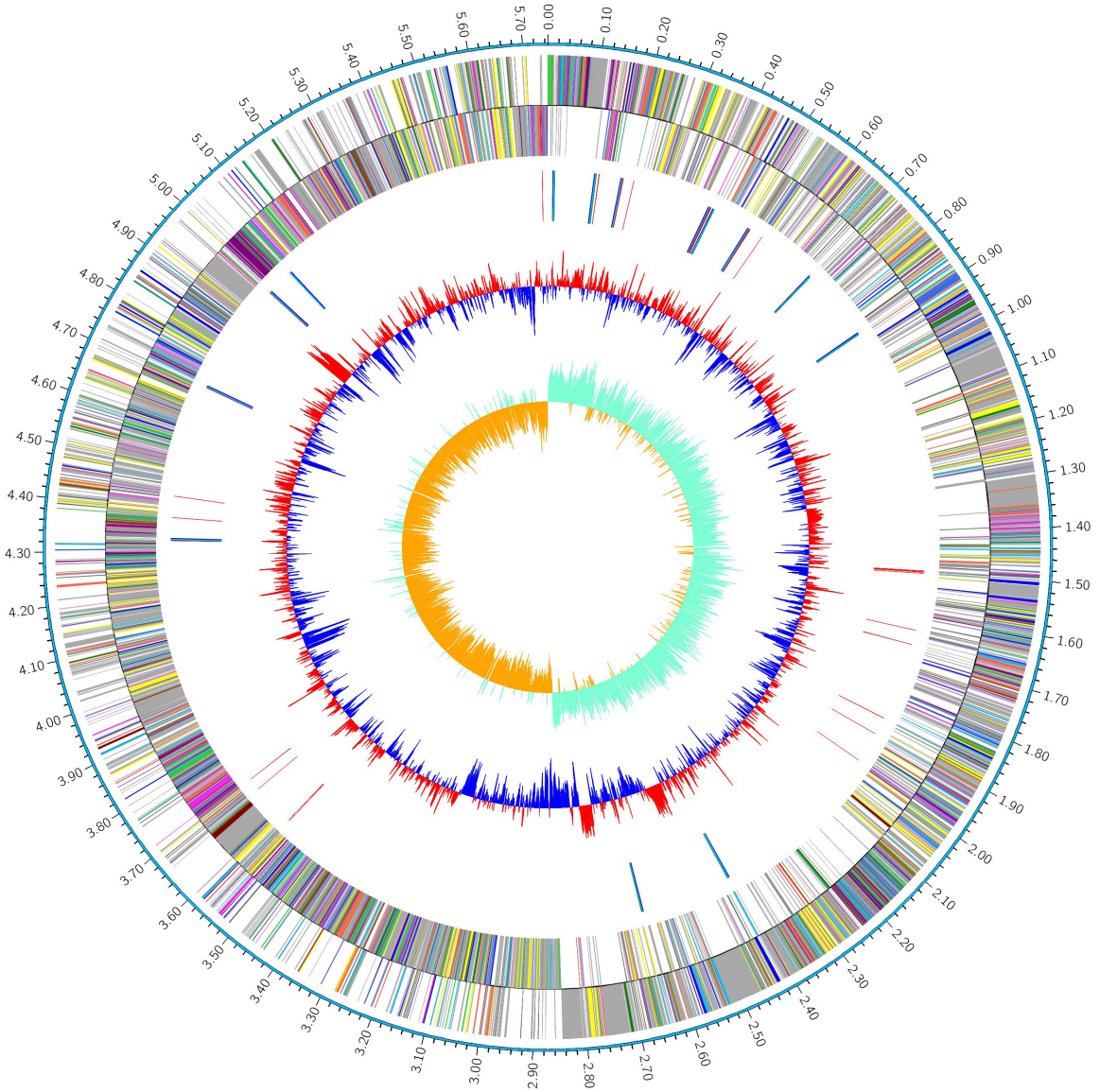


FIGURE 1 | Circular map of the genome of the *P. polymyxa* strain HY96-2. The distribution of the circle from the outside to the inside indicates the genome size, forward CDS, reverse CDS, repeat sequences, tRNA (blue), rRNA (purple), GC ratio (red and blue indicate regions where the GC ratio is higher than average and lower than average, respectively), and GC skew (cyan and orange indicate regions where the G content is greater than and less than the C content, respectively).

To date, no studies have been reported on the biofilm formation pathway in *P. polymyxa*. We explored the possible pathways responsible for biofilm formation in *P. polymyxa* by combining genomes and the information from KEGG database. An important biofilm formation pathway in *Bacillus* is as follows: (1) the sensor histidine kinase KinB (or KinA, KinC, or KinD) phosphorylates its own conserved histidine residue in response to an environmental stimulus; (2) the phosphate group is transferred from the activated KinB to Spo0F; (3) because Spo0F lacks an effector domain, the phosphate group of Spo0F-P is then transferred to the intermediary molecule Spo0B; (4) finally, the receptor domain of the effector Spo0A is phosphorylated by the phosphate group of Spo0B-P and subsequently triggers biofilm formation (Fabert et al., 1999; Jiang et al., 2000). However, no *spo0B* gene was detected in the genomes of the *P. polymyxa* strains. By querying the KEGG database, another pathway involved in sporulation in *B. subtilis* was found, which revealed that Spo0F-P can directly transfer the phosphate group to the effector Spo0A without Spo0B acting as a mediator, and when a high concentration of Spo0A-P accumulates it will trigger the sporulation. Therefore, we propose that a biofilm formation pathway exists in *P. polymyxa* in which KinB autophosphorylates in response to an environmental stimulus and then subsequently phosphorylates the Spo0F response regulator, whereupon the phosphate group is directly transferred to Spo0A to trigger biofilm formation (**Figure 6A**). In addition, *degS* and *degU* genes

TABLE 2 | General features of the genome of the *P. polymyxa* strain HY96-2.

Attribute	Value
Genome size (bp)	5745779
G+C ratio (%)	45.61
Protein-coding genes	5207
Gene total length (bp)	4958220
rRNA	42
tRNA	110
Genes assigned to COGs	2605
Genomic islands	16
CRISPR	1

were detected in the genome of the three *P. polymyxa* strains. It is therefore presumed that another possible biofilm formation pathway in *P. polymyxa* involves activation of the sensor histidine kinase DegS by the external stimulus to phosphorylate the response regulation protein DegU, thereby triggering biofilm formation (**Figure 6B**). This biofilm formation pathway has already been reported in *B. subtilis* (Stanley and Lazazzera, 2005).

Comparison of Genes/Gene Clusters Involved in Antibiotic Synthesis

The annotation of gene clusters related to secondary metabolite synthesis was performed using the antiSMASH database. For strain HY96-2, 15 gene clusters related to secondary metabolites were retrieved, accounting for 15.77% of the genome; the corresponding values for strains SC2 and E681 were 12 (11.89%) and 11 (9.85%), respectively. Among these three strains, HY96-2 exhibited a greater variety and higher content of secondary metabolites in the genome. The comparison of genes/gene clusters involved in antibiotic synthesis showed that the identities between strains HY96-2 and SC2 were significantly higher than those between strains HY96-2 and E681 (**Table 4**).

Two gene clusters involved in fungicide synthesis (fusaricidins and paenilarvins) as well as six genes or gene clusters involved in bactericide synthesis (polymyxin, tridecaptin, mersacidin, kalimantacin, polyketide, and bacitracin) were found in the genomes of strains HY96-2 and SC2. However, no gene clusters for the biosynthesis of paenilarvins, tridecaptin, or kalimantacin were detected in the E681 genome. For the main antimicrobial agents in *P. polymyxa*, fusaricidins and polymyxin, the comparison revealed that the synthetic gene clusters in strains SC2 and E681 did not exhibit very high similarities with those in strain HY96-2, but the similarities of the corresponding synthetic gene clusters between SC2 and HY96-2 were still higher than those between E681 and HY96-2. The identity of the fusaricidin synthetic gene clusters between SC2 and HY96-2 was 81.54%, whereas that between E681 and HY96-2 was only 61.53%. Similarly, the identity of the polymyxin synthetic gene clusters between SC2 and HY96-2 was 63.21%, whereas that between E681 and HY96-2 was only 54.94%. These variations in the genes and gene clusters involved in antibiotic synthesis between the three strains may explain the differences in their target profiles and efficiency against plant diseases.

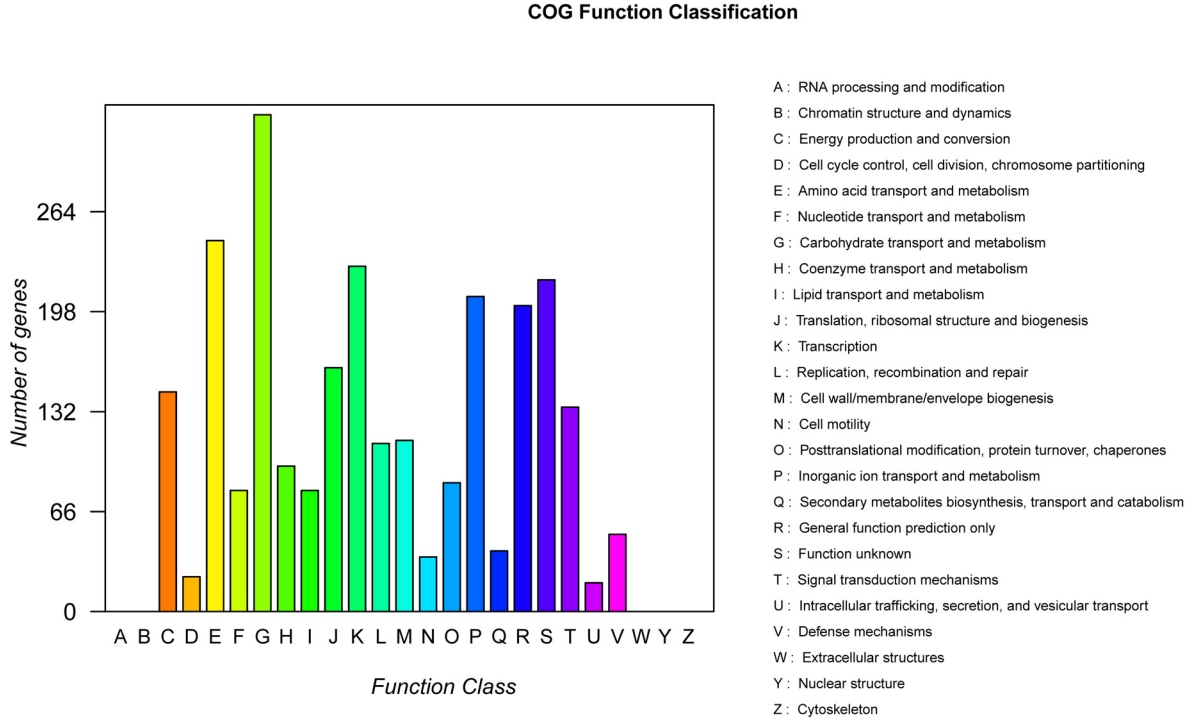


FIGURE 2 | Distribution of genes across COG functional categories in the chromosome of *P. polymyxa* strain HY96-2.

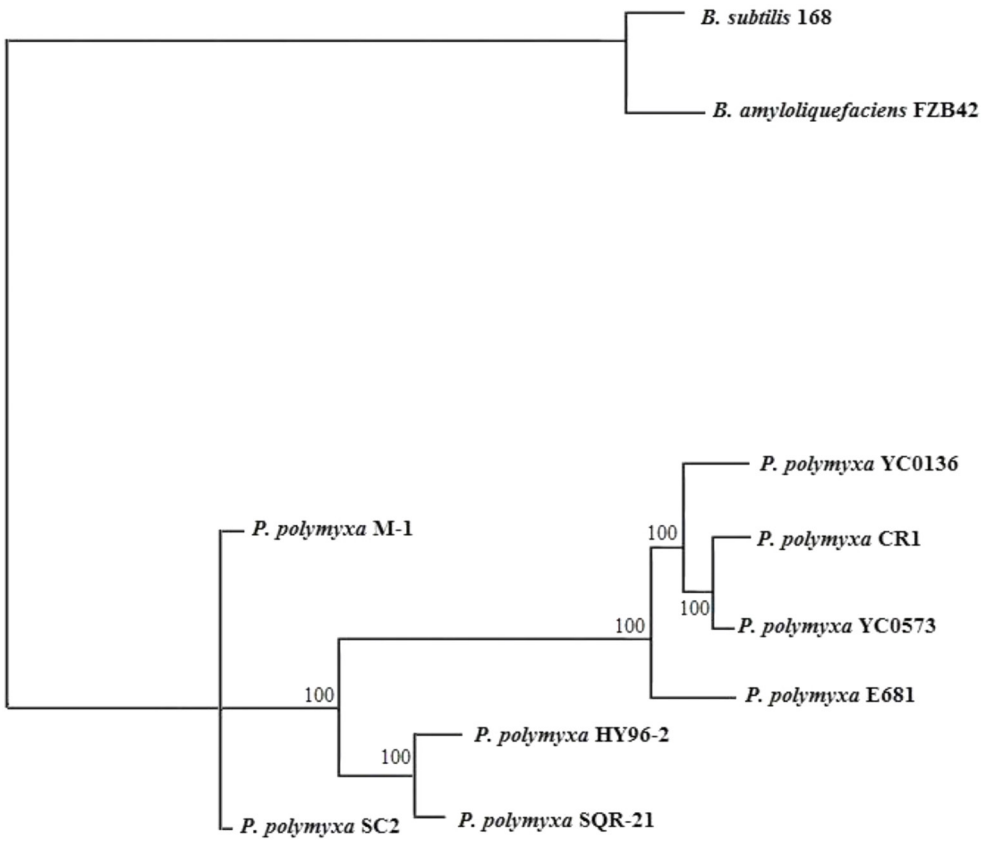


FIGURE 3 | Phylogenetic tree for *P. polymyxa* HY96-2 and the seven other *P. polymyxa* strains, *B. subtilis* 168, and *B. amyloliquefaciens* FZB42 based on homologous genes.

Comparison of Genes Involved in Resistance Inducer Synthesis

Key genes involved in the synthesis of resistance inducers in *P. polymyxa* were retrieved from the KEGG database, and the identities of these genes between HY96-2 and SC2 and E681 were also compared. The results revealed that the key genes for 2,3-butanediol, methanethiol, and isoprene were all found in the genomes of HY96-2, SC2, and E681, with sequence identities exceeding 86%. The identities of the corresponding genes between HY96-2 and SC2 (91.33–97.05%) were significantly higher than those between HY96-2 and E681 (86.83–92.05%) (**Table 5**). This indicates that the three strains can be expected to possess similar abilities to induce plant resistance, although their induction efficiencies may differ owing to the variation in the corresponding genes.

DISCUSSION

Microbial biopesticides have attracted increasing attention over recent years owing to their effectiveness, environmental friendliness, and safety toward humans and livestock (Berg, 2009). The development of microbial biopesticides in China is also proceeding rapidly and the number of registered and commercially available ones is growing sharply, in line with the Chinese government's policy of "two reductions" (i.e., reducing the amounts of chemical pesticides and fertilizers used). As an important biocontrol agent, *P. polymyxa* is a relatively novel species and the number of registered products based on this species is growing rapidly[8]. Microbial biopesticides based on *P. polymyxa* have also been produced industrially. However, only a few comprehensive studies of the biocontrol mechanism of *P. polymyxa* at the molecular level have been published to date (Eastman et al., 2014a; Xie et al., 2016). The previous reports concerning the complete genome of *P. polymyxa* predominantly focused on the general features of the genome and analysis of the effect of this species on promoting growth, but rarely involved the analysis of the biocontrol function and mechanism (Kim et al., 2010; Ma et al., 2011; Niu et al., 2011; Eastman et al., 2014b; Li et al., 2014; Liu et al., 2017a,b). In this study we have presented the complete genome of the industrial *P. polymyxa* strain HY96-2, which consists of a 5.75 Mb chromosome. The genome of HY96-2 was compared with those of seven other *P. polymyxa* strains (SC2, E681, YC0136, M-1, SQR-21, CR1, and YC0573). In particular, the biocontrol-related genes and gene clusters involved in the formation of biofilms, antibiotics, and

[8]http://www.icama.org.cn/fwb/index.jhtml

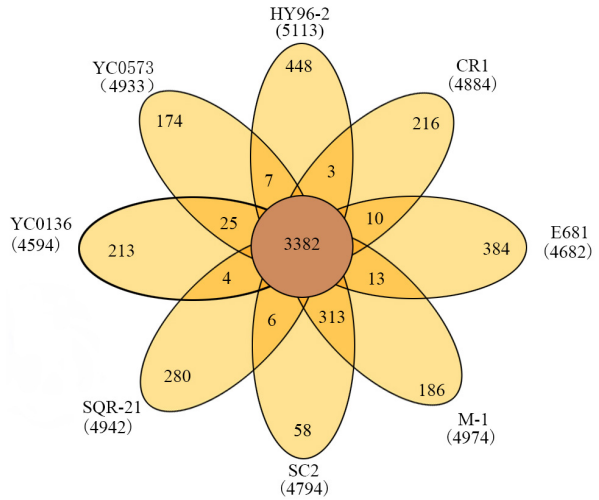


FIGURE 4 | Venn diagram showing numbers of specific and shared gene families among the eight different *P. polymyxa* strains.

systemic resistance inducers were compared between HY96-2 and the SC2 and E681 strains. The results of this comparison revealed that the genome of strain HY96-2 exhibits some degree of variation. And this finding possibly explained the relationship between genes relating to the biocontrol mechanism and the biocontrol target and efficacy.

According to the phylogenic tree, the homology between strains HY96-2 and E681 is greater than that between strains HY96-2 and SC2. However, comparison of the genes involved in biocontrol mechanism and 16s rRNA (Supplementary Table S4) suggested that strain HY96-2 is more similar to SC2 than E681. SC2 contains a large 0.51 Mb plasmid, whereas HY96-2 and E681 do not. This could be the main reason why the phylogenetic tree suggested that strain HY96-2 is closer to E681 than SC2. Strain SC2 was isolated from rhizosphere of pepper in Guizhou Province, China (latitude 24°37′–29°13′N), and strain HY96-2 was obtained from rhizosphere of tomato in Nanchang, Jiangxi Province, China (latitude 28°16′–28°58′N). Strain E681 was isolated from rhizosphere of winter barley in South Korea (latitude 33°–43°N). The biggest difference between these three strains were the regions and hosts where they were isolated. Strains HY96-2 and SC2 were all isolated from Solanaceae plants, and their geographic locations were the nearest, and they demonstrated the highest homology by comparing the genes related to biocontrol mechanism and 16s rRNA within the three strains. Thus, we speculated the host environment might be one of the reasons of the variation of strains HY96-2, SC2, and E681. Pan-genome analysis revealed that the number of specific gene families in strain HY96-2 was 448, corresponding to 468 genes, which was the highest among the eight *P. polymyxa* strains studied. These unique genes of strain HY96-2 were found to encode transcriptional regulators, helicase domain proteins, aminotransferases, transposases, etc. These functional genes might affect biofilm formation and the production of antibiotics and systemic resistance inducers. Thus, the variation and specialization of these genes could possibly result in the differences in biocontrol targets and efficacy between strain HY96-2 and the other *P. polymyxa* strains.

Biofilm formation is an important trait that has been linked to the colonization ability of biocontrol microorganisms (Ongena and Jacques, 2008). Comparison of the main genes involved in biofilm formation revealed that all 14 of the target genes could be found in strains HY96-2, SC2, and E681, and the similarities of these genes between strains SC2 and HY96-2 were much higher than those between strains HY96-2 and E681. In addition, quorum sensing has been reported to significantly affect biofilm formation in bacteria (Miller and Bassler, 2001). For *B. subtilis*, which can be considered a representative G^+ biocontrol bacterium, it has been reported that quorum sensing is mainly regulated by the *comA*, *comP*, *comX*, and *comQ* genes (Schneider et al., 2002). These *B. subtilis* quorum-sensing-related genes were deficient in the eight *P. polymyxa* strains studied, although another important quorum-sensing regulatory gene, *luxS*, was observed. *luxS* is a key regulatory gene in quorum sensing mediated by autoinducer 2 (AI-2) (Federle, 2009; Ma et al., 2017), which significantly affects biofilm formation in *P. polymyxa* (this conclusion was obtained in other study by our group, which has not yet been published). In addition, *luxS* was also found to be a key regulatory gene involved in quorum sensing in *P. polymyxa* CR1 (Eastman et al., 2014a). From the above results, it is possible to speculate that all three strains, HY96-2, SC2, and E681, could prevent disease by forming biofilms, but the biofilm formation ability of the three strains might differ owing to the variations in the genes involved in biofilm formation observed in their genomes.

In *B. subtilis*, Spo0A is a key transcriptional regulatory protein that controls the expression of over 100 genes, including those involved in biofilm formation and sporulation. DegU is also a global regulator in *B. subtilis* and controls multiple cellular processes such as competence, motility, and hydrolase secretion, without which biofilm formation would be unsuccessful (Kobayashi, 2007). As confirmed using the KEGG database, the genes related to the biofilm formation pathways regulated by these two key genes were found in all of the three tested *P. polymyxa* strains. Therefore, a possible pathway for biofilm formation in *P. polymyxa* can be preliminarily proposed as follows: in response to an environmental stimulus, KinB phosphorylates the Spo0F response regulator, which subsequently transfers the phosphate group directly to Spo0A to trigger biofilm formation. The main differences in the pathways mentioned above between *P. polymyxa* and *B. subtilis* are that the mediator Spo0B, which transfers the phosphate group between Spo0F and Spo0A, is present in *B. subtilis* but absent from *P. polymyxa*, and that the phosphate group can be directly transferred from Spo0F to Spo0A in *P. polymyxa*. Moreover, another possible pathway for biofilm formation in *P. polymyxa* was deduced, wherein the histidine kinase sensor DegS phosphorylates the response regulator protein DegU to form the biofilm in response to an external stimulus. To date, there have been no reports of the biofilm formation pathway in *P. polymyxa*. Thus, these two possible pathways for biofilm formation in *P. polymyxa* were first deduced and revealed in this

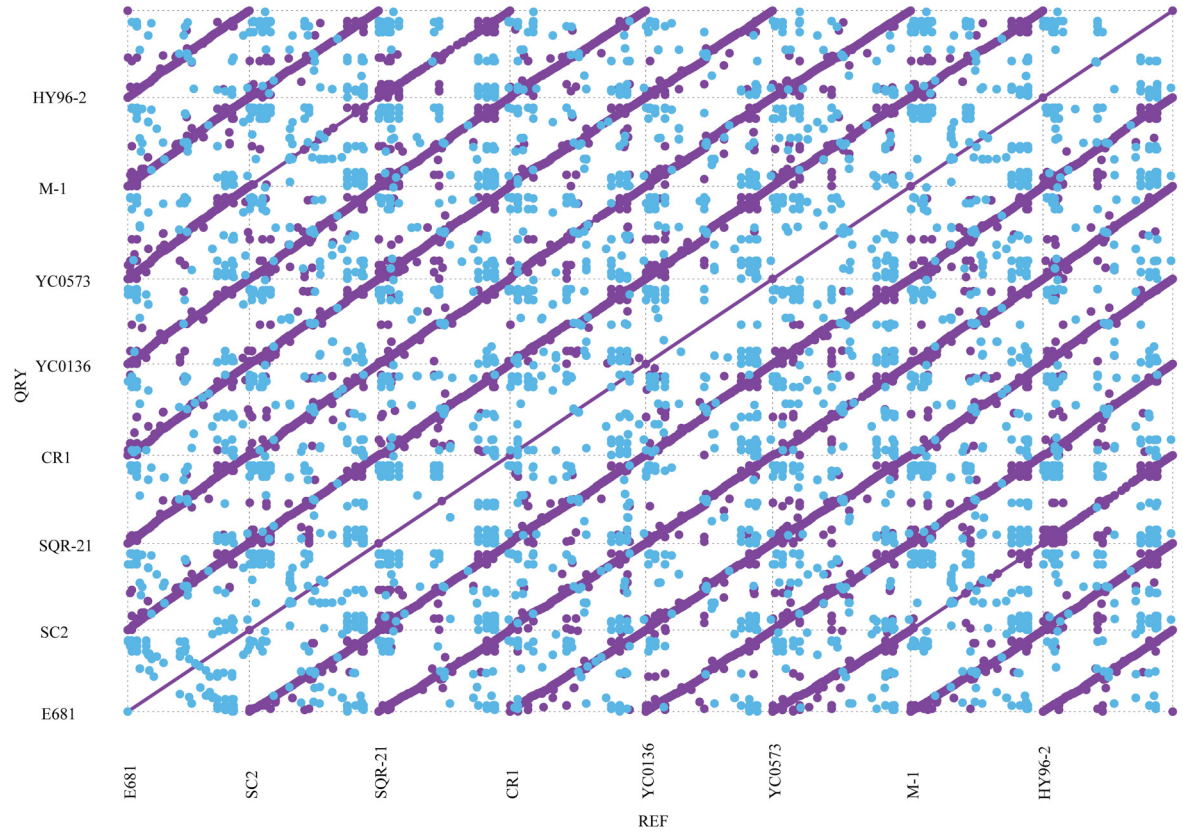


FIGURE 5 | Co-linearity results for strain HY96-2 and the seven other *P. polymyxa* strains. The purple dots represent the forward alignment of the two genome sequences, and the blue dots represent the reverse alignment of the corresponding segments.

TABLE 3 | Comparison of core genes involved in biofilm formation in strain HY96-2, as well as in strains SC2 and E681 (sequence similarity is expressed as a percentage of the nucleotide identity).

Gene name	Locus tag			Product	Identity (%)	
	HY96-2	SC2	E681		SC2	E681
luxS	C1A50_RS02845	PPSC2_RS31750	PPE_RS02675	*S*-ribosylhomocysteine lyase	97.19	91.99
Efp	C1A50_RS14785	PPSC2_RS43880	PPE_RS13870	Elongation factor P	98.57	93.19
degU	C1A50_RS22485	PPSC2_RS51810	PPE_RS21415	DNA-binding response regulator	95.99	93.64
degS	C1A50_RS22490	PPSC2_RS51815	PPE_RS21420	Histidine kinase	98.71	96.30
flgM	C1A50_RS22450	PPSC2_RS51780	PPE_RS21385	Flagellar biosynthesis anti-sigma factor FlgM	97.10	94.57
motA	C1A50_RS00510	PPSC2_RS29245	PPE_RS00510	Flagellar motor protein MotA	98.24	88.18
motB	C1A50_RS00505	PPSC2_RS29240	PPE_RS00505	Flagellar motor protein MotB	97.22	91.18
sacB	C1A50_RS03600	PPSC2_RS32495	PPE_RS03320	Levansucrase	95.00	93.60
sfp	C1A50_RS07585	PPSC2_RS36260	PPE_RS07145	4′-phosphopantetheinyl transferase	93.02	86.32
sigD	C1A50_RS09600	PPSC2_RS38730	PPE_RS09310	RNA polymerase sigma factor SigD	96.58	94.04
spo0A	C1A50_RS14655	PPSC2_RS43750	PPE_RS13740	Sporulation transcription factor spo0A	97.39	93.66
spo0F	C1A50_RS00680	PPSC2_RS29415	PPE_RS00675	Sporulation initiation phosphotransferase F	99.46	95.39
kinB	C1A50_RS19570	PPSC2_RS48955	PPE_RS18645	Two-component sensor histidine kinase	96.95	89.75
hfq	C1A50_RS13605	PPSC2_RS42680	PPE_RS12655	RNA chaperone Hfq	97.53	93.09

paper and found to possess high similarity with those reported in *B. subtilis*.

Comparison of the genes and gene clusters involved in antibiotic synthesis revealed more significant differences between the three *P. polymyxa* strains than those involved in biofilm formation. Strains HY96-2 and SC2 exhibited relatively high homology, whereas strains HY96-2 and E681 had relatively low homology in terms of the genes and gene clusters involved in

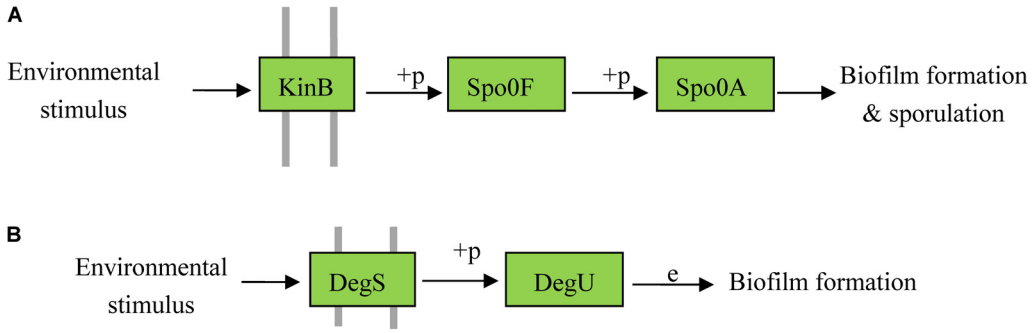


FIGURE 6 | Possible pathways of biofilm formation in *P. polymyxa*. **(A)** Pathway A. **(B)** Pathway B.

TABLE 4 | Comparison of gene clusters and core genes involved in antibiotic biosynthesis in strain HY96-2, as well as in strains SC2 and E681.

Antibiotic name	Activity	Locus tag			Type	Identity (%)	
		HY96-2	SC2	E681		SC2	E681
Fusaricidin	Broad-spectrum antifungal activity, especially against *Fusarium* sp.; also suppresses G^+ bacteria (Kajimura and Kaneda, 1995, 1996; Liu et al., 2011)	C1A50_RS00290-C1A50_RS00500	PPSC2_RS29015-PPSC2_RS29230	PPE_RS00290-PPE_RS00500	NRPS	81.54	61.53
Paenilarvins	Broad-spectrum antifungal activity (Sood et al., 2014)	C1A50_RS04650-C1A50_RS04845	PPSC2_RS33500-PPSC2_RS33695	–	TransATPKS–NRPS	81.88	–
Polymyxin	Broad-spectrum antibacterial activity, especially against G^- bacteria (Falagas and Kasiakou, 2005; Gounani et al., 2018)	C1A50_RS21330-C1A50_RS21525	PPSC2_RS50695-PPSC2_RS50860	PPE_RS20180-PPE_RS20350	NRPS	63.21	54.94
Polyketide	Suppresses G^- bacteria (Chakraborty et al., 2018)	C1A50_RS00805	PPSC2_RS29535	PPE_RS00800	PKS	94.93	90.29
Tridecaptin	Suppresses G^- bacteria (Cochrane et al., 2015)	C1A50_RS10840-C1A50_RS11035	PPSC2_RS40010-PPSC2_RS40205	–	NRPS	31.82	–
Bacitracin	Suppresses G^+ bacteria (Konzl et al., 1997)	C1A50_RS24385	PPSC2_RS53660	PPE_RS23255	NRPS	94.38	90.62
Kalimantacin	Suppresses G^+ bacteria (Thistlethwaite et al., 2017)	C1A50_RS15500-C1A50_RS15755	PPSC2_RS44500-PPSC2_RS44755	–	TransATPKS–Otherks-NRPS	68.75	–
Mersacidin	Suppresses G^+ bacteria (Sahl and Bierbaum, 1998)	C1A50_RS07480-C1A50_RS07590	PPSC2_RS36150-PPSC2_RS36265	PPE_RS06970-PPE_RS07075	Lantipeptide	83.54	72.92

TABLE 5 | Comparison of genes involved in the synthesis of resistance inducers in strain HY96-2, as well as in strains SC2 and E681.

Resistance inducer	Gene	Locus tag			Identity (%)	
		HY96-2	SC2	E681	SC2	E681
2,3-Butanediol	*alsD*	C1A50_RS10210	PPSC2_RS39445	PPE_RS09950	95.18	90.76
	ilvN	C1A50_RS07135	PPSC2_RS35800	PPE_RS06620	95.47	91.98
Methanethiol	*mtnE*	C1A50_RS13950	PPSC2_RS43025	PPE_RS13035	93.23	89.85
	metH	C1A50_RS13320	PPSC2_RS42420	PPE_RS12455	95.47	89.74
	metE	C1A50_RS23245	PPSC2_RS52550	PPE_RS22160	94.52	88.18
	mmuM	C1A50_RS24195	PPSC2_RS53470	PPE_RS23100	94.30	87.13
Isoprene	*idi*	C1A50_RS22765	PPSC2_RS52085	PPE_RS21690	92.55	86.83
	lytB	C1A50_RS07370	PPSC2_RS36040	PPE_RS06865	91.33	86.83
	gcpE	C1A50_RS18800	PPSC2_RS48225	PPE_RS17845	97.05	91.24
	ispF	C1A50_RS21940	PPSC2_RS51265	PPE_RS20840	94.76	89.10
	ispE	C1A50_RS00170	PPSC2_RS28895	PPE_RS00170	96.14	92.05

antibiotic production. The variations in these genes and gene clusters between the three strains might be responsible for the differences in the biocontrol targets and efficacies.

For the control of fungi, as the main antifungal metabolites in *P. polymyxa*, gene clusters for fusaricidins were found in all three of the tested *P. polymyxa* strains. To date, at least ten members of the fusaricidin family have been isolated from *P. polymyxa*, including fusaricidins A–D, LI-F03, LI-F04, LI-F05, LI-F06, LI-F07, and LI-F08 (Liu et al., 2011). Fusaricidins display excellent antifungal activities against many plant pathogenic fungi, especially *F. oxysporum*; fusaricidin B is particularly effective against *Candida albicans* and *Saccharomyces cerevisiae*. Fusaricidins also exhibit excellent germicidal activity against G^+ bacteria such as *Staphylococcus aureus* (Kajimura and Kaneda, 1995, 1996; Liu et al., 2011). The gene cluster for fusaricidin synthesis in strain HY96-2 showed 81.54% identity with that in strain SC2, which was higher than that for strain E681 (61.53% identity). Fusaricidin A was the main fusaricidin found in strain HY96-2 (Liu et al., 2011). Choi et al. (2008) discovered that the *fusA* gene in E681 plays an important role in fusaricidin biosynthesis, and the inactivation of this gene led to the complete loss of antifungal activity against *F. oxysporum*; moreover, *fusA* can produce more than one kind of fusaricidin. Though Mikkola et al. (2017) mention that fusaricidin A and B possess toxicity to mammalian cells at a certain concentration, the results of animal and environmental toxicology tests of the products with living cells of *P. polymyxa* HY96-2 showed that the animal and environmental toxicities of the products were slight and low, respectively, at the lowest level of toxicity in the corresponding test (Supplementary Figures S2, S3). Thus, we considered that as long as fusaricidins were not purified and developed as pesticide directly, microbial pesticides with living cells of *P. polymyxa* are environmentally friendly and safe toward animal. In addition, the gene clusters for paenilarvins, a class of iturin-like compounds with broad-spectrum antifungal activity, were also found in strains HY96-2 and SC2 but not in E681. The identity of the paenilarvin biosynthetic gene clusters between HY96-2 and SC2 was 81.88%. All three strains were reported to suppress the plant pathogenic fungus *F. oxysporum* and *B. cinerea*. Besides *F. oxysporum* and *B. cinerea*, the reported control targets of the three strains were different (**Table 1**). Strains HY96-2 and E681 were also found to suppress *R. solani*. The different biocontrol targets of these three strains may be attributable to the variation in their gene clusters responsible for the synthesis of antifungal metabolites.

For the control of bacteria, many differences were also observed between the three strains in terms of the genes or gene clusters involved in the synthesis of antibacterial metabolites. Using the antiSMASH database, six genes or gene clusters related to antibiotic synthesis were found in HY96-2 and SC2, including those involved in the biosynthesis of polymyxin, tridecaptin, polyketide, mersacidin, kalimantacin, and bacitracin. In contrast, only four were detected in strain E681, as the genes or gene clusters for tridecaptin and kalimantacin synthesis were absent. Among the six antibiotics, polymyxin, tridecaptin, and polyketide are able to suppress G^- bacteria (Cochrane et al., 2015; Chakraborty et al., 2018; Gounani et al., 2018), whereas mersacidin, kalimantacin, and bacitracin show activity against G^+ bacteria (Konzl et al., 1997; Sahl and Bierbaum, 1998; Thistlethwaite et al., 2017). The identities of the genes or gene clusters related to the six antibiotics were clearly higher between strains HY96-2 and SC2 than between strains HY96-2 and E681. All three of the *P. polymyxa* strains were found to contain polymyxin-related gene clusters. Polymyxin is another key antibiotic in *P. polymyxa* and possesses broad-spectrum antibacterial activity, especially against G^- bacteria (Falagas and Kasiakou, 2005). However, the polymyxin-related gene clusters in strains SC2 and E681 displayed low identities with that in strain HY96-2 of only 63.21 and 54.94%, respectively. Bacterial plant diseases are mainly caused by G^- bacteria, such as those belonging to the genera *Ralstonia* (Liu et al., 2017c), *Erwinia* (Bell et al., 2004), *Pseudomonas* (Wang et al., 2018), and *Xanthomonas* (Islam et al., 2018). Strain HY96-2 has been found to effectively control many G^- bacteria plant diseases in the field (**Table 1**), such as bacterial wilt, a soil-borne disease caused by *R. solanacearum* that has been referred to as "plant cancer," and angular leaf spot on cucumbers, a leaf disease caused by *P. syringae*. In the field, the biocontrol efficacy of 10^9 CFU/g *P. polymyxa* wettable powder (PPWP, developed with strain HY96-2) on tomato bacterial wilt was found to reach 91.03% with a dosage of 10.8 kg/hectare. Moreover, the biocontrol efficacy of PPWP against angular leaf spot on cucumbers reached 81.08% with 300-fold dilution, which was significantly higher than that obtained for a chemical pesticide (Supplementary Table S6). In contrast to strain HY96-2, neither strain E681 nor strain SC2 has found application as a bactericide. Strain E681 was only reported to inhibit the growth of the G^- plant pathogenic bacterium *P. syringae* and the G^- human pathogenic bacterium *Escherichia coli* (**Table 1**), while the detailed and definitive antibacterial spectrum of strain SC2 has not been reported. It can be deduced that the variations of the genes and gene clusters involved in antibacterial metabolite synthesis between these three strains might be responsible for the differences in their antibacterial spectra and control efficacy.

Comparison of the key genes involved in systemic resistance inducer production revealed that all three of the tested strains contained the key genes related to volatile organic compounds (2,3-butanediol, methanethiol, and isoprene). The genes responsible for these inducers in strains SC2 and HY96-2 exhibited 91.33–97.05% identity, which is higher than the 86.83–92.05% identity observed between strains E681 and HY96-2. It can be deduced that the three strains can induce similar systemic resistance in plants but with varying effectiveness owing to the variations in the related genes.

In summary, in this study we determined the complete genome sequence of *P. polymyxa* strain HY96-2 and performed a comparative genomic analysis between various *P. polymyxa* strains. Based on this comparison, especially in terms of biofilm formation, antibiotic synthesis, and systemic resistance inducer production, the biocontrol mechanisms of *P. polymyxa* strain HY96-2 were determined at the molecular level. These mechanisms can be deduced as follows: (1) biofilm formation to prevent infection of the plant; (2) synthesis of fusaricidins and paenilarvins to protect against fungal plant pathogens,

and the secretion of polymyxin, tridecaptin, and polyketide antibiotics with activity against G^- bacteria plant pathogens and the production of mersacidin, kalimantacin, and bacitracin with activity against G^+ bacteria plant pathogens; (3) induction of the systemic resistance of plants via inducing volatile organic compounds, including 2,3-butanediol, methanethiol, and isoprene, etc. The differences between the various *P. polymyxa* strains in terms of the control targets and efficacies might be attributable to the variations in the genes or gene clusters responsible for these three aspects of the biocontrol mechanism. The goal of this study was to provide a scientific basis for the further optimization of microbial biopesticides based on *P. polymyxa* strain HY96-2 in terms of field application and quality standards. For example, to develop a biopesticide based on strain HY96-2 for specifically controlling plant diseases caused by G^- bacteria, the levels of polymyxin in the product could be increased by altering the fermentation conditions or genetically modifying the producing strain. This study may also serve as a reference for future investigations into the differences in biocontrol targets and efficacy between different biocontrol agents.

AUTHOR CONTRIBUTIONS

YCL, YC, and YGL experimental design and authorship. YCL, YC, JY, ZZ, and QL experiments and data analysis. YCL, YC, YGL, and DZ manuscript revision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01520/full#supplementary-material

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Streptomyces globosus UAE1, a Potential Effective Biocontrol Agent for Black Scorch Disease in Date Palm Plantations

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Many fungal diseases affect date palm causing considerable losses in date production worldwide. We found that the fungicide Cidely® Top inhibited the mycelial growth of the soil-borne pathogenic fungus *Thielaviopsis punctulata*, the causal agent of black scorch disease of date palm, both *in vitro* and *in vivo*. Because the use of biocontrol agents (BCAs) can minimize the impact of pathogen control on economic and environmental concerns related to chemical control, we aimed at testing local actinomycete strains isolated from the rhizosphere soil of healthy date palm cultivated in the United Arab Emirates (UAE) against *T. punctulata*. The selected isolate can thus be used as a potential agent for integrated disease management programs. In general, the BCA showed antagonism *in vitro* and in greenhouse experiments against this pathogen. The most promising actinomycete isolate screened showed the highest efficacy against the black scorch disease when applied before or at the same time of inoculation with *T. punctulata*, compared with BCA or fungicide application after inoculation. The nucleotide sequence and phylogenetic analyses using the 16*S* ribosomal RNA gene with other *Streptomyces* spp. in addition to morphological and cultural characteristics revealed that the isolated UAE strain belongs to *Streptomyces globosus* UAE1. The antagonistic activity of *S. globosus* against *T. punctulata*, was associated with the production by this strain of diffusible antifungal metabolites i.e., metabolites that can inhibit mycelial growth of the pathogen. This was evident in the responses of the vegetative growth of pure cultures of the pathogen when exposed to the culture filtrates of the BCA. Altogether, the pathogenicity tests, disease severity indices and mode of action tests confirmed that the BCA was not only capable of suppressing black scorch disease symptoms, but also could prevent the spread of the pathogen, as a potential practical method to improve disease management in the palm plantations. This is the first report of an actinomycete, naturally occurring in the UAE with the potential for use as a BCA in the management of the black scorch disease of date palms in the region.

Keywords: actinomycetes, antibiosis, biocontrol, black scorch, date palm, *Thielaviopsis punctulata*, UAE

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is cultivated for its edible fruit and for its value as a shelter to humans, animals, and plants. In the United Arab Emirates (UAE) and in many other countries in the region, this plant also has social, traditional and heritage values (Mahmoudi et al., 2008). Date palm trees are exposed to various pathogenic infections, mainly fungi and phytoplasma, causing serious deleterious diseases as well as significant economic losses (Carpenter and Elmer, 1978). Black scorch disease, also locally known as Medjnoon or Fool, is caused by the fungus *Thielaviopsis paradoxa* (De Seyeres) Hohn or *Thielaviopsis punctulata* (Hennebert) Paulin, Harrington and McNew (de Beer et al., 2014). These soil-borne wound pathogens affect date palm tissues at all ages of growth, over a wide range of date growing areas in the world, causing losses of >50% in newly plantations and fruits (Gariani et al., 1994; Abdelmonem and Rasmy, 2007; Saeed et al., 2016). Previous reports have associated *T. paradoxa* with black scorch disease on date palm in Iraq (Abbas et al., 1997), Saudi Arabia (Al-sharidy and Molan, 2008), Kuwait (Mubarak et al., 1994), Italy (Polizzi et al., 2006) and the United States (Garofalo and McMillan, 2004). Recent studies have identified *T. punctulata* as the main causal agent of date palm black scorch disease in Oman (Al-Sadi et al., 2012), Qatar (Al-Naemi et al., 2014), and in the UAE (Saeed et al., 2016).

Depending on the time of infection and the stage of the disease development, typical symptoms of black scorch are hard black lesions on leaves, inflorescence blight, and trunk and bud rot (Suleman et al., 2001; Zaid et al., 2002; Abbas and Abdulla, 2003; Al-Raisi et al., 2011). Severe symptoms are well-characterized by "neck bending" of the regions affected by the fungal invasions in the terminal bud and heart, leading to the death of the tree. Poor horticultural practices and environmental stresses can also exacerbate the disease development. Studies on *T. paradoxa*- and *T. punctulata*-colonized palm tissues under salinity and drought stresses showed increased severity of black scorch, which eventually resulted in plant death (Suleman et al., 2001). Traditional field management practices such as avoidance of wounds in tissues or tree parts, cutting and burning affected palms have been reported to limit disease incidence (Chase and Broschat, 1993). Appropriate irrigation and fertilization programs are also recommended to minimize disease severity (Zaid et al., 2002).

Chemical application continues to be the major strategy to mitigate the menace of most crop diseases, despite its negative impact on the environment and human health. For example, the fungicide benomyl is highly effective against early infections by date palm pathogens (Zaid et al., 2002). Recently, it has been reported that Score® (difenoconazole) is an effective chemical treatment for black scorch on date palm (Saeed et al., 2016). On the other hand, integrated disease management (IDM) aims at reducing chemical inputs by delivering lower amounts and/or less frequent treatments with chemical fungicides, applied only when necessary (Lopez-Escudero and Mercado-Blanco, 2011). Biological control, also known as biocontrol, can limit the increases in pathogen populations, and often suppress the plant tissue destroying activities of pathogens by other organism(s) (AbuQamar et al., 2017). *In vitro* and *in vivo* experiments using species of *Trichoderma*, *Chaetomium* or their antagonistic products were applied to control black scorch pathogens (Soytong et al., 2005; Sánchez et al., 2007; Ammar, 2011; Al-Naemi et al., 2016).

Actinomycetes are a diverse group of Gram-positive bacteria that exhibit wide morphological differences that range from relatively simple rods and cocci to complex mycelial organization (Locci and Sharples, 1984). Reproduction is by fragmentation of the hyphae or by production of spores. Actinomycetes are ubiquitous, found in habitats such as soils, composts, freshwater, seawater, and cold- and warm-blooded animals. *Streptomyces* spp., a common genus in the order Actinomycetales, are a biologically active component of the soil microflora (Williams and Wellington, 1982). They are mostly found in relatively dry, humic, calcareous soils. *Streptomyces* spp. have been investigated predominantly, because of their dominance, ease of isolation and antibiotics production (Goodfellow and Williams, 1983).

Actinomycetes have also been identified as active biocontrol agents (BCAs) effective against many pathogenic fungi and oomycetes (Lee et al., 2005; El-Tarabily et al., 2009). They are known for their bioactive metabolites, including antibiotics, plant growth factors, vitamins, alkaloids, enzymes and enzyme inhibitors active against their natural enemies (Doumbou et al., 2001; Bressan, 2003; Shahidi et al., 2004; El-Tarabily and Sivasithamparam, 2006; Verma et al., 2011; Baltz, 2016). In general, biological control is an environmentally sustainable strategy that can be employed to manage plant pathogens and improves crop productivity.

In addition, rhizosphere microorganisms can activate the plant's programmed defense pathways, resulting in reducing the effects of subsequent biotic attack, known as induced systemic resistance (ISR) (Bakker et al., 2013). Actinomycetes, including *Streptomyces* spp., are soil-borne beneficial bacteria that have been shown to trigger ISR *in planta* and inhibit pathogen growth via induction of plant defense mechanisms (Martínez-Hidalgo et al., 2015). Induced systemic resistance induces plant resistance to a broad spectrum of root and foliar pathogens. In this study, we aimed to identify a new antifungal actinomycete from the UAE soil environment; and to determine the effect of the antifungal activity of the metabolites of the promising actinomycete against *T. punctulata in vitro* and *in vivo*. We also compared it to the activity of Cidely® Top, a foliar fungicide treatment, which has been shown to provide protection against black scorch disease on date palms in greenhouse trials. This study has explored the potential to use both biocontrol and fungicides to develop an IDM strategy against this disease.

MATERIALS AND METHODS

Fungal Growth and Disease Assays

The fungus, *T. punctulata* (DSM-102798), was previously identified by Saeed et al. (2016) as the causal agent of the date palm scorch disease in the UAE. *T. punctulata* was cultured on potato dextrose agar (PDA; Lab M Limited, Lancashire, United

Kingdom) plates (pH 6.0); supplemented with ampicillin (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) used at a rate of 25 mg l^{-1} of agar medium, to inhibit the bacterial contaminants. The pathogen was subcultured on fresh PDA plates every 10 days and incubated at 28°C.

For disease assays, leaf bases of 8-month-old date palm (cv. Chichi) seedlings, obtained from the Date Palm Development Research Unit (DPDRU), United Arab Emirates University, Al-Ain, UAE, were surface-sterilized with 70% ethanol, and mechanical wounding was performed with sterilized scalpels prior to inoculation. These date palm seedlings were inoculated with agar plugs (8-mm in diameter) colonized by fungal mycelium from 10-days old *T. punctulata* cultures at the leaf base region, and the area of inoculation was wrapped with parafilm (Sigma–Aldrich) (Saeed et al., 2016). Inoculated seedlings were maintained in a greenhouse at 28°C, and examined for disease development.

Isolation and Detection of the Antifungal Activity of Actinomycete Isolates

Five random rhizosphere soil samples limited to 30 cm-depth obtained with a clean spade near the roots of healthy date palm trees, were collected in plastic bags. Rhizosphere soil samples were air-dried for 4 days at 28°C to reduce the numbers of contaminant bacteria (Williams et al., 1972), passed through a 5-mm mesh sieve to remove small stones and root fragments and stored in sterile screw-capped jars at 25°C in the dark, for a week prior to microbiological processing. Actinomycetes were isolated using the soil dilution plate method (Johnson and Curl, 1972) on inorganic salt starch agar (ISSA) (Küster, 1959) amended with cycloheximide and nystatin (each 50 μg ml^{-1}; Sigma–Aldrich) with specific soil pre-treatments (Hayakawa and Nonomura, 1987). Briefly, the soil pre-treatments involved preparing serial dilutions of the soil suspension by suspending the sample in 6% yeast extract (YE) (Lab M Limited) and 0.05% sodium dodecyl sulfate (SDS) (Sigma–Aldrich) for 20 min at 40°C, and diluting with water to remove other factors promoting bacterial growth or injurious to germinating actinomycete propagules. The YE and SDS were included to increase and decrease the numbers of actinomycetes and bacteria, respectively (Nonomura and Hayakawa, 1988). Seven replicate plates were used per dilution, which were incubated at 28°C in dark for 7 days. Actinomycete colonies were counted, transferred onto oatmeal agar plates (ISP medium 3) supplemented with 0.1% yeast extract (OMYEA) (Küster, 1959) and stored in 20% glycerol (cryoprotectant) at −20°C (Wellington and Williams, 1978). All isolates were tentatively identified and grouped to the genus level on the basis of their standard morphological criteria and according to the absence or presence of aerial mycelium, distribution (aerial/substrate) and form of any spores present and stability or fragmentation of substrate mycelium (Cross, 1989).

We screened all 47 rhizosphere actinomycete isolates obtained, for their potential to produce diffusible antifungal metabolites active against *T. punctulata* using the cut-plug technique (Pridham et al., 1956). Actinomycete isolates were inoculated on OMYEA plates and incubated at 28°C in dark for 7 days. Plugs were then cut from the growing margins of the actinomycete colonies with a sterilized 11-mm cork-borer and transferred aseptically to the center of PDA plates freshly seeded with *T. punctulata* and further incubated at 28°C in dark for 4 days. The diameters of zones of inhibition were determined in mm. Three replicates were used for each actinomycete isolate.

Inocula for the preparation of the PDA seeded plates were prepared by cultivating *T. punctulata* on PDA slants at 28°C until abundant sporulation occurred. The slant surfaces were then flooded with 50 mM phosphate buffer (pH 6.8), and spores as well as some mycelial fragments were dislodged by scraping the surface with a sterilized scalpel; and were homogenized in an Omni-mixer (OCI Instruments, Omni Corporation International, Waterbury, CT, United States) at 4000 rpm for 20 min. The resultant suspensions were diluted and added to sterile cooled PDA prior to the pouring of the plates. A suspension of approximately 10^8 CFU ml^{-1} was used as inoculum. The control consisted of PDA plates with OMYEA plugs without any actinomycete growth.

Based on the results obtained from this experiment, only the most promising antagonistic BCA (isolate #7) that produced the strongest inhibition of *T. punctulata* was selected for further analysis. The rest of the isolates were considered either to be non-producers of antifungal metabolites active against *T. punctulata* or those that produced only low or non-detectable levels of inhibition against *T. punctulata*, and were therefore not included in the subsequent studies.

Identification and Detection of the Antifungal Activity of the Promising BCA

Identification of the BCA isolate #7 to the species level was based on morphological, cultural, and physiological characteristics as described by Locci (1989). Light microscopy (100X) was carried out using Nikon-Eclipse 50i light microscope (Nikon Instruments Inc., Melville, NY, United States), whilst scanning electron microscopy (SEM) was carried out using the Philips XL-30 SEM (FEI Co., Eindhoven, The Netherlands). We further confirmed the identification by 16*S* rRNA gene sequence analysis done by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ), Braunschweig, Germany. The partial 16*S* rRNA gene sequence (about 1501 nucleotides) was determined by direct sequencing of PCR-amplified 16*S* rRNA as described by Rainey et al. (1996). PCR conditions were: initial denaturation of 3 min at 95°C, followed by 28 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min. A final extension step consisted of 5 min at 72°C was also included. Phylogenetic tree was constructed to predict the species level characterization of the studied isolate using the neighbor-joining method implemented in Molecular Evolutionary Genetics Analysis 7.0 (MEGA7) software (Saitou and Nei, 1987; Kumar et al., 2016).

We also tested the ability of the BCA (isolate #7) for its potential to produce diffusible antifungal metabolites active against *T. punctulata* using the cup plate method (Bacharach and Cuthbertson, 1948). Individual 250 ml Erlenmeyer flasks containing 50 ml of sterile fish meal extract broth (FMEB; El-Tarabily et al., 1997) were inoculated with 1 ml of 10% glycerol

suspension of the BCA (approximately 10^8 CFU ml^{-1}) and incubated in a gyratory shaker (Model G76, New Brunswick Scientific-Edison, Edison, NJ, United States) at 200 rpm at 28°C in dark for 5 days. After incubation, the suspensions from each flask were centrifuged for 30 min at 2000 *g*. The supernatant (crude culture filtrate) was filtered through sterile Millipore membranes of pore size 0.22 μm (Millipore Corporation, Billerica, MA, United States) and collected in sterilized tubes and stored at 4°C.

Inocula for the preparation of the *T. punctulata* seeded PDA plates were prepared as described above for the cut-plug technique. Wells were cut in the centers of the fresh PDA plates seeded with *T. punctulata* using a sterilized 11-mm cork-borer. Aliquots (0.3 ml) of the filter-sterilized crude culture filtrate were pipetted into the wells using a sterilized syringe. The plates were incubated at 28°C in dark for 4 days; and the diameters of zones of inhibition were measured in mm. Filter-sterilized inorganic salt starch broth without the BCA was similarly pipetted into the wells in the PDA plates seeded with *T. punctulata* as a control.

In addition, we conducted a dialysis membrane overlay technique to assay inhibition of *T. punctulata* by the BCA. The single thickness dialysis membrane overlay technique (Gibbs, 1967) was used on fish meal extract agar (FMEA; El-Tarabily et al., 1997). The 90-mm dialysis membrane (Type 45311; Union Carbide Corporation, Alsip, IL, United States) was overlaid on FMEA and the membrane surface was inoculated with the BCA by evenly streaking cells and/or spores of a 7-day old culture of the BCA grown on OMYEA. The plates were incubated at 28°C in dark for 10 days. The membranes with the adhering colonies were subsequently removed from the agar plates and the center of each plate was inoculated with a disk (5-mm in diameter) of *T. punctulata* culture grown for 7 days on FMEA. Plates were incubated at 28°C in dark. The colony diameter of *T. punctulata* was measured in mm after 8 days and compared to that of FMEA plates (control) where the pathogen was grown without the BCA. At the end of the incubation period, if the pathogen had not grown from the agar plugs, these plugs were further transferred to a fresh PDA plate and incubated at 28°C for 5 days to determine whether the diffused metabolites were fungicidal (no pathogen growth from the plug) or fungistatic (pathogen growth from the plug).

Assays of Producing Volatile Antifungal Compounds, Hydrocyanic Acid and Siderophores by the BCA

Production of volatile antifungal metabolites by the BCA was tested on FMEA as described by Payne et al. (2000). Briefly, FMEA plates were inoculated with the BCA by evenly streaking cells and/or spores from a 7-day old culture onto the whole surface of the agar. These cultures were grown at 28°C in dark for 14 days. At this time, plates of the same medium were inoculated with an actively growing *T. punctulata* mycelial plug (5-mm in diameter). The lids were removed and the plates containing the pathogen were inverted over the BCA plates. The two plate bases were taped together with a double layer of Parafilm (American National Can TM, Greenwich, CT, United States). Control plates were prepared in the same way except that a non-inoculated FMEA plate was used instead of a plate containing the BCA. After a further 7 days of incubation, the colony diameter of *T. punctulata* growing in the presence of the BCA was measured and compared to that of the control.

Hydrogen cyanide (hydrocyanic acid) production by the BCA was detected as described by Bakker and Schippers (1987). The change in color from yellow to orange–brown on the filter paper impregnated with 0.5% picric acid and 2% sodium carbonate indicated the production of cyanide.

Plates of chrome azurol S (CAS) agar developed by Schwyn and Neilands (1987) and modified by Alexander and Zuberer (1991) known as modified M9 agar, were inoculated with the BCA and incubated at 28°C in dark for 10 days. Development of yellow–orange halo zone around the culture was considered as positive for siderophore production (Alexander and Zuberer, 1991).

Determination of Chitinase and β-1,3-glucanase Activities of the BCA

The BCA was inoculated onto a colloidal chitin agar (Gupta et al., 1995) plate and incubated at 28°C in dark until zones of chitin clearing were seen around and beneath the colonies. Colloidal chitin was prepared from crab shell chitin (Sigma–Aldrich) (Hsu and Lockwood, 1975). Clear zone diameters were measured in mm to indicate the chitinase activity of the isolate.

Quantitative production of chitinase and β-1,3-glucanase by the BCA were also determined as described previously (Singh et al., 1999) using the minimal synthetic medium (Tweddell et al., 1994) amended with 2 mg ml^{-1} of either colloidal chitin or laminarin (Sigma–Aldrich), respectively.

Chitinase specific activity was calculated by measuring the release of *N*-acetyl-D-glucosamine (NAGA) from colloidal chitin. Specific activity (U = 1 unit of chitinase) was defined as the amount of the enzyme that released 1 μmol of NAGA mg^{-1} protein h^{-1} (Reissig et al., 1955). The specific activity of β-1,3-glucanase was determined by measuring the amount of reducing sugars liberated from laminarin using dinitrosalicylic acid (DNS) solution (Miller, 1959). Specific activity (U = 1 unit of β-1,3-glucanase) was defined as the amount of the enzyme that released 1 μmol of glucose mg^{-1} protein h^{-1} (Miller, 1959). Protein content of the enzyme solution was determined by the Folin phenol reagent method (Lowry et al., 1951).

Effect of BCA Crude Culture Filtrate on *Thielaviopsis punctulata* Mycelial Growth, Spore Germination and Germ Tube Elongation

The assay for inhibition of colony and mycelial growth was conducted on PDA plates as described by Lorito et al. (1993). The crude culture filtrate was mixed with sterilized PDA (45°C at 10, 25, 50, 75 and 100% proportions) and poured into Petri plates. Control plates contained 0% crude culture filtrate. The medium was inoculated with a 5-mm in diameter agar plug with actively growing *T. punctulata* mycelium (placed colonized surface down) in the center of the plates. The colony growth of *T. punctulata* was

compared with that of the control after 5 days of incubation in the dark at 28°C.

The assay for inhibition of mycelial growth was also conducted in potato dextrose broth (PDB) (Lorito et al., 1993). Prepared crude culture filtrate was mixed with sterilized PDB at 0, 10, 25, 50, 75, and 100% proportions. The PDB was inoculated with a 5-mm diameter agar plug with actively growing *T. punctulata* mycelium. The mycelial dry weight of *T. punctulata* was measured in g after 10 days of incubation in the dark at 28°C.

The effect of the filter-sterilized crude culture filtrate of the BCA on aleuroconidia germination and germ tube elongation of *T. punctulata* was conducted in PDB as described by Lorito et al. (1993). Briefly, the crude culture filtrate was prepared as described above using FMEB. Aliquots (20 μl) of the crude culture filtrate were mixed with 20 μl of spore suspension of *T. punctulata* and 60 μl of PDB. The control consisted of the FMEB without the BCA replacing the crude culture filtrate. The reaction mixture was incubated at 28°C in dark. After 24 h, the percent spore germination and average length of germ tubes in μm were microscopically determined at 40X using a light microscope (Nikon-Eclipse 50i) and compared with the control.

For the effect of the crude culture filtrate of the BCA on hyphal morphology, *T. punctulata* was grown in 100-ml PDB at 28°C in dark for 10 days. The culture broth was removed and the mycelial mats were aseptically washed four times with sterile distilled water (Sneh, 1981). A 100-ml carbon-deficient salt solution was added to the living mycelium of *T. punctulata*. A 50-ml of the filter-sterilized crude culture filtrate of the BCA was also added to the mycelial suspension. The flasks were incubated at 28°C in dark for 4 days, and the culture inspected daily. Controls consisted of 100 ml carbon-deficient salt solution containing *T. punctulata* mycelium incorporated with 50 ml FMEB, without the BCA.

At each sampling, a sub-sample of *T. punctulata* hyphae was retrieved and any subsequent changes in the hyphal morphology observed at 100X using a light microscope (Nikon-Eclipse 50i) connected with Nikon camera (DS – Flic). Three replicates were used at each sampling.

In Vitro Evaluation of the Minimum Effective Concentration of Cidely® Top Fungicide

An *in vitro* evaluation of the fungicide Cidely® Top (difenoconazole and cyflufenamid; Syngenta International AG, Basel, Switzerland) was carried out as previously described (Jonathan et al., 2012). The fungicide, obtained at a dissolved solution of 25, 75, 125, 250, 500, or 1000 ppm final concentration in sterile water, was introduced aseptically into sterilized molten PDA at 25°C. The molten PDA was amended with ampicillin (Sigma–Aldrich) to inhibit bacterial growth. The solution was carefully swirled to attain homogenization status. The resulting mixtures were aseptically dispensed into sterile Petri dishes. A sterile cork-borer measuring 5-mm in diameter was used to introduce the tested pathogen onto the control (without fungicide) and treatment (with fungicide) media. Cultures were incubated at 28°C in dark for 15 days after which radial growth measurements were recorded daily. The percentage of the mycelial growth was measured and growth inhibition was calculated according to the following equation: Mi % = (Mc – Mt)/Mc × 100%; where; Mi, Inhibition of the mycelial growth; Mc, colony diameter (in mm) of control set; and Mt, colony diameter (in mm) of the target fungus on the medium with fungicide.

In Vivo Experiments with the BCA and the Fungicide

In vivo evaluations of the BCA and the fungicide were carried out on 8-month-old date palm plants (cv. Chichi). Seedlings were divided into two experiments conducted in parallel:

Experiment I: The purpose of this experiment was to compare the minimum effective dosage concentration of Cidely® Top fungicide with the BCA in controlling black scorch disease. For the fungicide treatment, only one cylindrical wound was made in the leaf base of seedlings to introduce one agar plug of the inoculum. At 2 weeks post inoculation (wpi) (when disease symptoms were evident), we sprayed the inoculated seedlings with 100 ml of Cidely® Top (250 ppm) fungicide, as previously described by Saeed et al. (2016). Healthy controls were wounded and treated with PDA disks; disease controls were wounded and treated with PDA disks colonized with the pathogen. For BCA treatments, three cylindrical wounds (8-mm in diameter) were made in each leaf base of date palm seedlings in order to introduce one agar plug of the *T. punctulata* inoculum at 2 wpi preceding the insertion of two agar plugs of the BCA treatment. Six plants in separate pots were used for each group/treatment; and pots were arranged in a completely randomized design. The plants were grown for another 16 weeks before the number of dead plants in each treatment was counted. The experiment was repeated three times. Groups/treatments for Experiment I were as follows:

Healthy controls (C): Non-inoculated- or non-treated-seedlings;

Diseased controls (*Tp*): Inoculated-seedlings with *T. punctulata* only;

BCA controls (BC): Inoculated-seedlings with BCA only;

Chemical fungicide treatment (*Tp*+CC): Sprayed-seedlings with Cidely® Top 2 weeks after inoculation with *T. punctulata*; and

BCA treatment: Inoculated-seedlings with BCA 2 week after *T. punctulata* inoculation (*Tp*+BC).

Experiment II: The purpose of this experiment was to investigate the effectiveness of various timings of BCA treatment to manage black scorch disease. Similar to the BCA treatment described above, methods of inoculation with *T. punctulata* and BCA application was used. In this experiment, the timing of the BCA treatment was the important factor (preventive, concurrent or curative). The set-up of the treatments/groups for Experiment II was as follows:

Diseased controls (*Tp*): Inoculated-seedlings with *T. punctulata* only;

BCA controls (BC): Inoculated-seedlings with BCA only;

Preventive treatment: Inoculated-seedlings with BCA 1 week before *T. punctulata* inoculation;
Concurrent treatment: Inoculated-seedlings with BCA at the same time of *T. punctulata* inoculation; and
Curative treatment: Inoculated-seedlings with BCA 1 week after *T. punctulata* inoculation.

Six plants in separate pots, arranged in a completely randomized design, were used for each treatment/group. Plants were grown for another 16 weeks before the number of dead plants in each treatment was counted. The experiment was conducted three times.

Control and inoculated seedlings were further kept in a greenhouse with a photoperiod extended to 15 h under fluorescent lights (160 W mol^{-1} m^{-2} s^{-1}) at 28°C until disease symptoms were evident, and frequent observations were made until 16 wpi with *T. punctulata*. The schedule of the timing of *T. punctulata* inoculation, the actinomycete BCA isolate and chemical fungicide treatments, and disease evaluation are presented in **Figure 1**.

Spore Counts and Disease Severity Index in Inoculated Plants

The growth of *T. punctulata* in inoculated plants was determined on the basis of the number of fungal spores (total spore counts) at 16 wpi. Three tender leaf bases from six inoculated seedlings per treatment were collected, cut into small pieces (2–5 mm in diameter), soaked in 10 ml distilled water and vigorously agitated for 30 min. Harvested spores were counted using haemocytometer (Agar Scientific Limited, Essex, United Kingdom).

Disease severity index (DSI) was recorded at 8 and 16 wpi using a scale of 0–5: 0 = no apparent symptoms, 1 = 1–10% necrotic or dark brown area around the point of infection, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% (Molan et al., 2004). All experiments were repeated three times with similar results.

Statistical Analysis

For the *in vitro* evaluation of Cidely® Top fungicide and BCA against *T. punctulata*, data were analyzed using the analysis of variance (ANOVA) while means were separated using Duncan's multiple range test at 5% level of significance. These experiments were repeated in triplicates using five plates/treatment for each time with similar results.

For the fungal spore counts and DSI of the *in vivo* treatments against *T. punctulata*, three replicates for each group were examined. Data represent the mean ± SD from a minimum of six plants. ANOVA and Duncan's multiple range test were performed to determine the statistical significance at $P < 0.05$. Similar results were obtained in each replicate. SAS Software version 9 was used for all statistical analyses performed (SAS Institute, 2002).

RESULTS

Isolation, Identification and Screening of Actinomycetes Isolates

The population of actinomycetes in the date palm rhizosphere was found to be 6.73 ± SE 1.28 log_{10} CFU g^{-1} dry soil. Forty-seven streptomycete and non-streptomycete actinomycete strains were isolated, of which 37 isolates (78.8%) belonged to the genus *Streptomyces*, whilst only 10 isolates (21.2%) belonged to genera of non-streptomycete actinomycetes. The latter isolates were found to be affiliated to the genera *Micromonospora*, *Rhodococcus*, *Streptoverticillium,* and *Nocardia*. We also found that 25.5% (12/47) of the rhizosphere actinomycete isolates (9 streptomycete and 3 non-streptomycete) produced strong diffusible antifungal metabolites active against *T. punctulata* using the cut-plug technique (**Table 1**). All the 12 isolates produced large zones of inhibition (>20 mm) against *T. punctulata*. The remaining isolates were either non-producers of diffusible antifungal metabolites active against *T. punctulata* or produced very low levels of inhibition against *T. punctulata* (inhibition

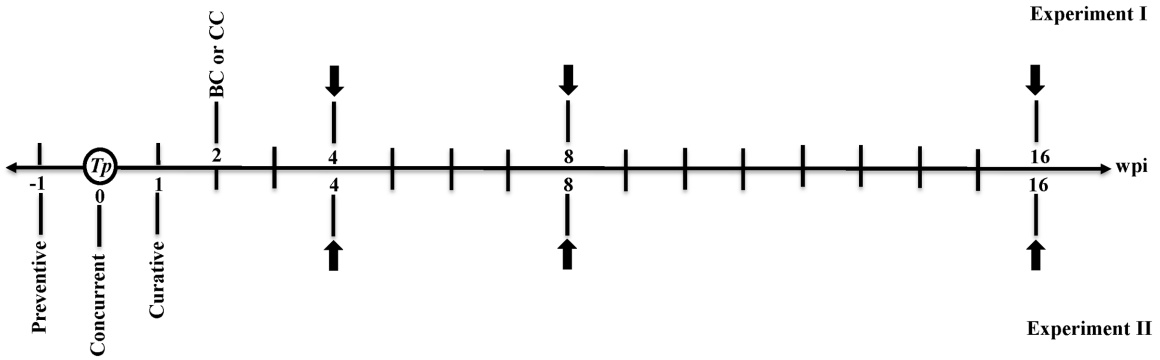


FIGURE 1 | Timeline of microbial inoculation, BCA and fungicide treatments, and disease evaluation schedule. Preventive: BCA treatment 1 week before *Thielaviopsis punctulata* inoculation; Concurrent: BCA treatment at the same time of *T. punctulata* inoculation; Curative: BCA treatment 1 week after *T. punctulata* inoculation; BC or CC: BCA or Cidely® Top fungicide (chemical treatment) at 2 wpi with *T. punctulata*. White circle, establishment of *T. punctulata* disease assay on date palm (cv. Chichi) seedlings; black arrow, disease progress and treatment evaluation at 4, 8, and 16 wpi with *T. punctulata*; *Tp*, *T. punctulata*; CC, chemical control using Cidely® Top fungicide; BC, biocontrol treatment using the BCA candidate (*Streptomyces globosus* UAE1); BCA, biocontrol agent; wpi, weeks post inoculation.

TABLE 1 | Diameter of the inhibition zone of tested actinomycete isolates against *Thielaviopsis punctulata* using the cut-plug technique.

Actinomycete		Diameter of inhibition zone (mm)[a]
Genera	**Isolate**	
Streptomyces sp.	#3	22.50 ± 0.64 *fg*
	#7 (BCA)	33.80 ± 0.44 *a*
	#10	26.00 ± 0.70 *e*
	#18	21.62 ± 0.74 *g*
	#21	28.87 ± 0.42 *cd*
	#23	30.57 ± 0.44 *b*
	#33	29.05 ± 0.41 *cd*
	#38	23.57 ± 0.44 *f*
	#45	31.27 ± 0.32 *b*
Micromonospora sp.	#15	29.87 ± 0.42 *bc*
Streptoverticillium sp.	#29	25.37 ± 0.34 *e*
Rhodococcus sp.	#42	28.25 ± 0.41 *d*

[a]*Values are means of three replicates ± SE. Values with the same letter are not significantly different at P = 0.05. Isolate #7 represents the biocontrol agent (BCA), Streptomyces globosus UAE1.*

zones < 20 mm); they were therefore not included in the subsequent studies. Based on the results obtained, the most promising inhibitory and antagonistic BCA candidate (isolate #7) producing the strongest inhibition against *T. punctulata* was selected for further analyses (**Table 1**; Supplementary Figure S1).

Identification of the BCA Isolate #7 to the Species Level

The potential antagonistic BCA candidate (isolate #7) was isolated and preliminarily identified by nucleotide sequence of its 16*S* rRNA gene. The resulting sequence data from this strain was deposited in NCBI (GenBank Accession Number: KY980677). In addition, a comparison with representative 16*S* rRNA gene sequences of organisms belonging to the Actinobacteria was carried out using phylogenetic analysis. Comparison of the 16*S* rRNA gene of isolate #7 (∼1501 bp) with sequences in the GenBank database revealed that the BCA candidate was a streptomycete sp. with 100% similarity to *Streptomyces globosus* (AJ781330) and *S. toxytricini* (AB184173) (**Figure 2A**). The rest of the *Streptomyces* spp. showed less than 99.7% similarity with the target antagonistic strain. This suggests that the isolated strain could possibly be either *S. globosus* or *S. toxytricini*; thus, it was necessary to obtain a more definitive identification of the isolate.

To confirm the species of isolate #7, pure cultures were cultivated on ISP medium 3 supplemented with yeast extract (OMYEA). Cultures typical white, turning to beige, aerial mycelium and yellow substrate mycelium were evident after 14 days of cultivation (**Figure 2B**). Using SEM, the configuration of the spore chains of the isolate revealed spore chain morphology which belongs to Rectiflexibiles (RF) form, consisting of cylindric or elongated smooth spores (0.75 × 1.0 μm) on an aerial mycelium (**Figure 2C**). Together, this suggests that the outstanding isolate #7 can be identified as *S. globosus* (Krassilnikov, 1941) Waksman in Waksman and Lechevalier (1953) Strain UAE1.

In Vitro Evaluation of Antagonistic Properties of the BCA Isolate

The incorporation of the filter-sterilized crude culture filtrate of the BCA into the wells using the cup-plate technique resulted in significant ($P < 0.05$) retardation of the growth of *T. punctulata*, producing large zones of inhibition (45 mm ± SE 0.88), caused by the diffused antifungal metabolites, when compared to the control treatment (**Figure 3A**). Following the removal of the dialysis membranes from the FMEA, the growth of the inoculum of *T. punctulata* was clearly inhibited by the diffused metabolites of the BCA, when compared to the control or the non-diffusible antifungal metabolite-producing *Streptomyces* sp. (isolate #25) (Supplementary Figure S1). The pathogen did not grow from the plugs transferred from treatment plates to the fresh PDA medium in the absence of diffused metabolites. This confirmed that the BCA showed fungicidal activities to *T. punctulata*.

In order to determine whether the BCA produced volatile antifungal compounds, isolate #7 along with the positive (isolate #25) and negative controls were grown on FMEA. Similar to the control which did not contain any actinomycete isolates, the growth of *T. punctulata* over the BCA was not affected with any visible effect on colony morphology when compared to the volatile antifungal producing *Streptomyces* (isolate #25) which caused total inhibition of the growth of the pathogen (Supplementary Figure S2). The BCA also failed to produce hydrogen cyanide or siderophores. The BCA did not produce clear inhibition zones around or beneath the colonies when grown on colloidal chitin agar, indicating of the failure of the tested BCA to produce chitinase compared to the chitinase-producing *Micromonospora* sp. (isolate #4) (Supplementary Figure S2). In addition, the BCA did not produce any detectable levels of chitinase or β-1,3-glucanase, when grown in the liquid medium containing colloidal chitin or laminarin, respectively. Together, our data suggest that the inhibition of the causal agent of black scorch is the result of the activities of the diffusible antifungal metabolites of the BCA.

Inhibition of *Thielaviopsis punctulata* Mycelial Growth and Spore Germination by the Crude Culture Filtrate of the BCA

We demonstrated that the culture filtrates of the BCA were effective in inhibiting growth of *T. punctulata*. On PDA plates, the increasing levels of the BCA culture filtrates increasingly and significantly inhibited the colony and mycelial growth of *T. punctulata* after 5 days of incubation at 28°C (**Figure 3B** and **Table 2**). A total inhibition of *T. punctulata* mycelial growth was observed when culture filtrates were supplied with 75% or above. In PDB, the assay for inhibition of mycelial growth showed a similar trend to the effect of the BCA filter-sterilized crude culture filtrate on the mycelial growth of the pathogen on PDA plates. The culture filtrates of the BCA significantly inhibited the mycelial growth of *T. punctulata* when incorporated into PDB with increasing proportions compared to the control after 5 days of incubation at 28°C (**Table 2**). Moreover, the germination of aleuroconidia and the average length of germ tubes produced by *T. punctulata* were significantly reduced in the presence of

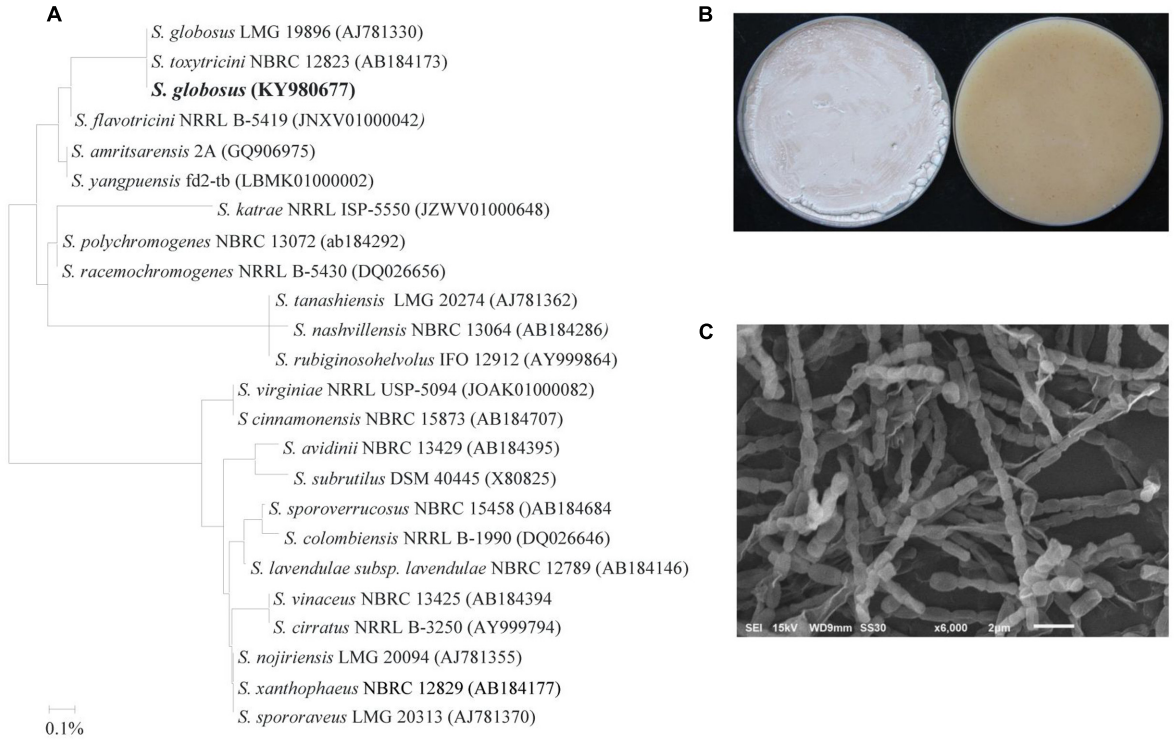


FIGURE 2 | Identification of *Streptomyces globosus* UAE1 based on phylogenetic, cultural and morphological characteristics. **(A)** The dendrogram showing the phylogenetic relationships between *S. globosus* UAE1 (KY980677) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences prepared by the neighbor-joining method. **(B)** Beige aerial mycelium (left) and yellow substrate mycelium (right) growing on (ISP medium 3) supplemented with yeast extract, and **(C)** Scanning electron micrograph (6,000X) of the Rectiflexibiles spore chains and smooth-surfaced spores of *S. globosus* UAE1. In **(A)** the scale bar on the rooted tree indicates a 0.01 substitution per nucleotide position.

the filter-sterilized crude culture filtrate of the BCA after 24 h of incubation compared with those without BCA (**Table 2**). This suggests that the culture filtrates that supported the BCA not only inhibited spore germination and germ tube elongation, but also mycelial growth of *T. punctulata in vitro*.

There was also noticeable hyphal abnormalities, including hyphal swelling (ballooning), septum malformation and abnormal branch formation in *T. punctulata* treated with the filter-sterilized crude culture filtrate of the BCA (**Figure 3C**). In addition, the hyphal cells underwent cytoplasmic coagulation in crude BCA culture filtrate-treated flasks. Mycelial mats in control flasks remained healthy and intact.

In Vitro Inhibitory Effect of the Fungicide Treatment on Mycelial Growth of *Thielaviopsis punctulata*

In order to evaluate the effect of the fungicide Cidely® Top (difenoconazole and cyflufenamid) to inhibit the mycelial growth of *T. punctulata*, six concentrations (25, 75, 125, 250, 500, or 1000 ppm) of the selected fungicide were applied *in vitro* (Supplementary Figure S3). The data obtained from this study revealed that up to 250 ppm of the fungicide tested; there were significant differences associated with the concentrations applied, in inhibiting the mycelial growth of the pathogen (**Figure 4A**). A significantly increased fungal inhibition zone was evident at the 250 ppm concentration (**Figure 4B**) as well as at the other two high concentrations tested (500 or 1000 ppm), ranging between 68 and 73% mycelial growth inhibition (stationary growth state). Thus, there were no significant differences between the 250 ppm and higher concentrations of treatments (**Figure 4A**). This suggests that the systemic fungicide (Cidely® Top) was adequately successful in inhibiting the mycelial growth of *T. punctulata*, at 250 ppm and therefore is considered to be the most appropriate fungicide concentration to serve as a minimum inhibitory dose for used in greenhouse or field trials.

Effect of the Minimum Dose of the Fungicide (Cidely® Top) and BCA on Mycelial Growth of *Thielaviopsis punctulata*

A previous study detailed the disease symptoms and the identity of the causal pathogen of the black scorch disease on date palm in the UAE (Saeed et al., 2016). The responses of the pathogen *in vitro* clearly indicated the BCA and the minimum dosage concentration (250 ppm) of Cidely® Top fungicide to be effective against date palm black scorch caused by *T. punctulata*. In order to evaluate the chemical Cidely® Top fungicide as a possible IDM component, and compare that with the BCA in

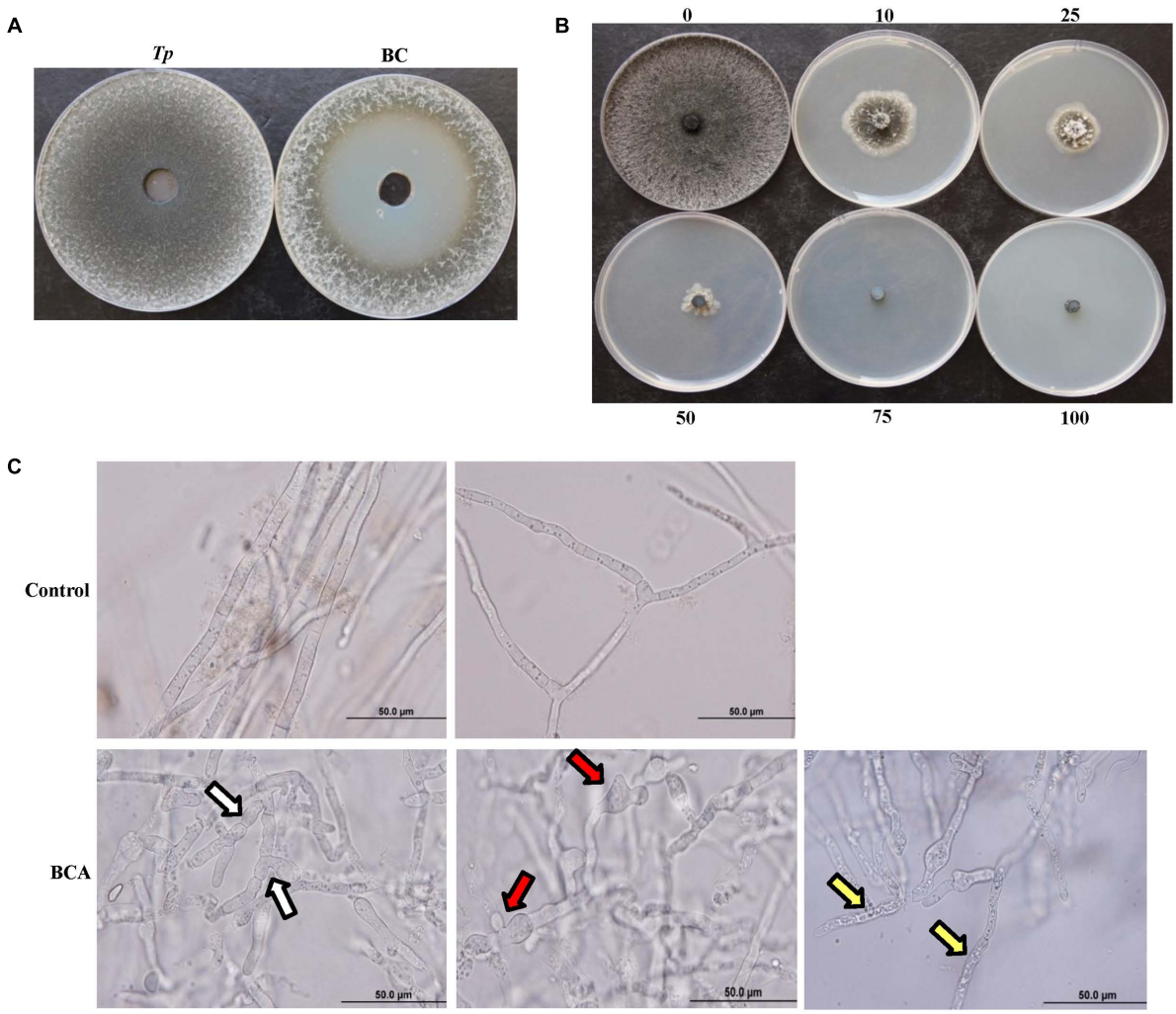


FIGURE 3 | Inhibitory effect of the BCA candidate on *Thielaviopsis punctulata*. **(A)** Inhibition of *T. punctulata* mycelial growth by *Streptomyces globosus* UAE1 (isolate #7) using the cup plate technique, **(B)** Gradual inhibition of *T. punctulata* colony growth on PDA plates containing different proportions (%) of crude culture filtrate of *Streptomyces globosus* UAE1 (isolate #7), and **(C)** Abnormalities evident in hyphal morphology and cytoplasmic contents of *T. punctulata*, following exposure to *S. globosus* UAE1 (isolate #7) metabolites (bottom panel), compared to control (top panel). White arrows point to hyphal septum malformation and branch deformation; while red and yellow arrows point to hyphal swellings and cytoplasmic coagulation, respectively.

TABLE 2 | Inhibition of *Thielaviopsis punctulata* mycelial growth, spore germination and germ tube elongation by the crude culture filtrate of the BCA.

Culture filtrate (%)	Colony diameter (mm)[a]	Mycelial dry weight (g)[a]	Spore germination (%)[a]	Germ tube length (µm)[a]
0	99.03 ± 0.28 *a*	70.28 ± 2.94 *a*	88.11 ± 1.98 *a*	54.66 ± 1.54 *a*
10	39.63 ± 1.68 *b*	39.36 ± 2.79 *b*	32.88 ± 1.52 *b*	40.65 ± 2.21 *b*
25	22.08 ± 1.34 *c*	18.93 ± 1.49 *c*	19.04 ± 1.69 *c*	21.83 ± 1.64 *c*
50	13.03 ± 0.09 *d*	9.35 ± 1.19 *d*	9.16 ± 1.01 *d*	12.01 ± 1.06 *d*
75	0.00 ± 0.00 *e*	1.98 ± 0.18 *e*	3.95 ± 0.51 *e*	7.64 ± 1.05 *e*
100	0.00 ± 0.00 *e*	0.21 ± 0.10 *e*	0.30 ± 0.14 *e*	0.49 ± 0.20 *f*

[a]*Values are means of three replicates ± SE. Values with the same letter within a column are not significantly different at P = 0.05.*

suppressing *T. punctulata*, an *in vivo* experiment was conducted in the greenhouse (Experiment I). Firstly, a pathogenicity test was done to determine the effect of *T. punctulata* on date palm seedlings. Typical symptoms of black scorch disease after 4 wpi with *T. punctulata* (*Tp*) were observed (**Figure 5A**). The disease progressed with time and leaves of infected seedlings showed distinct bending at 16 wpi. No disease symptoms were noticed in inoculated BCA alone (BC) or non-inoculated

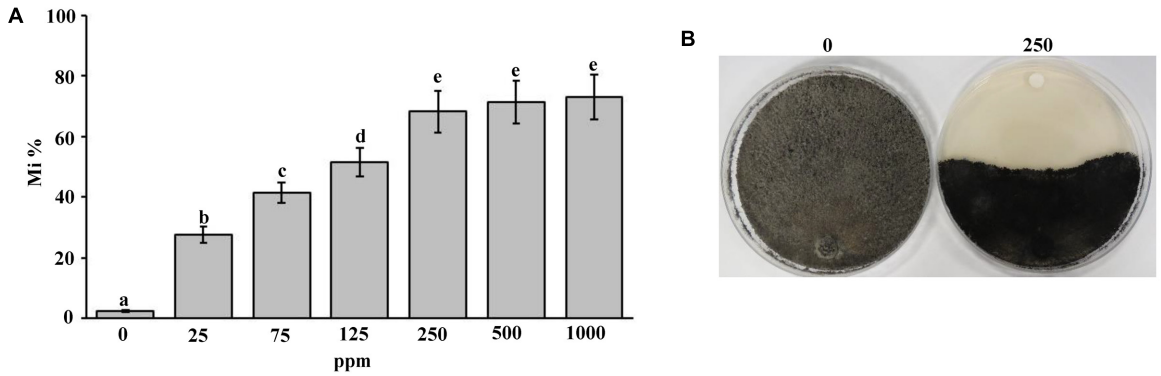


FIGURE 4 | Sensitivity of *Thielaviopsis punctulata* to Cidely® Top fungicide. **(A)** Growth inhibition rate (Mi %) of *T. punctulata* using different concentrations of Cidely® Top after 15 days. Values with different letters are significantly different at $P = 0.05$, and **(B)** Effect of Cidely® Top (250 ppm) on *in vitro* mycelial growth.

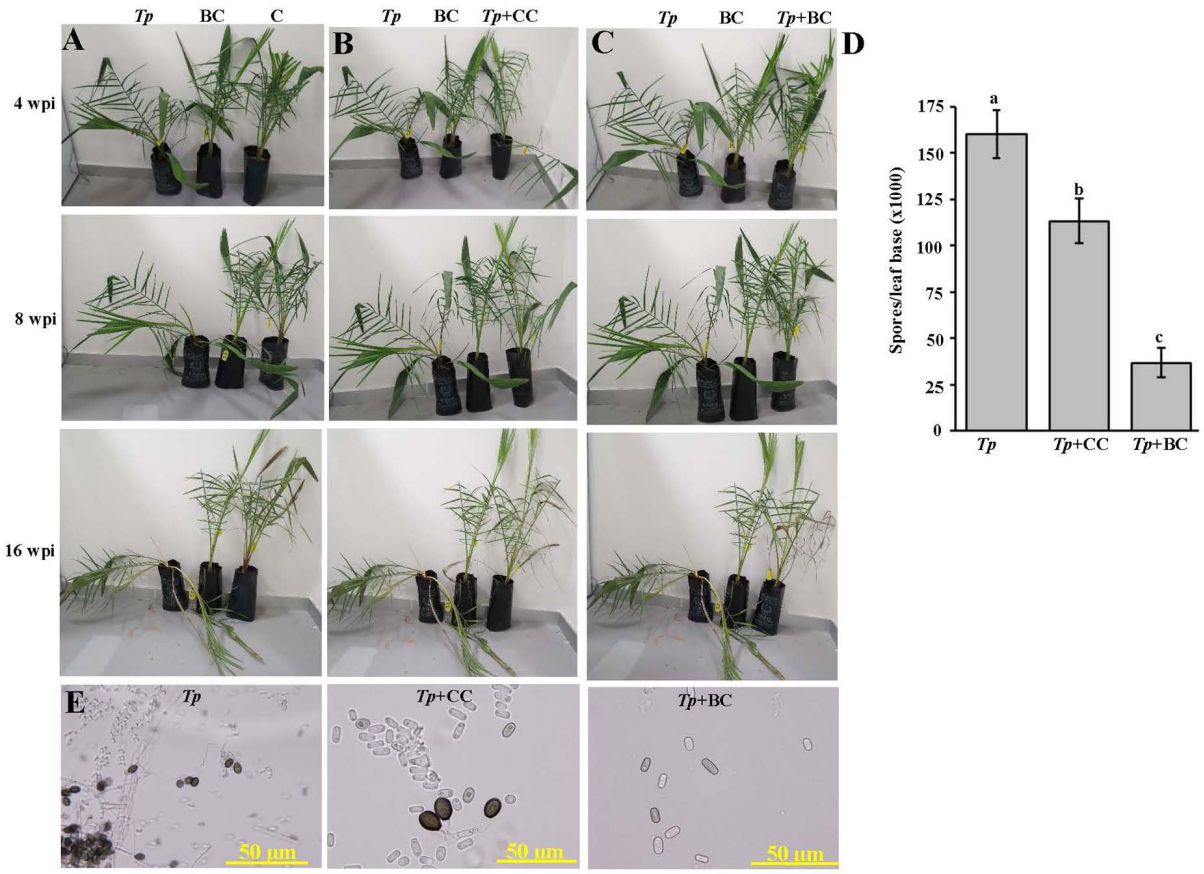


FIGURE 5 | Effect of the biocontrol agent and Cidely® Top fungicide on *Thielaviopsis punctulata*-infected plants. **(A)** Pathogenicity test on *T. punctulata*-inoculated date palm seedlings (*Tp*). Effect of **(B)** Cidely® Top fungicide treatment after *T. punctulata* inoculation (CC + *Tp*), and **(C)** biocontrol treatment in controlling black scorch disease on date palm (BC + *Tp*) at 4, 8, and 16 wpi on *T. punctulata*-inoculated seedlings. **(D)** Number of spores ml^{-1} at 16 wpi after inoculation with *T. punctulata*. Data for spores ml^{-1} represent the mean ± SE from a minimum of 12 inoculated regions; and values with different letters are significantly different at $P = 0.05$. **(E)** Pathogen conidia reisolation from affected tissues of the sick *T. punctulata*-inoculated seedlings (left), and the recovering fungicide- and BC-treated plants. In **(B–E)** seedlings inoculated with *T. punctulata* at 2 weeks before the fungicide or biocontrol treatment. Experiments were repeated at least three times with similar results. *Tp*, inoculated-seedlings with *T. punctulata* only; BC, inoculated-seedlings with BCA only; C, control (no inoculation or treatment); *Tp* + BC, inoculated-seedlings with BCA 2 weeks after *T. punctulata* inoculation, *Tp* + CC, sprayed-seedlings with the chemical control (Cidely® Top) 2 weeks after inoculation with *T. punctulata*; wpi, weeks post inoculation.

seedlings (C) (**Figure 5A**). Secondly, we sprayed Cidely® Top on diseased seedlings 2 weeks after inoculation with *T. punctulata* (CC + *Tp*) and assessed the efficacy of the fungicide for another 14 weeks post treatment (wpt; corresponding to 16 wpi with *T. punctulata*). Since we did not find major differences in the inhibition zone of mycelial growth at higher concentrations of Cidely® Top fungicide *in vitro*, we used only 250 ppm (the minimum effective concentration) in the greenhouse. At 4 wpi, the inoculated plants treated with the fungicide (CC + *Tp*) started to recover, which was in contrast to *T. punctulata*-inoculated plants (*Tp*) (**Figure 5B**). We also observed that fresh leaves emerged from the heart of date palm of inoculated seedlings treated with Cidely® Top at 8 wpi, and all dried leaves dropped by 16 wpi (**Figure 5B**). This confirmed our *in vitro* results of the inhibitory effect of the lowest effective dose of Cidely® Top on mycelial growth of *T. punctulata*. Thirdly, we applied the actinomycete BCA on seedlings after 2 wpi with *T. punctulata* (BC + *Tp*). Plants inoculated with the BCA candidate following inoculation with *T. punctulata*, recovered when compared with seedlings inoculated with *T. punctulata* (*Tp*) at all time points of inoculation with the pathogen, and appeared to be healthy and were comparable to plants that were inoculated with BCA alone (BC) (**Figure 5C**). This suggests that this BCA candidate also effectively inhibits *T. punctulata* growth *in vivo*.

We also compared the responses of the pathogen to the fungicide and the biological treatments to determine their effects on spore numbers and conidial morphology. Therefore, the spore counts at leaf base of treated date palm plants were determined. The BCA candidate (BC + *Tp*) caused a greater reduction of the number of spores, followed by Cidely® Top-treated plants (CC + *Tp*) (**Figure 5D**). At least a three-fold reduction in total spore numbers of *T. punctulata* in BCA-treated seedlings were observed compared with the fungicidal treatment and was also associated with the absence of the thick-walled, dark brown and oval aleuroconidia (chlamydospores) (**Figure 5E**). Only the second type of smooth-walled, hyaline and cylindrical phialoconidia (endoconidia) was observed in the BCA treatment (BC + *Tp*); even these occurred in lesser amount than in *T. punctulata*-inoculated (*Tp*) or fungicide-treated seedlings (CC + *Tp*) (**Figure 5E**). In general, the pathogen appeared not to be adequately aggressive to support disease progression when BCA was applied, while only a moderate inhibitory effect was observed in the case of the Cidely® Top treatment.

Effect of Application Timing of the BCA on the Pathogenicity of *Thielaviopsis punctulata*

In parallel to experiment (I), we evaluated the impact of timing of the application of the BCA on the aggressiveness of *T. punctulata*. For this purpose, three timing intervals of the BCA treatment (Experiment II) were applied to determine the best time management of the BCA on date palm black scorch. They were: (i) 1 week before inoculation with *T. punctulata* (preventive; **Figure 6A**); (ii) at the same time of inoculation (concurrent; **Figure 6B**); or (iii) 1 week after inoculation with the pathogen (curative; **Figure 6C**). All application treatments of the BCA tested suppressed the black scorch disease to varying degrees albeit their timing (**Figure 6**).

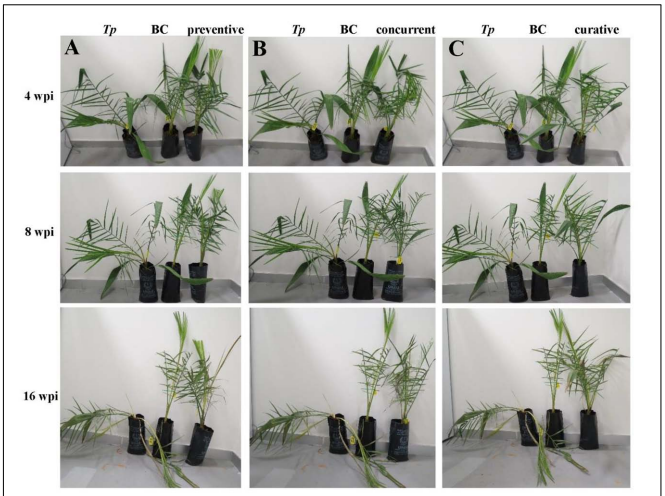


FIGURE 6 | Antagonistic effect of the biocontrol agent against *Thielaviopsis punctulata*. Effect of **(A)** Preventive, **(B)** Concurrent, and **(C)** Curative biocontrol treatment on date palm (cv. Chichi) at 4, 8, and 16 wpi with *T. punctulata*. inoculated-seedlings with *T. punctulata* only; BC, inoculated-seedlings with BCA only; preventive, inoculated-seedlings with BCA 1 week before *T. punctulata* inoculation; concurrent, inoculated-seedlings with BCA at the same time of *T. punctulata* inoculation; curative, inoculated-seedlings with BCA 1 week after *T. punctulata* inoculation. wpi, weeks post inoculation.

We also found significant differences between all treatments (Experiments I and II) when the DSI was calculated. As expected, plants infected with *T. punctulata* (*Tp*) progressed with disease from 8 wpi until they eventually died at 16 wpi (**Table 3**). There was a drastic decrease in DSI in the Cidely® Top-treated seedlings (CC + *Tp*) between 8 and 16 wpi, when compared with that of the same plants inoculated with the pathogen but with no fungicide treatment (*Tp*). Although the two curative BCA treatments at 1 wpi (curative) and 2 wpi (BC+*Tp*) with *T. punctulata* did not show significant difference in the DSI measurements between each other and the fungicide-treated plants, the results obtained from the concurrent application were clearly distinguishable. It is evident that the DSI of concurrent application of BCA and *T. punctulata* was significantly lower than that of the fungicide treatment or any of the BCA curative treatments. In comparison with the *T. punctulata*-infected plants, the DSI of the preventive applications dropped from 4.63 to 2.25 at 8 wpi and 4.88 to 1.88 at 16 wpi, providing 45–65% reduction in disease development. Control (C) seedlings not inoculated with *T. punctulata*, persistently showed no disease symptoms at any time tested. In general, the preventive application a week before inoculation with *T. punctulata* was the most effective treatment in suppressing the pathogen invasion, followed by the concurrent, and then by any of the BCA curative or fungicide treatment. Our data also clearly suggest that the appropriate timing of application of the BCA isolate is a critical factor and should precede *T. punctulata* infection for best results.

TABLE 3 | Disease severity index (DSI) of *Thielaviopsis punctulata*-inoculated-date palm seedlings (cv. Chichi) treated with fungicide or BCA (*Streptomyces globosus* UAE1) at 8 and 16 wpi ($n = 6$).

Treatment		DSI[a]	
		8 wpi	16 wpi
	Tp	4.63 *a*	4.88 *a*
Experiment I	CC + *Tp*	3.50 *b*	3.25 *bc*
	BC + *Tp*	3.00 *bc*	2.88 *c*
Experiment II	Curative	3.00 *bc*	2.50 *cd*
	Concurrent	2.75 *c*	2.00 *d*
	Preventive	2.25 *d*	1.88 *e*
	BC	0.00 *f*	0.00 *f*
	C	0.00 *f*	0.00 *f*

[a]*DSI is on a scale of 5: 0 = no infection, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% damage necrotic or dark brown area around the point of inoculation. Values with similar letters are not significantly different at P = 0.05.*

Tp, inoculated-seedlings with T. punctulata only; Tp + CC, sprayed-seedlings with Cidely® Top 2 weeks after inoculation with T. punctulata; Tp + BC, inoculated-seedlings with biocontrol agent (BCA) 2 week after T. punctulata inoculation; curative, inoculated-seedlings with BCA 1 week after T. punctulata inoculation; concurrent, inoculated-seedlings with BCA at the same time of T. punctulata inoculation; preventive, inoculated-seedlings with BCA 1 week before T. punctulata inoculation; BC, inoculated-seedlings with BCA only; C, control (non-inoculated; non-treated seedlings). wpi, weeks post inoculation.

DISCUSSION

Date palm (*Phoenix dactylifera* L.) is a popular crop in the semi-arid and arid regions. Harsh environmental (abiotic) conditions affect plant growth and production of date palm. In addition, organismal (biotic) challenges are significant limiting factors for date yields during critical developmental stages. *T. punctulata*, the main fungal pathogen causing black scorch disease on date palm, has been recorded in various date growing regions in the Arabian Peninsula (Al-Sadi et al., 2012; Al-Naemi et al., 2014) including the UAE (Saeed et al., 2016). The fungus infects the outside leaves, and rapidly kills the younger leaves and the terminal buds (Suleman et al., 2001; Zaid et al., 2002; Abbas and Abdulla, 2003). Therefore, there is an urgent need to develop novel methods to control this disease. In our efforts to develop environmentally sustainable solutions to combat this damaging disease in the UAE, we aimed to isolate an actinomycete strain from the date palm rhizosphere in the UAE; and proceeded to determine its antifungal activity against *T. punctulata*. Moreover, application of fungicides was used as a comparison and also as a part of the IDM strategy to protect plantations against serious invasions by the fungal pathogen.

Fungicides often have "curative" properties, which means they are capable of suppressing the invasion by the pathogen of the host, post-infection. Despite this ability, these chemicals can be active against a pathogen within a few days of infection. In an attempt to search for a successful fungicide for sustained inhibition of *T. punctulata*, we selected Cidely® Top fungicide and tested its efficacy under different conditions. This systemic fungicide was effective in inhibiting the fungus at the tested minimum effective dosage concentration (250 ppm) both *in vitro* and *in vivo*. Previously, we found that three systemic fungicides (Score®, Phyton®, and Naturame®) also reduced mycelial growth *in vitro*; although contact fungicides such as Ortiva® failed to have an impact on fungal growth (Saeed et al., 2016). Similar to Score®, the chemical-based fungicide Cidely® Top contains difenoconazole. This indicates that fungicides containing the active ingredient difenoconazole are highly effective in inhibiting mycelial growth of *T. punctulata*. The results obtained from difenoconazole (Score® and Cidely® Top) seem to differ with certain other aspects. A previous report indicated that this fungicide did not stimulate seed germination in sugarcane infected with *T. paradoxa* (Croft, 1998), may be attributable to the different fungicide application methods, plant species or strain differences. Foliar application of Cidely® Top also reduced the number of spores produced by *T. punctulata* in the greenhouse experiment (**Figure 5**) which may be related to the significant reduction in disease symptoms and DSI in the chemical-treated seedlings after 8–16 wpi. Hence, these findings supported our hypothesis that Cidely® Top may serve as a suitable candidate for consideration as a fungicide and a potential method in the IDM strategy against *T. punctulata*.

For decades, control and management of fungal plant diseases have been dependent on the synthetic fungicides. However, frequent use of such fungicides may cause accumulation of toxic compounds potentially hazardous to humans and the environment, and in addition may result in a high risk of pathogens developing resistance to the fungicide. Pretty and Bharucha (2015) have reported that results obtained from IDM approaches by lowering fungicide use will benefit not only farmers, but also global environment and human health. In India, it was found that the most effective component to integrate IDM and control powdery mildew caused by *Leveillula taurica* was to minimize the use of fungicides, resulting in significant yield increase of bell pepper (Kumar et al., 2008). In addition, elimination of early insecticide sprays in irrigated rice areas in Vietnam, saved farmers money and reduced pesticide use (Price, 2001). These are some successful examples of the benefits of adopting this IDM of minimizing chemical applications, and supports our finding of the potential use of the minimal effective spray of Cidely® Top as a component of IDM. Policies, laws and regulations, through authorized agencies or even governments, shall provoke the implementation of IDM to minimize reliance on fungicides, prompting European Union to intervene and promote IDM (Pretty and Bharucha, 2015).

To eliminate the judicious use of these "risky" chemicals, we evaluated the efficacy of actinomycete strains isolated from healthy date palm habitats to inhibit the phytopathogen *T. punctulata*, *in vitro* and *in vivo*. In this study, over 75% of the isolated actinomycetes from the rhizosphere soil of healthy date palms belong to the genus *Streptomyces*. This study is in accordance with the previous reports that streptomycete actinomycetes are known to be predominant among actinomycetes on isolation plates and commonly produce useful antibiotics (∼80% of the total antibiotic production) and active secondary metabolites (Thenmozhi and Krishnan, 2011). The identity of the BCA (isolate #7) was further confirmed by the ribosomal gene (16*S* rRNA) sequence analysis, and the isolate

revealed 100% sequence similarity with *S. globosus* (Krassilnikov, 1941) Waksman in Waksman and Lechevalier (1953) and *Streptomyces toxytricini* (Preobrazhenskaya and Sveshnikova, 1957; Pridham et al., 1958). The 16*S* rRNA sequencing has been used as a basic approach for the identification of microbial communities as well as for assessing microbial diversity in natural environments (Solanki et al., 2014). Based on morphological, cultural and physiological characterizations, our results confirmed that the selected species was *S. globosus* (Strain UAE1).

Emphasis was made to look for promising BCAs among actinomycetes as they are known to be relatively suited to be active in dry hot environments such as those in the UAE. They are also known to include many strains capable of functioning as BCAs (El-Tarabily and Sivasithamparam, 2006). *S. globosus* UAE1 exhibited strong antifungal activity against *T. punctulata*, mainly attributable to the production of diffusible antifungal metabolites, but not mycolytic cell-wall degrading enzymes, volatile metabolites, hydrocyanic acid, or iron-chelating siderophores. Multiple dual-culture assays (Bacharach and Cuthbertson, 1948; Pridham et al., 1956; El-Tarabily et al., 1997, 2009) were used in the current study in order to evaluate the nature of the antagonistic activity of this BCA against the black scorch causing agent *in vitro*. In this study, microscopic examination was performed to find out the mode of action and interaction of the antagonistic isolate with the pathogen. The observations revealed that the strain *S. globosus* was capable of causing considerable morphological alternations of hyphae such as cytoplasmic coagulation, shriveled and swelling mycelia, and septal malformations. Similar morphological changes in hyphae due to the activity of antifungal compounds have been demonstrated with other phytopathogens (Wang et al., 2010; Lu et al., 2013). Although many researches have noted promising results relating to microbial antagonism using actinomycetes under laboratory conditions, many of those isolates failed to repeat their performance under greenhouse and field conditions (Doumbou et al., 2001; El-Tarabily and Sivasithamparam, 2006).

To eliminate the discrepancies between *in vitro* and *in vivo* assays, we tested the effect of the strain *S. globosus* UAE1 on *T. punctulata* under greenhouse conditions. In general, our results demonstrated that biological control was more effective in reducing disease development than the chemical fungicide; and this reduction was dependent on the time of application. For example, curative treatments using BCA to established infections by the pathogen would limit damage to the tree and prevent *T. punctulata* spores from germinating within the date palm grove (**Figure 5**). Competition between the BCA and *T. punctulata* applied together led to a greater control efficacy, evident in reduced DSI. We argue that prevention of infection is the best management strategy when it comes to dealing with black scorch disease on date palm plantations. Application of BCA in advance pathogen invasion had more profound effects of biocontrol efficacy, as the main mechanism of protection appears to help establish the required biomass of the BCA ahead of the pathogen invasion and prevent the systemic ingression of *T. punctulata* within the host. The diffusible compounds produced by the BCA candidate were clearly related to the inhibition, destruction and suppression of the invading pathogen within the plant host. There is, however, a possibility of the production by the BCA of compounds capable of inducing host resistance to the pathogen. This possibility was not explored at this stage but certainly will be investigated in future studies. One should not eliminate the possibility that the "preventive" treatment may also induce ISR in plants to manage the black scorch disease on date palm. This form of resistance by the BCA candidate, can be brought through fortifying the physical and mechanical strength of cell wall (Knoester et al., 1999), leading to the synthesis of defense chemicals against *T. punctulata* attack. Defense reactions may activate a diverse array of plant defense genes encoding pathogenesis-related (PR) proteins i.e., chitinase, β-1,3-glucanases, chalcone synthase, phenylalanine ammonia lyase, peroxidase and phytoalexins (Ahn et al., 2002).

The commercial product, Mycostop®, is a biological fungicide that contains spores and mycelium from *Streptomyces griseoviridis*. Application of Mycostop® to the root zone of crop plantations, including date palm, reduced spore germination and inhibited hyphal growth of plant pathogenic fungi including *T. punctulata* (Suleman et al., 2002; Minuto et al., 2006). Other commercial *Streptomyces* biocontrol agents, such as *Streptomyces lydicus* (Actinovate®, Micro108® or Actino-iron®) and *Streptomyces saraceticus* (YAN TEN), have been released to the market (Elliott et al., 2009; Palaniyandi et al., 2013). This suggests that the *Streptomyces* strain isolated in this study can serve as a potential antifungal product against *T. punctulata*. Whilst a number of recent reports have focused on biological controls using species of *Trichoderma* or *Chaetomium* against *T. punctulata* or *T. paradoxa* growth (Soytong et al., 2005; Chakrabarty et al., 2013; Al-Naemi et al., 2016), the current study demonstrates, for the first time, the feasibility of using a streptomycete actinomycete isolate as a BCA against black scorch disease caused by *T. punctulata*.

Application of a BCA can be considered as a successful practice only if it is relatively safe to humans (U. S. Environmental Protection Agency, 2005), is effective over a long duration (Clewley et al., 2012), survives under adverse conditions (McFadyen, 1998) and, if possible, improves plant growth (Mefteh et al., 2017). *Streptomyces* spp. are inexpensive, long lasting, safe, and can survive various harsh conditions (Ningthoujam et al., 2009). Similarly, the isolated *S. globosus* UAE1 strain has the capability to produce spores under extreme heat and drought conditions common to the UAE environment. In this study, actinomycetes were specifically targeted because they are likely better adapted to the UAE environment compared to other bacteria or fungi; in addition of having the ability to be active under conditions prevalent in the dry and arid environments (Goodfellow and Williams, 1983).

Reports focusing on biological control often provide partial or full protection. Thus far, many studies recommend using IDM as an applied disease control strategy combining chemical and biological antagonists on crops (AbuQamar et al., 2017) along with breeding and biotechnology programs (AbuQamar et al., 2016; Sham et al., 2017). We propose that isolates of *S. globosus* such as the one we studied is an excellent candidate as BCA for the management of this devastating disease.

Future 'omics' analyses will advance our understanding of the biology of *T. punctulata*, and will shed light on the complex *T. punctulata*-date palm interaction. Ultimately, our long-term goal is to develop future strategies to effectively manage the black scorch disease using environmentally sustainable strategies. Although many actinomycete isolates have been identified from a wide range of environmental habitats to manage other fungal pathogens in other countries (Goodfellow and Williams, 1983; Doumbou et al., 2001; El-Tarabily and Sivasithamparam, 2006), this is the first report of the antagonistic activity of an actinomycete strain against any *Thielaviopsis* species, isolated from UAE soils, or elsewhere.

AUTHOR CONTRIBUTIONS

ES, RI, KE-T, and SAQ designed the research. KE-T and SAQ supervised the study. ES, AS, ZS, YA, and KE-T performed *in vitro* and *in vivo* experiments. ES, AS, KE-T, and SAQ performed *in vivo* greenhouse experiments. AS and SAQ developed the phylogenetic analysis. RI, KE-T, and SAQ analyzed the data. ZS and YA assisted with experiments and/or data evaluation. K-ET and SAQ wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

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Biological Control of Mango Dieback Disease Caused by *Lasiodiplodia theobromae* Using Streptomycete and Non-streptomycete Actinobacteria in the United Arab Emirates

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Dieback caused by the fungus *Lasiodiplodia theobromae* is an important disease on mango plantations in the United Arab Emirates (UAE). In this study, 53 actinobacterial isolates were obtained from mango rhizosphere soil in the UAE, of which 35 (66%) were classified as streptomycetes (SA) and 18 (34%) as non-streptomycetes (NSA). Among these isolates, 19 (12 SA and 7 NSA) showed antagonistic activities against *L. theobromae* associated with either the production of diffusible antifungal metabolites, extracellular cell-wall-degrading enzymes (CWDEs), or both. Using a "novel" mango fruit bioassay, all isolates were screened *in vivo* for their abilities to reduce lesion severity on fruits inoculated with *L. theobromae*. Three isolates, two belonging to *Streptomyces* and one to *Micromonospora* spp., showed the strongest inhibitory effect against this pathogen *in vitro* and were therefore selected for tests on mango seedlings. Our results revealed that the antifungal action of *S. samsunensis* UAE1 was related to antibiosis, and the production of CWDEs (i.e., chitinase) and siderophores; whilst *S. cavourensis* UAE1 and *M. tulbaghiae* UAE1 were considered to be associated with antibiotic- and CWDE-production, respectively. Pre-inoculation in greenhouse experiments with the most promising actinobacterial isolates resulted in very high levels of disease protection in mango seedlings subsequently inoculated with the pathogen. This was evident by the dramatic reduction in the estimated disease severity indices of the mango dieback of individual biocontrol agent (BCA) applications compared with the pathogen alone, confirming their potential in the management of mango dieback disease. *L. theobromae*-infected mango seedlings treated with *S. samsunensis* showed significantly reduced number of defoliated leaves and conidia counts of *L. theobromae* by 2- and 4-fold, respectively, in comparison to the other two BCA applications. This indicates that the synergistic antifungal effects of *S. samsunensis* using multiple modes of action retarded the *in planta* invasion of *L. theobromae*. This is the first report of BCA effects against *L. theobromae* on mango seedlings by microbial antagonists. It is also the first report of actinobacteria naturally existing in the soils of the UAE or elsewhere that show the ability to suppress the mango dieback disease.

Keywords: actinobacteria, antibiosis, biocontrol, chitinase, dieback, mango, *Lasiodiplodia theobromae*, UAE

INTRODUCTION

Mango (*Mangifera indica* L.), frequently recognized as "the king of fruits," is a popular fruit in tropical and subtropical regions (Usman et al., 2001; Berardini et al., 2005). Due to its delicious taste, high nutritional value and economical importance in international markets, mango has increasingly been cultivated not only in its traditional producing areas, but also in non-traditional production countries such as the United Arab Emirates (UAE) (Nelson, 2008; Saeed et al., 2017a). Mango can be attacked by a number of bacterial and fungal pathogens causing several diseases in all parts of the tree and at all stages of its life (Ploetz, 2004). Studies have identified the pathogenic fungus, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. (Zambettakis, 1954; Sutton, 1980), as the causal agent of mango dieback disease in different areas of the world, including Brazil, Korea, India, Oman, Pakistan, USA (Sharma et al., 1994; Ploetz et al., 1996; Al Adawi et al., 2003; Khanzada et al., 2004; de Oliveira Costa et al., 2010; Hong et al., 2012) and the UAE (Saeed et al., 2017a). Thus, dieback is considered to be the most destructive disease, leading to significant yield loss and low fruit quality of mango (Ploetz, 2003).

The disease symptoms of dieback on mango are commonly associated with drying and withering of twigs from top downwards, followed by discoloration, drying and eventual dropping of leaves (Khanzada et al., 2004). Other symptoms can also be observed on other parts of the tree, including reproductive structures (Naqvi et al., 2014). In advanced stages of the disease, branches dry one after another, resulting in the appearance of bare twigs and the decline of trees. Typically, a complete wilting and death of the affected mango trees may occur within weeks or few months after infestation with *L. theobromae* (Saeed et al., 2017a). Regrettably, once the symptoms of dieback are present, it is very hard to save the mango orchard or reverse the disease development. In the field, poor orchard management and unfavorable environmental stresses such as drought, heat, sun scorch, water stress, salinity and nutritional deficiency, can also provoke the progress of disease (Kazmi et al., 2005; Paolinelli-Alfonso et al., 2016). Studies have shown that most common varieties of mango are highly susceptible to dieback disease caused by *L. theobromae* (Ramos et al., 1997). In general, dieback is a serious disease of mango, which causes damage to tree health and considerable loss of fruit yield. Thus, there is an urgent need for research to find innovative and safe solutions for this destructive disease.

Unfortunately, strategies involving early applications with chemical fungicides on affected plants are still the main means to lessen the severity of most diseases on crops (Saeed et al., 2016). The increasing awareness of fungicide-related risks have further highlighted the need for adopting alternative and sustainable methods, such as proper horticultural practices, biological control (or biocontrol) agents (BCAs) and safe natural compounds, to replace the dependence on chemical fungicides for disease control (Ma and Michailides, 2005). Integrated pest management (IPM) aims at using a combination of practices and minimizing chemical inputs that are only applied when needed (López-Escudero and Mercado-Blanco, 2011). In that regard, efforts toward using natural enemies native to the same environment can effectively reduce or exterminate pathogen populations (AbuQamar et al., 2017; Syed Ab Rahman et al., 2018). *In vitro* and field studies were previously applied against *L. theobromae* using species of *Bacillus* to control seed and seedling rot of bottle gourd (Sultana and Ghaffar, 2010), *Trichoderma* and *Aspergillus* to control inflorescence blight of cashew (Adeniyi et al., 2013), and *Pseudomonas* to control stem-end rot on mango fruits (Seethapathy et al., 2016). No information exists on potential antagonistic microorganisms that have been identified to be capable of managing mango dieback disease under greenhouse/field conditions caused by *L. theobromae*, as a component of an IPM strategy.

Actinobacteria are a group of Gram-positive bacteria that include some of the most common soil bacteria (Locci and Sharples, 1984). *Streptomyces*, a common actinobacterial genus, is also a biologically active "vehicle" of the soil microbiota (Barka et al., 2016) that can play a vital role against phytopathogens including fungi and oomycetes (El-Tarabily et al., 2009; Saeed et al., 2017b). In comparison, other genera of actinobacteria, such as *Actinoplanes*, *Microbispora*, *Micromonospora* and *Streptosporangium* have rarely been investigated as BCAs and/or plant growth promoters (PGPs) (El-Tarabily et al., 1997, 2009; El-Tarabily and Sivasithamparam, 2006).

The biological control of soil-borne fungal pathogens by actinobacteria may involve several mechanisms (Doumbou et al., 2001; Whipps, 2001; Kinkel et al., 2012), including inhibition of pathogen growth through the production of antifungal metabolites (Getha et al., 2005; Palaniyandi et al., 2013; Saeed et al., 2017b), production of siderophores (Xue et al., 2013), destructive parasitism (El-Tarabily et al., 1997), competition for infection sites and/or nutrient resources (Cook and Baker, 1983), and lysis of fungal hyphae by the production of cell-wall-degrading enzymes (CWDEs; glucanases and chitinases) (Valois et al., 1996; Mahadevan and Crawford, 1997; El-Tarabily et al., 2000; El-Tarabily, 2006; Singh and Gaur, 2016). Soil-borne beneficial bacteria, such as actinobacteria, may also trigger induced systemic resistance (ISR) in plants and reduce the effects of pathogen attacks through the induction of their defense mechanisms (Martínez-Hidalgo et al., 2015). As a result, application of BCAs represents an environmentally-friendly strategy for sustainable agriculture and cost-efficient protection that can enhance crop productivity.

Previously, we identified *L. theobromae* as the mango dieback pathogen in the UAE, and evaluated the activity of systemic fungicide treatments in providing protection against this disease on mango plants in the greenhouse and in the field (Saeed et al., 2017a). In the current study, we specifically targeted actinobacteria as potential candidates as they are mostly well-adapted to be active in the arid environment, in comparison to other bacteria and fungi (Goodfellow and Williams, 1983). We isolated a wide spectrum of streptomycete actinobacteria (SA) and non-streptomycete actinobacteria (NSA) from mango rhizosphere. The objectives of the present investigation were to: (i) screen all the actinobacterial isolates for their ability to produce *in vitro*, diffusible antifungal metabolite(s) and/or CWDEs capable of inhibiting *L. theobromae*, (ii) select the

promising isolates using a novel mango fruit bioassay which we developed, to assess their potential to reduce disease progression on fruits, and (iii) evaluate the differences in the potential of the promising isolates with known mode(s) of antagonism under greenhouse conditions, for their effectiveness in controlling mango dieback disease. Our results demonstrate the potential to use selected actinobacteria with more than a single mode of action as antagonists to be incorporated into sustainable IPM strategies to manage dieback disease in mango orchards in the UAE and elsewhere.

MATERIALS AND METHODS

Fungal Growth and Culture

The pathogen, *L. theobromae* (DSM 105134), was previously reported as the cause of mango dieback disease in the UAE (Saeed et al., 2017a). Strain DSM 105134 was isolated from tissues sampled from diseased mango trees affected by *L. theobromae*, and identified using combined *ITS* sequences (GenBank accession number: MF114110). The pathogen was cultured on potato dextrose agar (PDA, pH 6.0; Lab M Limited, Lancashire, United Kingdom) plates, supplemented with 25 mg l^{-1} ampicillin (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) to suppress bacterial contaminants. The fungus was sub-cultured every 10 days on PDA plates at 28°C.

Isolation of SA and NSA From Mango Rhizosphere

Five rhizosphere soil samples were collected from 30 cm depth under healthy mango trees in sealable plastic bags. The rhizosphere soil samples were air-dried for 4 days at 25°C (Williams et al., 1972), passed through a 5 mm mesh sieve, and stored prior further analyses.

The soil dilution plate method was used to isolate SA from each rhizosphere sample using inorganic salt starch agar (Küster, 1959) supplemented with the antifungal antibiotics nystatin and cycloheximide (50 μg ml^{-1} each; Sigma-Aldrich). In order to increase and decrease the populations of filamentous actinobacteria and other bacteria, respectively, the soil pre-treatments described by Hayakawa and Nonomura (1987) was used. For each dilution, seven plates were used and incubated at 28°C in dark for 7 days.

For the NSA recovery, four polyvalent *Streptomyces* phages (El-Tarabily, 2006) were used to reduce the dominance of SA on inorganic salt starch agar plates (Kurtböke et al., 1992). The stock phage suspension (10^{12} plaque forming units ml^{-1}) was prepared by mixing high-titer phage suspensions of each polyvalent *Streptomyces* phage. Seven plates were inoculated with 0.2 ml aliquots of the phage-treated soil suspension, dried and incubated in dark at 28°C for 14 days. Control treatments were considered as plates without phages.

Colonies of SA and NSA, expressed as $\log_{10}$ colony forming units (cfu) g^{-1} dry soil, were purified on oatmeal agar plates (ISP medium 3) amended with 0.1% yeast extract (Küster, 1959). Colonies were identified based on morphological features, distribution of aerial/substrate mycelia, presence/absence of aerial mycelia, and the stability/fragmentation of substrate mycelia (Cross, 1989).

Detection of the Antifungal and CWDE Activities

We characterized all actinobacterial isolates based on their ability to secrete diffusible antifungal metabolites active against *L. theobromae* using the cut-plug method (Pridham et al., 1956). The actinobacterial isolates were inoculated on fish meal extract agar (El-Tarabily et al., 1997) plates and incubated at 28°C in dark for 7 days. PDA-seeded plates were prepared by initially cultivating *L. theobromae* on PDA slants at 28°C until sporulation, which were then flooded with 50 mM phosphate buffer (pH 6.8) (Saeed et al., 2017b). Spores and some mycelial fragments were homogenized at 4,000 rpm for 20 min; and the resulting supernatants were diluted in PDA plates. The inoculum consisted of approximately 10^8 cfu ml^{-1}. PDA-seeded plates with non-inoculated agar plugs served as control. Plugs were transferred from the actinobacterial cultures on fish meal extract agar with a sterilized 11 mm cork-borer onto PDA plates seeded with *L. theobromae* kept at 28°C in dark for 5 days. The diameters of zones of inhibition were determined. Five plates were used for each actinobacterial isolate. The most promising antifungal metabolite-producing isolates showing the largest zone of inhibition were picked for further experiments; and the remaining of the isolates were not used in the subsequent tests.

All isolates were also tested for their abilities to produce clearing zones on *L. theobromae* mycelial fragment agar as an indicator of preliminary production of CWDEs according to Valois et al. (1996). Large (>30 mm) and small (<30 mm) diameters represented high and low CWDE activities, respectively. In addition, all obtained isolates were evaluated for their potential to produce chitinase enzyme. Each isolate was inoculated onto colloidal chitin agar plates, and incubated at 28°C in dark for 7 days (Gupta et al., 1995). The clearing zones surrounding the colonies were measured and used to detect the chitinase activity. Large (>30 mm) and small (<30 mm) diameters represented high and low chitinase activities, respectively. Five replicate plates were used for each actinobacterial isolate. The most promising, highly active CWDE-producing isolates showing the largest clearing zones on both mycelial fragment agar and colloidal chitin agar plates were chosen for further experiments.

Mango Fruit Bioassay

A novel mango fruit bioassay was developed in our laboratory to determine the ability of the most promising candidates to suppress or reduce disease development (lesion formation) following inoculation with *L. theobromae in vivo*. The mango fruit bioassay was modified according to previous tests of carrot bioassay against *Pythium coloratum* (El-Tarabily et al., 1997) and mango fruit pathogenicity against *L. theobromae* (Saeed et al., 2017a).

Mature mango fruits (cv. Badami) were placed in plastic trays on sterile paper towels moistened with sterile distilled water, and were inoculated by placing the agar plugs (11 mm) colonized by the actinobacterial isolates and/or *L. theobromae*,

described above, onto each mango fruit according to the following combinations: (i) a sterile non-inoculated PDA agar plug (control; C); (ii) the antagonist alone (BCA) with a sterile PDA agar plug above it; (iii) *L. theobromae* (*Lt*) alone with a sterile PDA agar plug below it; and (iv) pairing *L. theobromae* and the antagonists together (BCA+*Lt*), with the BCA on the mango surface and *L. theobromae*-inoculated plug on top of the BCA. The antagonists were inoculated onto the mango surface 24 h prior the pathogen in order to allow time for the secretion of antifungal metabolites and/or chitinase onto the mango surface. Each mango fruit was inoculated with the four treatment combinations for each BCA of four fruits/tray in triplicates. Trays were covered with aluminum foil and incubated under humid conditions in dark at 28°C for 4 days. The lesion diameters were measured in order to determine disease indices. To fulfill Koch's postulates, all diseased fruit tissues were incubated on PDA plates at 28°C in dark for 5 days.

Assays of Producing Diffusible Antifungal Metabolites or Chitinase

We assessed the three most promising antagonistic BCAs for their ability to secrete diffusible antifungal metabolites active against *L. theobromae* using the cup plate technique as previously described (Bacharach and Cuthbertson, 1948). Inocula for the preparation of the *L. theobromae*-seeded PDA plates were prepared, as described above, for the cut-plug method. In order to assess the inhibition of *L. theobromae* by the diffusible antifungal metabolites on fish meal extract agar (El-Tarabily et al., 1997) or by the chitinase on colloidal chitin agar (El-Tarabily et al., 2000), a dialysis membrane overlay technique (Gibbs, 1967) was used.

Briefly, dialysis membrane (Type 45311; Union Carbide Corporation, IL, USA) with adhering colonies were removed from the agar plates and the center of each plate was inoculated with a disc (5 mm diameter) of *L. theobromae* culture. At the end of the incubation period, the colony diameter of *L. theobromae* was measured. The agar plugs were further transferred to a fresh PDA plate and incubated at 28°C for 5 days to determine whether the diffused metabolites/chitinase were fungistatic (pathogen growth from the plug) or fungicidal (no pathogen growth from the plug).

Volatile Antifungal Compounds, Hydrocyanic Acid and Siderophore Production

Production of volatile antifungal compounds (Payne et al., 2000) by the BCAs was examined using fish meal extract agar. For the production of hydrogen cyanide (hydrocyanic acid), the BCAs were inoculated on tryptic soy agar medium (Lab M Limited) supplemented with 4.4 g glycine l^{-1}. The plates were inverted and a piece of filter paper (soaked in 0.5% picric acid in 2% sodium carbonate) was placed in the lid of each Petri dish, and incubated at 28°C for 5 days (Bakker and Schippers, 1987). Discoloration of the filter paper to orange brown after incubation indicates production of hydrogen cyanide (Castric, 1975).

For siderophore production, plates of chrome azurol S (CAS) agar developed by Schwyn and Neilands (1987), were inoculated with the BCAs and incubated at 28°C in dark for 7 days. Development of yellow-orange halo zone around the colony was considered positive for siderophore production.

Determination of CWDE Activities of the BCA Candidates

Erlenmeyer flasks containing 50 ml of minimal synthetic medium (Tweddell et al., 1994) supplemented with 2 mg ml^{-1} of either *L. theobromae* cell wall fragments, colloidal chitin, or laminarin (Sigma-Aldrich) were prepared. Flasks containing each substrate were inoculated with 2 ml of a 20% glycerol suspension of each BCA (10^8 cfu ml^{-1}), incubated on a rotary shaker (Model G76, New Brunswick Scientific, NJ, USA) at 250 rpm for 7 days, and further centrifuged at 12,000 × *g* for 30 min. The supernatant was filtered using 0.22 μm Millipore membranes (Millipore Corporation, MA, USA) and used as a source of crude enzymes (El-Tarabily, 2003).

Chitinase and ß-1,3-glucanase activities were determined by measuring the release of N-acetyl-D-glucosamine and the amount of reducing sugars liberated using dinitrosalicylic acid solution (Miller, 1959), respectively. The protein content of the enzyme solution was determined as described by Lowry et al. (1951) using Folin phenol reagent.

Effect of BCA Crude Culture Filtrates on Mycelia and Conidia of *L. theobromae*

The filter-sterilized crude culture filtrate for each BCA (section Determination of CWDE Activities of the BCA Candidates) using fish meal extract broth or colloidal chitin broth (Gupta et al., 1995) was proportionally poured in PDA plates. The medium was inoculated with a 5 mm diameter agar plug colonized with *L. theobromae* mycelium (placed upside down). The colony diameter (mm) of *L. theobromae* was measured after 5 days at 28°C.

The crude culture filtrate prepared from fish meal extract broth or colloidal chitin broth was also proportionally mixed with potato dextrose broth (PDB; Lab M) (Lorito et al., 1993). The PDB was inoculated with a 5 mm diameter agar plug colonized with *L. theobromae*. The dry weight of *L. theobromae* was measured after 10 days of incubation in dark at 28°C.

The effect of the crude culture filtrate of each BCA on mature conidia germination and germ tube elongation of *L. theobromae* was carried out in PDB according to Lorito et al. (1993). The percentage spore germination and average germ tubes lengths were microscopically determined after 24 h at 40X using Nikon-Eclipse 50i light microscope (Nikon Instrument Inc., NY, USA) and compared with the control (non-inoculated filter-sterilized fish meal extract broth or colloidal chitin broth).

The effect of the crude culture filtrate of the three BCAs on the morphology of *L. theobromae* hyphae was assessed (Sneh, 1981). At sampling, *L. theobromae* hyphae treated with the BCA was microscopically examined at 100X using a light microscope. *L. theobromae* mycelium incorporated with non-inoculated filter-sterilized fish meal extract broth or colloidal chitin broth served as control treatments. Three replicates were used at each sampling.

Identification and Phylogenetic Analysis of the BCA Candidates

The identification of the three promising BCAs, BCA1 (isolate #12), BCA2 (isolate #29) and BCA3 (isolate #44), was carried out using 16*S* rRNA gene sequence analysis done by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ), Braunschweig, Germany, using the primers 900R (5′-CCGTCAATTCATTTGAGTTT-3′); 357F (5′-TACGGGAGGCAGCAG-3′) and 800F (5′-ATTAGATACCCTGGTAG-3′) (Rainey et al., 1996). Sequences for BCA1, BCA2 and BCA3 were deposited in Genbank with accession numbers MG548382, MG461691, and MF872601; respectively. Phylogenetic tree was constructed to predict the species level characterization of the studied isolates using the maximum likelihood method implemented in Molecular Evolutionary Genetics Analysis 7.0 (MEGA7) software (Felsenstein, 1981; Kumar et al., 2016) after multiple alignments of the data by CLUSTAL_X (Thompson et al., 1997). In each case, bootstrap values were calculated based on 1,000 resamplings.

Identification of BCA1 and BCA2 isolates was further confirmed based on cultural, morphological, and physiological characteristics as described by Locci (1989). Scanning electron microscopy (SEM) was carried out for the three BCA isolates (BCA1, BCA2, and BCA3) using Philips XL-30 SEM (FEI Co., Eindhoven, The Netherlands) to determine the morphology of the spore chains and surface.

Disease Assays and Greenhouse Trials

For disease assays on mango seedlings under greenhouse conditions, the growing tip region of the stem of 12-month-old mango (cv. Badami) seedlings were surface-sterilized with 70% ethanol, mechanically wounded, and inoculated with 5 mm PDA plugs colonized with *L. theobromae* culture or non-colonized plugs (controls) (Saeed et al., 2017a). The area of inoculation was covered with Parafilm. Inoculated seedlings were kept at 28°C under greenhouse conditions, and monitored for disease development.

In vivo evaluations of the BCAs were also carried out on mango seedlings. We aimed to investigate the efficacy of the three BCA treatments to manage dieback disease. Similar to the *L. theobromae* treatment described above, methods of inoculation with the pathogen and BCA application were used. All BCA treatments were preventive (seedlings treated with each BCA 1 week before *L. theobromae* inoculation). The treatments/groups used for this experiment were as follows:

Healthy controls (C): Non inoculated control seedlings;

Diseased controls (*Lt*): Seedlings inoculated with *L. theobromae* only;

Biocontrol treatment without pathogen (*Ss*, *Sc,* or *Mt*): Seedlings inoculated with either *S. samsunensis, S. cavourensis* or *M. tulbaghiae,* respectively;

Preventive biocontrol treatment (*Ss*+*Lt*; *Sc*+*Lt*, or *Mt*+*Lt*): Seedlings inoculated with either *S. samsunensis, S. cavourensis* or *M. tulbaghiae*, respectively, 1 week before *L. theobromae* inoculation.

For each treatment/group, six plants in separate pots, arranged in a completely randomized design, were used. Control (healthy and diseased) and inoculated seedlings were maintained under controlled greenhouse conditions of 15 h light/9 h dark under fluorescent lights (160 W mol^{-1} m^{-2} s^{-1}) at 28°C. Corresponding with the disease symptoms/recovery, disease severity index (DSI) was recorded at 3 and 9 weeks post inoculation (wpi) using the following scale: 0 = no apparent symptoms, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% necrotic or dark brown area around the point of inoculation (Saeed et al., 2017a). The number of falling leaves and the total number of fungal conidia in inoculated plants were determined at 6 and 9 wpi, respectively. Harvested conidia from three leaf bases of 6 inoculated seedlings per treatment were counted using a haemocytometer (Agar Scientific Limited, Essex, UK) (Saeed et al., 2017a).

Statistical Analyses

For mango fruit bioassay, the effect of actinobacteria on lesion formation was evaluated and analyzed using Analysis of Variance (ANOVA). Each mango fruit was inoculated with the four treatments and each tray contained four fruits with three replicate trays for each isolate. Significant differences between means at $P = 0.05$ were determined by Duncan's multiple range test.

ANOVA and Duncan's multiple range test at 5% level of significance were used to analyze the *in vitro* evaluation of BCA against *L. theobromae*. Experiments were repeated in triplicates using five plates per treatment for each time with similar results.

For the falling leaves and fungal conidia counts of the *in vivo* treatments against *L. theobromae* in the greenhouse trial, six plants were used for each treatment. ANOVA and Duncan's multiple range test were used to determine the statistical significance ($P < 0.05$). All experiments were repeated independently three times with similar results.

Three replicates for each group (6 plants each) were tested for the DSI of the *in vivo* treatments. ANOVA and Duncan's multiple range test were conducted to determine the statistical significance at $P < 0.05$. Similar results were obtained in each replicate. For all statistical analyses, SAS Software version 9 was used (SAS Institute, 2002).

RESULTS

Production of Diffusible Antifungal Metabolites and CWDEs by BCAs

Fifty-three SA and NSA strains were isolated from mango rhizosphere, of which 35 SA (66.1%) and 18 NSA (33.9%) were obtained from inorganic salt starch agar plates. The *Streptomyces* phages with high polyvalency were used to facilitate the isolation of NSA from rhizosphere samples on inorganic salt starch agar plates (**Figure 1**). Consequently, the numbers of SA were significantly ($P < 0.05$) reduced, but the numbers of NSA increased on the plates treated with the four phages (**Table 1**). Therefore, SA and NSA (*Actinoplanes, Actinomadura, Microbispora*, *Micromonospora*, *Nocardia*, *Rhodococcus*, and *Streptosporangium* spp.) were readily isolated and identified to the genus level based on morphological features, distribution

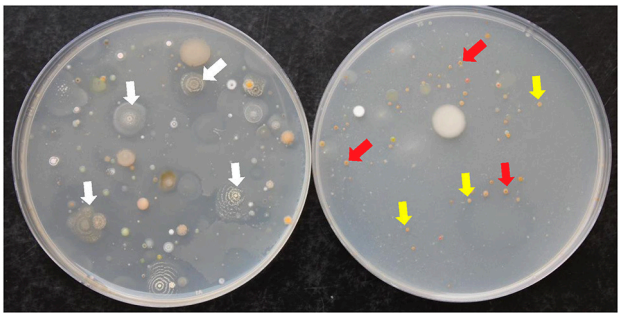


FIGURE 1 | Colonies of actinobacteria isolated from mango rhizosphere grown on inorganic salt starch agar plates. Soil dilutions tubes were treated without **(Left)** and with **(Right)** four polyvalent *Streptomyces* phages. White arrows represent the dominance of streptomycete actinobacterial colonies **(Left)**; whereas yellow and red arrows represent the dominance of non-streptomycete actinobacterial colonies i.e., *Actinoplanes* and *Micromonospora* spp., respectively **(Right)**.

of aerial and/or substrate mycelia, presence/absence of aerial mycelia, and the stability or fragmentation of substrate mycelia.

We found that 11 out of 53 of the rhizosphere actinobacterial (7 SA and 4 NSA) isolates were capable of producing strong antifungal metabolites active against *L. theobromae* using the cut-plug method (**Table 2**). Eleven isolates (#3, 7, 9, 12, 16, 21, 25, 29, 42, 49, and 50) produced large zones of pathogen inhibition (>30 mm), and were considered as the most promising BCA candidates (**Table 2**; **Figure 2A**); thus were selected for further analyses. The rest of the isolates that caused very low levels of inhibition (<30 mm) were not included in the subsequent studies.

Of the 53 isolates, 8 SA and 4 NSA (isolates #3, 10, 12, 18, 31, 33, 41, 44, 45, 49, 50, and 51) were ranked as highly active chitinase-producing isolates. These 12 isolates produced large clearing zones (>30 mm) around the colony on colloidal chitin agar plates and on *L. theobromae* mycelial fragment agar (**Table 2**; **Figure 2B**). The remainder of the isolates produced small clearing zones (<30 mm) and were not further assessed. It is noteworthy to mention that four BCA candidates (isolates #3, 12, 49, and 50) produced both the diffusible antifungal metabolites and CWDEs (**Table 2**). This suggests that the isolated SA and NSA from the local mango rhizosphere soil samples may have antifungal activities of single or multiple modes of action against plant pathogens, including *L. theobromae*.

Selection of the Most Promising Antagonistic BCA Candidates

We used the mango fruit bioassay method to evaluate the most effective 19 BCA candidates against *L. theobromae* (**Table 2**; **Figure 3A**). Lesions produced on the fruits by the pathogen (*Lt*) alone were relatively large, brownish, round to elliptical, water-soaked and depressed, with clear margins (**Figures 3B–D**). When certain isolates were paired with the pathogen (BCA+*Lt*) on the mango fruit surface, they completely suppressed the pathogen with no lesions formed compared with the treatment

TABLE 1 | The effect of introducing four polyvalent *Streptomyces* phages on the colony-forming units of streptomycete and non-streptomycete actinobacteria from mango rhizosphere soil.

Actinobacteria	Without phage	With phage
	$\log_{10}$ cfu g soil^{-1}	
SA	5.96 ± 0.45*a*	2.04 ± 0.31*b*
NSA	1.86 ± 0.18*a*	3.70 ± 0.26*b*

Values are means of seven replicates ± SE. Within rows, values followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range test. SA, streptomycete actinobacteria; NSA, non-streptomycete actinobacteria.

with the pathogen plug alone (*Lt*) (**Table 2**; **Figure 3**). Certain isolates significantly ($P < 0.05$) reduced lesion development in comparison to the treatment with the pathogen alone (**Table 2**).

In general, ten isolates (6 SA and 4 NSA) totally prevented or reduced lesion development to varying degrees, whilst the remaining 9 isolates failed to reduce lesion formation (**Table 2**). The isolates that showed production of antifungal metabolites and/or CWDEs, but failed to totally prevent lesion development on mango fruits were excluded. The mango fruit bioassay led to the selection of only three most promising antagonistic isolates: 2 SA (#12 and #29) and 1 NSA (#44) which completely prevented lesion formation on mango fruit.

Our data suggest that the three BCA candidates selected are highly effective against *L. theobromae*, and that the preventive effect of isolates #12, #29 and #44 could have the potential to manage dieback disease on mango seedlings. The actinobacteria tested alone (BCA treatment) did not cause any harmful effects on mango fruits (**Figure 3**). This clearly showed that the antagonists and/or their metabolites(s) are able to inhibit the pathogen preventing it from producing lesions on the mango fruit surface.

In Vitro Evaluation of Antagonistic Properties of the BCA Candidates

The filter-sterilized crude culture filtrate of either BCA1 or BCA2 introduced into the wells using the cup plate technique, caused significant ($P < 0.05$) retardation of the growth of *L. theobromae*, when compared to the antifungal metabolite non-producing BCA3 or control (**Table 3**; Figure S1). Notably, the effect of diffused antifungal metabolites by BCA1 was significantly ($P < 0.05$) higher than those produced by BCA2 (**Table 3**).

The growth of the pathogen was clearly inhibited by the diffused metabolites of BCA1 and BCA2 only after removing the dialysis membranes from the fish meal extract agar, compared to the control or BCA3 (**Table 3**; Figure S1). In addition, the pathogen failed to grow from the plugs transferred from the treatment plates to fresh PDA in the absence of diffused metabolites, confirming that the metabolites of BCA1 and BCA2 were clearly fungicidal to *L. theobromae*.

The diffused metabolites of BCA1 and BCA3 from the colloidal chitin agar plates, inhibited the growth of *L. theobromae*

TABLE 2 | *In vitro* and *in vivo* antagonism shown by 19 isolates of streptomycete and non-streptomycete actinobacteria against *Lasiodiplodia theobromae*.

Treatment	Isolate	*In vitro*			*In vivo*
		Diameter of inhibition zone (mm)[a]	Diameter of clearing zone (mm)[b]	Diameter of clearing zone (mm)[c]	Lesion diameter (mm)[d]
Streptomyces sp.	3	46.41 ± 0.49*b*	46.10 ± 0.98*d*	48.79 ± 0.35*d*	15.88 ± 1.03*b*
	7	40.40 ± 0.58*e*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	32.21 ± 0.65*a*
	10	0.00 ± 0.00*i*	54.50 ± 2.16*cb*	54.96 ± 1.70*c*	33.13 ± 0.31*a*
	12 (BCA1)	49.16 ± 0.88*a*	56.04 ± 0.87*b*	57.97 ± 0.98*b*	0.00 ± 0.00*e*
	18	0.00 ± 0.00*i*	52.59 ± 0.45*c*	54.80 ± 0.65*c*	33.32 ± 0.49*a*
	21	44.40 ± 0.55*c*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	32.31 ± 0.65*a*
	29 (BCA2)	42.40 ± 0.49*d*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	0.00 ± 0.00*e*
	31	0.00 ± 0.00*i*	35.98 ± 0.72*f*	37.52 ± 0.55*f*	0.00 ± 0.00*e*
	41	0.00 ± 0.00*i*	40.81 ± 0.77*e*	42.06 ± 1.35*e*	33.03 ± 0.69*a*
	42	37.10 ± 0.86*f*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	12.73 ± 1.74*c*
	49	31.73 ± 0.95*h*	41.72 ± 0.95*e*	43.98 ± 0.79*e*	0.00 ± 0.00*e*
	51	0.00 ± 0.00*i*	56.22 ± 1.05*b*	57.29 ± 0.53*b*	32.38 ± 0.42*a*
Actinoplanes sp.	9	31.23 ± 0.76*h*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	0.00 ± 0.00*e*
Microbispora sp.	16	49.60 ± 0.60*a*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	32.81 ± 0.73*a*
Micromonospora sp.	25	47.13 ± 0.84*b*	0.00 ± 0.00*g*	0.00 ± 0.00*g*	7.45 ± 0.57*d*
	44 (BCA3)	0.00 ± 0.00*i*	63.90 ± 1.43*a*	65.62 ± 1.30*a*	0.00 ± 0.00*e*
Nocardia sp.	33	0.00 ± 0.00*i*	38.18 ± 0.40*f*	39.66 ± 0.37*f*	31.90 ± 0.62*a*
Rhodococcus sp.	45	0.00 ± 0.00*i*	52.18 ± 1.04*c*	54.37 ± 1.18*c*	32.22 ± 0.98*a*
Streptosporangium sp.	50	34.53 ± 0.60*g*	54.10 ± 1.36*cb*	56.15 ± 1.29*bc*	12.01 ± 1.20*c*
Diseased control (*L. theobromae*)		ND	ND	ND	32.51 ± 0.57*a*
Healthy control (no*L. theobromae*)		ND	ND	ND	0.00 ± 0.00*e*

[a] *Production of diffusible antifungal metabolites active against L. theobromae using the cut-plug method.*
[b] *Production of chitinase on colloidal chitin agar.*
[c] *Production of cell-wall-degrading enzymes on mycelial fragment agar.*
[d] *Effect of the antagonistic BCA on L. theobromae using the in vivo mango fruit bioassay.*
Values are means of three replicates ± SE for the in vitro experiments and in vivo experiments. Values within each column, followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range test.
Isolates #12, #29 and #44 represent Streptomyces samsunensis UAE1 (BCA1), S. cavourensis UAE1 (BCA2) and M. tulbaghiae UAE1 (BCA3); respectively.
ND, not determined.

inoculum; in contrast to isolate BCA2 or control, after removing the dialysis membranes (**Table 3**; Figure S1). The pathogen did not recover from the plugs when transferred from treated plates to fresh PDA. This indicated that BCA1 and BCA3 showed fungicidal activities to *L. theobromae*.

To determine whether the BCA produced volatile antifungal compounds, the three BCA candidates were grown on fish meal extract agar. BCA2 and BCA3 failed to produce any volatile antifungal compounds, capable to inhibit the growth of the pathogen (**Table 3**; Figure S2). However, BCA1 produced volatile antifungal compounds and caused complete suppression of the pathogen growth. None of the three BCA candidates produced hydrogen cyanide; while only BCA1 produced siderophores (**Table 3**; Figure S2).

Chitinase production by BCA1 and BCA3 was significantly ($P < 0.05$) higher in the media amended with colloidal chitin or on media amended with the pathogen cell walls (**Table 3**). Both BCA1 and BCA3 also produced ß-1,3-glucanase when grown on media amended with laminarin or *L. theobromae* cell walls. The production of ß-1,3-glucanase was found to be significantly ($P < 0.05$) higher on laminarin-amended medium. On the other hand, there were no detectable levels of chitinase or ß-1,3-glucanase by BCA2 when it was grown in media containing either colloidal chitin or *L. theobromae* cell walls, or in media containing laminarin or *L. theobromae* cell walls, respectively (**Table 3**). The production of chitinase and ß-1,3-glucanase by BCA3 were, however, significantly ($P < 0.05$) higher than those produced by BCA1.

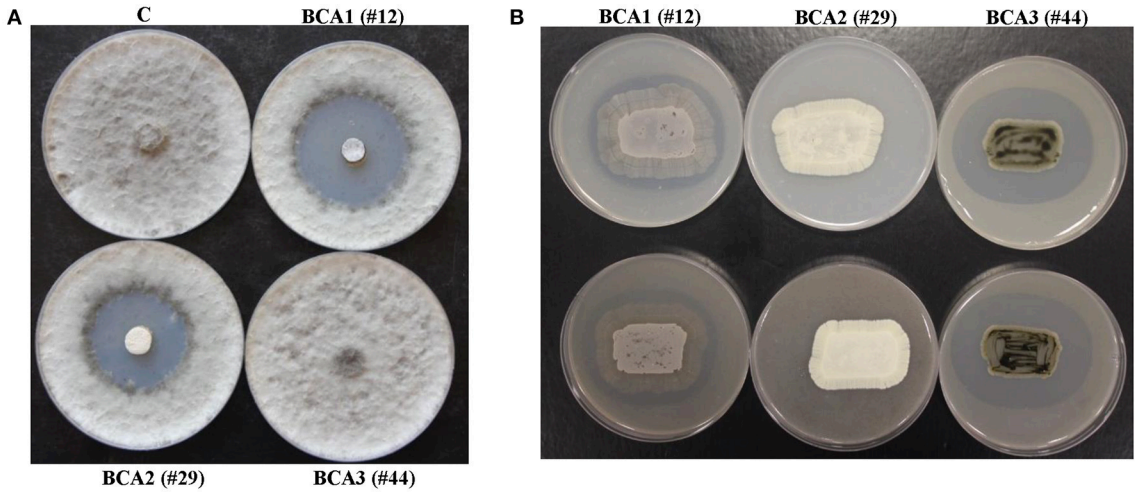


FIGURE 2 | Production of diffusible antifungal metabolites and cell-wall-degrading enzymes by BCA candidates active against *Lasiodiplodia theobromae*. Inhibition of *L. theobromae* mycelial growth by the BCA1 and BCA2 using cut-plug method **(A)**; and production of chitinase enzymes by BCA1 and BCA3 grown on colloidal chitin agar (upper panel) and production of CWDEs on mycelial fragment agar (lower panel) **(B)**. In **(A)** the diffusible antifungal metabolite-producing isolate BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) and BCA2 (isolate #29; *S. cavourensis* UAE1) compared to the non-diffusible antifungal metabolite-producing isolate BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1). In **(B)** production of CWDEs by BCA1 and BCA3 isolates compared to the non- CWDE producing BCA2 isolate. C, a sterile non-inoculated PDA agar plug (control).

Effect of Crude Culture Filtrates of the BCA Candidates on *L. theobromae*

We showed that the filter-sterilized crude culture filtrates of either BCA1 or BCA2 from fish meal extract broth were effective in inhibiting growth of *L. theobromae* (**Table 4**). On PDA plates, the increasing levels of the BCA1 and BCA2, but not BCA3, crude culture filtrates significantly ($P < 0.05$) inhibited the colony and mycelial growth of *L. theobromae* (**Table 4**; Figure S3). Mycelial growth was totally inhibited when crude culture filtrates were incorporated into PDA at 75% or above. In PDB, the assay of the mycelial growth inhibition of the pathogen by BCA1 and BCA2 was similar to that in PDA plates. Crude culture filtrates of BCA1 and BCA2 from fish meal extract broth significantly decreased the mycelial dry weight of the pathogen when proportionally added into PDB (**Table 4**). When compared with the control, the crude culture filtrates of BCA1 and BCA3 from colloidal chitin broth increasingly inhibited colony growth on PDA plates and mycelial dry weight of *L. theobromae* on PDB, with the increasing levels of crude culture filtrates after 5 days of incubation at 28°C (**Table 4**).

Similarly, a significant ($P < 0.05$) reduction in the germination of the thick walled, mature conidia and the average length of germ tubes produced by *L. theobromae* was found when the pathogen was exposed to the crude culture filtrate of BCA1 and BCA2 (in fish meal extract broth), and BCA1 and BCA3 (in colloidal chitin broth) after 24 h of incubation (**Table 4**). This indicates that the crude culture filtrates of BCA1, BCA2, and BCA3 inhibited not only mycelial growth, but also spore germination and germ tube elongation of *L. theobromae*.

We also observed hyphal abnormalities such as hyphal swelling (ballooning), cytoplasmic coagulation and hyphal lysis in *L. theobromae* treated with the crude culture filtrate of BCA1 obtained from fish meal extract broth and colloidal chitin broth, respectively (**Figure 4**). There were hyphal abnormalities, including hyphal swelling and cytoplasmic coagulation without hyphal lysis in *L. theobromae* exposed to the crude culture filtrate of BCA2 from fish meal extract broth (**Figure 4A**). The pathogen treated with the crude culture filtrate of BCA3 obtained from colloidal chitin broth showed only hyphal lysis (**Figure 4B**). Mycelial mats in control flasks remained healthy and unaffected (**Figure 4**).

Identification of the Promising BCA Candidates to the Species Level

The promising antagonists BCA1, BCA2, and BCA3 were identified by determining the nucleotide sequence of their 16*S* rRNA gene. The 16*S* rRNA gene sequences of BCA1 (*Streptomyces samsunensis*; GenBank accession number MG548382), BCA2 (*S. cavourensis*; MG548383) and BCA3 (*Micromonospora tulbaghiae*; MG548384) were compared with that of other actinobacteria. Comparisons of the 16*S* rRNA gene of BCA1, BCA2 and BCA3 with sequences in the GenBank database showed that these BCA candidates were streptomycete spp. for isolates #12 (BCA1) and #29 (BCA2) and a non-streptomycete sp. for isolate #44 (BCA3). BCA1 showed above 99% similarity to *S. samsunensis* (EU077190) and *S. malaysiensis* (AB249918) (**Figure 5A**), although, the remaining isolates of *Streptomyces* spp. showed less than 98.8% similarities. The phylogenetic analysis of BCA2 showed 100% similarity to both *S. cavourensis* (AB184264) and *S. albolongus* (AB184425) (**Figure 6A**); while the rest showed <99.6% similarity with the strain of interest. This may suggest that BCA1 may possibly be either *S. samsunensis* or *S. malaysiensis*; while BCA2 could be either *S. cavourensis* or *S. albolongus*; thus it was necessary to

TABLE 3 | *In vitro* antagonistic activities of the three BCA candidates against *Lasiodiplodia theobromae*.

Activities	BCA1	BCA2	BCA3
Production of diffusible metabolites using the cup plate technique (diameter of zone of inhibition measured in mm)	67.97 ± 0.91 *a*	53.82 ± 0.93 *b*	0.00 ± 0.00 *c*
Production of diffusible metabolites using the dialysis membrane technique from the fish meal extract agar plates[a]	+	+	−
Production of chitinase using the dialysis membrane technique from the colloidal chitin agar plates[a]	+	−	+
Production of volatile compounds[a]	+	−	−
Production of hydrogen cyanide[b]	−	−	−
Production of siderophores[b]	+	−	−
Chitinase from colloidal chitin (U ml^{-1})[c]	5.56 ± 0.17 *a*	0.00 ± 0.00 *b*	7.75 ± 0.22 *c*
Chitinase from *L. theobromae* cell wall (U ml^{-1})[c]	3.73 ± 0.14 *a*	0.00 ± 0.00 *b*	5.60 ± 0.21 *c*
ß-1,3-glucanase from laminarin (U ml^{-1})[d]	3.57 ± 0.16 *a*	0.00 ± 0.00 *b*	5.54 ± 0.18 *c*
ß-1,3-glucanase from *L. theobromae* cell wall (U ml^{-1})[d]	2.79 ± 0.20 *a*	0.00 ± 0.00 *b*	3.76 ± 0.21 *c*

[a] *+, fungicidal effect; −, no fungicidal effect.*
[b] *+, produced; −, not produced.*
[c] *A unit of chitinase was expressed as the amount of the enzyme that released 1 μmol of N-acetyl-D-glucosamine mg^{-1} protein h^{-1}.*
[d] *A unit of ß-1,3-glucanase was expressed as the amount of the enzyme that released 1 μmol of glucose mg^{-1} protein h^{-1}.*
Values are means of three replicates ± SE. Values with the same letter within a row are not significantly ($P > 0.05$) different according to Duncan's multiple range test.
BCA1, isolate #12; Streptomyces samsunensis UAE1; BCA2, isolate #29; S. cavourensis UAE1; BCA3, isolate #44; Micromonospora tulbaghiae UAE1.

obtain a more reliable identification of these isolates. Based on the 16*S* rRNA gene comparisons, the third identified BCA3 was considered as *Micromonospora tulbaghiae* (Kirby and Meyers, 2010) Strain UAE1, due to the high similarity (100%) with *M. tulbaghiae* (DSM 45142). The rest of the *Micromonospora* spp. showed 99.4% or less similarity than that with this specific strain (**Figure 7A**).

To confirm the identity of BCA1, the pure cultures produced grayish black aerial mycelia with grayish yellow substrate mycelial growth on ISP medium 3 for 14 days (**Figure 5B**). Using SEM, the configuration of the spore chains showed spiral chains of rugose ornamented spores (**Figure 5C**). Together, the genotypic, morphological, cultural and phenotypic data showed that BCA1 can be recognized as *Streptomyces samsunensis* (Sazak et al., 2011) Strain UAE1 (Table S1).

On the other hand, typical yellow aerial mycelia and yellowish-brown substrate mycelia were observed when BCA2 was cultivated (**Figure 6B**). BCA2 showed straight to flexuous (Rectiflexibiles) chains and smooth-surfaced spores (**Figure 6C**). Our data demonstrated that BCA2 (isolate #29) can be recognized as *Streptomyces cavourensis* (Giolitti, 1958) (in Waksman, 1961) Strain UAE1 (Table S2). Pure cultures of BCA3 (*M. tulbaghiae*) on ISP medium 3 produced typical brownish-black charcoal-like substrate mycelia without the formation of aerial mycelia (**Figure 7B**); with the formation of single oval to spherical smooth-surfaced spores (**Figure 7C**).

Effect of the Promising BCA Candidates on *L. theobromae* in the Greenhouse

The responses of the pathogen to these selected antagonists *in vitro* clearly indicated the three BCA candidates to be effective against mango dieback caused by *L. theobromae*. With an aim of evaluating and comparing the outcome of the application of the BCAs in suppressing *L. theobromae* using different antifungal activities, an *in vivo* experiment involving greenhouse grown plants was conducted. For this purpose, "preventive" treatments with the three BCA candidates 1 week before inoculation with *L. theobromae* were applied to determine their impact on mango dieback.

Initially, a pathogenicity test was carried out to determine the effect of inoculation with *L. theobromae* on mango seedlings. Symptoms typical of the mango disease after 3 wpi with *L. theobromae* (*Lt*) were observed (**Figures 8A–C**). The disease progressed with time, initially with leaves of infected seedlings showing distinct defoliation at 6 wpi. No disease symptoms were evident in any of the plants inoculated with BCA candidate alone (*Ss*, *Sc*, or *Mt*) or on non-inoculated seedlings (C) (**Figures 8A–C**). Secondly, we individually applied the BCA candidates *S. samsunensis*, *S. cavourensis* or *M. tulbaghiae* on seedlings 1 week before inoculation with *L. theobromae*, designated as *Ss*+*Lt*, *Sc*+*Lt*, or *Mt*+*Lt*, respectively. Plants inoculated with the BCA candidates prior to inoculation with *L. theobromae* (*Ss*+*Lt*, *Sc*+*Lt*, or *Mt*+*Lt*) recovered when compared with seedlings inoculated with *L. theobromae* only (*Lt*) at all time points of assessments. These plants appeared to be healthy and were comparable to those that were inoculated with any of the corresponding BCA candidate alone (*Ss*, *Sc*, or *Mt*) or without infection with *L. theobromae* (C) (**Figures 8A–C**; Figure S4). This suggests that these BCA candidates effectively inhibit *L. theobromae* growth *in vivo*.

We found significant differences between treatments when the DSI was assessed. Plants inoculated with *L. theobromae* (*Lt*) showed disease progression until 9 wpi (Table S3). Seedlings not inoculated with *L. theobromae* (control; C) showed no disease symptoms at any of the assessment time-points. There was a dramatic decrease in DSI in all *L. theobromae*-infected seedlings that were previously treated with any of the BCA candidates

TABLE 4 | Inhibition of mycelial growth, spore germination and germ tube elongation of *Lasiodiplodia theobromae* by the crude culture filtrate of the three BCA candidates either obtained from fish meal extract broth or colloidal chitin broth.

BCA	Culture filtrate (%)	Colony diameter (mm)	Mycelial dry weight (g)	Conidia germination (%)	Germ tube length (μm)
(A) FISH MEAL EXTRACT BROTH					
BCA1	0	99.60 ± 0.22*a*	81.05 ± 2.76*a*	90.06 ± 1.77*a*	59.86 ± 0.74*a*
	10	37.76 ± 1.39*b*	28.40 ± 2.86*b*	34.25 ± 1.74*b*	47.24 ± 0.92*b*
	25	18.87 ± 0.95*c*	9.86 ± 1.08*c*	21.31 ± 1.34*c*	29.35 ± 0.94*c*
	50	9.72 ± 0.84*d*	3.88 ± 0.76*d*	8.96 ± 0.36*d*	18.59 ± 1.58*d*
	75	0.00 ± 0.00*e*	0.40 ± 0.10*d*	3.19 ± 0.32*d*	11.81 ± 0.84*e*
	100	0.00 ± 0.00*e*	0.00 ± 0.00*d*	0.54 ± 0.09*d*	3.50 ± 0.61*f*
BCA2	0	99.51 ± 0.12*a*	78.60 ± 3.60*a*	89.17 ± 1.91*a*	53.26 ± 1.53*a*
	10	39.72 ± 1.83*b*	35.87 ± 2.08*b*	40.36 ± 3.03*b*	36.09 ± 1.91*b*
	25	21.57 ± 0.89*c*	15.92 ± 2.35*c*	26.12 ± 1.41*c*	28.17 ± 1.79*c*
	50	11.33 ± 1.21*d*	7.82 ± 1.15*d*	16.19 ± 0.91*d*	16.36 ± 0.01*d*
	75	0.00 ± 0.00*e*	3.15 ± 0.59*de*	6.68 ± 0.37*e*	6.32 ± 1.27*e*
	100	0.00 ± 0.00*e*	0.15 ± 0.08*e*	0.97 ± 0.02*f*	0.42 ± 0.14*f*
(B) COLLOIDAL CHITIN BROTH					
BCA1	0	98.70 ± 0.68*a*	80.39 ± 2.97*a*	93.10 ± 2.13*a*	60.69 ± 1.77*a*
	10	48.13 ± 2.05*b*	40.49 ± 1.66*b*	49.49 ± 2.86*b*	36.58 ± 1.85*b*
	25	26.94 ± 1.14*c*	22.80 ± 1.82*c*	28.55 ± 0.90*c*	25.11 ± 1.28*c*
	50	16.25 ± 1.23*d*	13.58 ± 1.03*d*	18.87 ± 0.93*d*	13.21 ± 1.29*d*
	75	0.00 ± 0.00*e*	4.24 ± 0.92*e*	8.48 ± 0.75*e*	3.41 ± 0.51*e*
	100	0.00 ± 0.00*e*	0.48 ± 0.16*e*	2.19 ± 0.42*f*	0.00 ± 0.00*e*
BCA3	0	99.84 ± 0.12*a*	83.27 ± 2.64*a*	93.32 ± 1.47*a*	66.34 ± 1.81*a*
	10	29.27 ± 1.45*b*	30.84 ± 2.79*b*	28.04 ± 0.86*b*	42.55 ± 1.49*b*
	25	16.79 ± 1.05*c*	13.12 ± 1.34*c*	18.42 ± 1.03*c*	17.44 ± 1.30*c*
	50	6.85 ± 1.00*d*	4.96 ± 0.95*d*	10.03 ± 0.81*d*	6.07 ± 0.83*d*
	75	0.00 ± 0.00*e*	0.00 ± 0.00*e*	2.49 ± 0.45*e*	0.00 ± 0.00*e*
	100	0.00 ± 0.00*e*	0.00 ± 0.00*e*	0.00 ± 0.00*e*	0.00 ± 0.00*e*

Values are means of three replicates ± SE. Values with the same letter within a column for each BCA are not significantly ($P > 0.05$) different, according to Duncan's multiple range test. BCA1, isolate #12; Streptomyces samsunensis UAE1; BCA2, isolate #29; S. cavourensis UAE1; BCA3, isolate #44; Micromonospora tulbaghiae UAE1.

at 3 and 9 wpi, when compared with plants inoculated with the pathogen alone. In comparison with the *L. theobromae*-infected seedlings, the DSI of the preventive applications of individual BCA candidate (*Ss*+*Lt*, *Sc*+*Lt*, and *Mt*+*Lt*) dropped from 2.83 to 0.17 at 3 wpi and from 4.33 to 0.17 at 9 wpi; providing 94.0 and 96.1% reduction in disease development, respectively. The three preventive treatments with the BCA candidates at 3 and 9 wpi did not show significant ($P > 0.05$) difference among the DSI measurements, and the BCA-treated plants without the pathogen. This suggests that the preventive treatment with BCA candidates a week before inoculation with *L. theobromae* effectively suppresses the pathogen invasion.

It is evident that the DSI values in the case of BCA applications were significantly lower than those with *L. theobromae*; however, the differences of the DSI were indistinguishable among these BCA treatments. For that reason, we compared the responses of the biological control treatments to determine their effects on leaf defoliation and the production of conidia on the host plant. The number of falling leaves in seedlings inoculated with *L. theobromae* dramatically increased compared to any BCA-inoculated or non-inoculated seedlings at 6 wpi (**Figure 8D**). Mango seedlings treated with BCA before *L. theobormae* infection significantly reduced the number of defoliated leaves; thus seedlings of *Ss*+*Lt* showed the lowest number of falling leaves among the other two treatments and were comparable to its corresponding *Ss* treatment. In addition, conidia counts of *L. theobromae* at the leaf base of treated mango seedlings were made. The BCA treatment (*Ss*+*Lt*) caused a greater reduction in the number of conidia, followed by the other BCA-treated plants, *Sc*+*Lt* and *Mt*+*Lt* at the end of the greenhouse experiment (**Figure 8E**). At least 4-fold reduction in total conidia numbers of *L. theobromae* in *S. samsunensis*-treated plants was measured when compared with other BCA treatments (**Figure 8E**). In general, the pathogen appeared not to be sufficiently aggressive to support disease progression in the presence of *S. cavourensis* or *M. tulbaghiae*, while a strong inhibitory effect on the pathogen was observed in the case of the *S. samsunensis* treatment. Together, this suggests that *S. samsunensis*, which exhibited multiple modes of action, successfully controlled mango dieback disease.

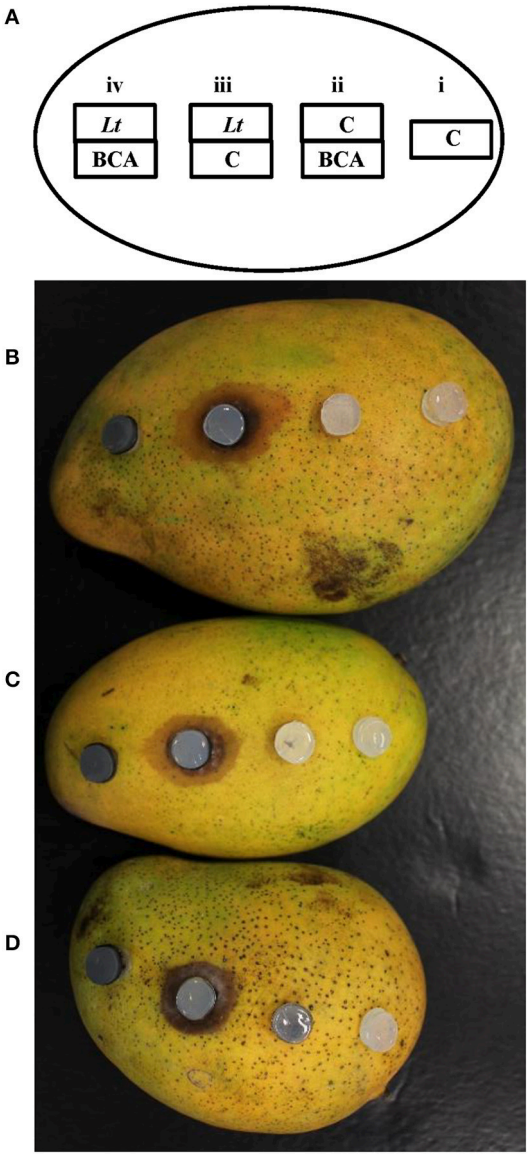


FIGURE 3 | *In vivo* inhibitory effect of the BCA candidates against *Lasiodiplodia theobromae* using the "mango fruit bioassay". An illustration showing an inoculated-mango fruit with the colonized BCA and/or *L. theobromae* agar plugs in combinations onto each mango fruit **(A)**. Mango fruit bioassays using BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) **(B)**; BCA2 (isolate #29; *S. cavourensis* UAE1) **(C)**; or BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1) **(D)** as potential BCAs. (i) C, a sterile non-inoculated PDA agar plug (control); (ii) BCA, the antagonist alone with a sterile agar plug above it; (iii) *Lt*, *L. theobromae* inoculum alone with a sterile agar plug below it; and (iv) BCA+*Lt*, pairing *L. theobromae* and the antagonist together, with the BCA on the mango surface and *L. theobromae*-inoculated plug on top of the BCA.

DISCUSSION

Nowadays, seeking an alternative strategy to the use of chemicals by native microorganisms as a source material for plant growth promotion and disease amelioration in agriculture has become a priority (Saeed et al., 2017b; Syed Ab Rahman et al., 2018). Actinobacteria could be potential targets as BCA candidates since they have many properties that control diseases, increase nutrient supply and enhance growth of plants (Doumbou et al., 2001; Barka et al., 2016). Ultimately, this approach can be employed as a main component in IPM, and may be combined with others to prevent losses and damages caused by plant diseases (Saeed et al., 2017b; Syed Ab Rahman et al., 2018). These outcomes led us to explore local UAE soils for the actinobacterial communities and screen their potential under *in vitro* and *in vivo* greenhouse conditions for their effectiveness in the control of the mango dieback disease caused by *L. theobromae*. We hypothesized that exposure to microbial metabolites produced by the soil-inhabiting actinobacteria can be exploited for the management of this devastating mango disease.

In this study, we aimed at identifying SA and NSA isolates that are capable of restricting invasions of *L. theobromae* on mango plantations. A total of 53 actinobacterial strains were obtained from the rhizosphere of healthy mango plants. Due to their dominance as a biologically active component of the soil microflora, many researchers have successfully isolated SA from soil environments (Goodfellow and Williams, 1983; Palaniyandi et al., 2013). The NSA, however, are rarely isolated actinobacteria whose isolation frequency using commonly used techniques is usually lower than the numbers of SA (Jose and Jebakumar, 2013). For that reason, the isolation of the uncommon actinobacteria was achieved by the application of *Streptomyces* polyvalent phages that selectively permitted the appearance of the genera of NSA on isolation plates, a technique not commonly used in screening for BCA candidates. Some studies have recommended the use of *Streptomyces* phages to omit "weedy" *Streptomyces* colonies (Kurtböke et al., 1992; Kurtböke, 2012). In the current study, the isolated NSA group comprised of *Actinoplanes, Actinomadura, Microbispora*, *Micromonospora*, *Nocardia*, *Rhodococcus,* and *Streptosporangium* spp. (**Table 1**).

The development of new BCA and/or biocontrol products for use against plant diseases requires screening of large numbers of antagonistic candidates (Köhl et al., 2011). In our effort to conduct an appropriate performance evaluation of BCA candidate(s) against *L. theobromae*, a series of screening steps were performed. A first round of *in vitro* screening on agar plates allowed rapid and clear discriminating results. Secondly, a selection of antagonists to *L. theobromae*, using the novel *in vivo* mango fruit bioassay was also evaluated. Thirdly, selected candidates were identified to the genus and species levels. Finally, an assessment of the feasibility of the selected BCA candidates in controlling dieback disease on mango seedlings under greenhouse conditions was carried out. Accordingly, 11 were regarded as highly diffusible antifungal metabolite-producing isolates on fish meal extract agar plates. In addition, an agar medium incorporating *L. theobromae* mycelial fragments helped to select 12 antagonists which had the required enzymes to destroy the components of *L. theobromae* cell wall. Mycelial fragment agar has been previously used to isolate glucanolytic BCA candidates against *Phytophthora*

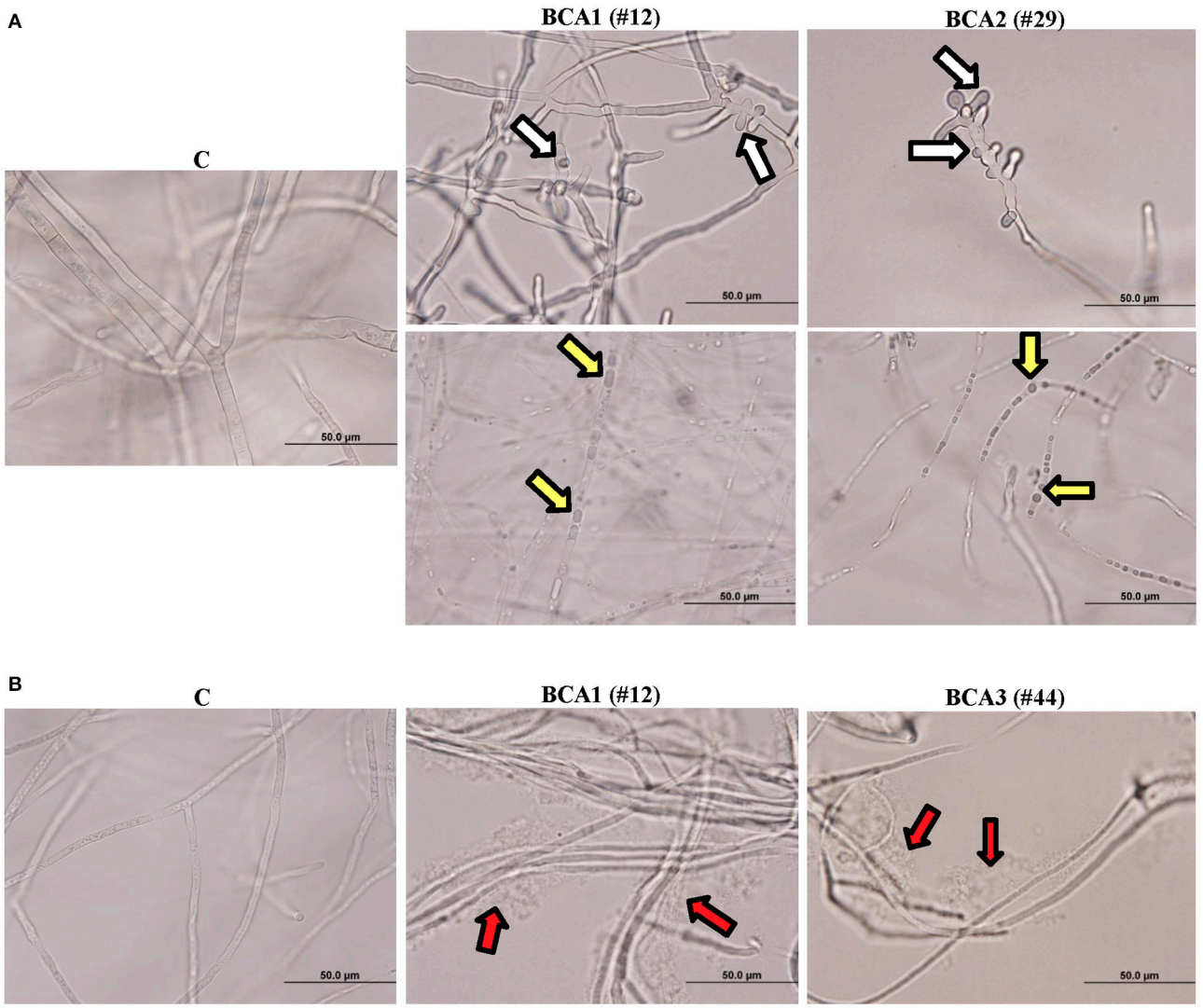


FIGURE 4 | Effect of the BCA candidates on hyphae and cytoplasm of *Lasiodiplodia theobromae*. Abnormalities observed in hyphal morphology and cytoplasmic contents of *L. theobromae*, following exposure to **(A)** filter-sterilized crude culture filtrate of BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) and BCA2 (isolate #29; *S. cavourensis* UAE1) on fish meal extract broth, or **(B)** BCA1 and BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1) on colloidal chitin broth compared to control. White arrows point to hyphal septum malformation and branch deformation; while yellow and red arrows point to cytoplasmic coagulation and lysis of cytoplasm, respectively.

fragariae (Valois et al., 1996) and *Pythium aphanidermatum* (El-Tarabily, 2006). Many promising antagonistic isolates obtained in the current investigation that showed clearing zones on *L. theobromae* mycelial fragment agar and on colloidal chitin agar, may have secreted ß-1,3-glucanases and chitinase which hydrolyzed glucans and chitin present in the pathogen cell wall and helped to lyse *L. theobromae* hyphae. This study clearly indicates that SA and NSA can serve as potential BCAs against *L. theobromae*.

Because testing of isolates under greenhouse and field conditions for efficacy requires additional labor and time, the antagonistic potential of candidates were further assessed in a second screening round of laboratory bioassays. We argue that the mango fruit antagonism bioassay would provide a "clear-cut" prediction of what may occur in the field. Such laboratory *in vivo* assays using plant material are of value since they provide a rapid screening of large numbers of antagonistic candidates in the presence of the pathogen. Thus, the results must be confirmed by greenhouse/field trials. Previous studies have used similar "bioassay" approach as a practical step in the selection of BCAs for major pathogens and pests on crops. In assays carried out *in vitro* as well as on the carrot roots or mango fruits, the BCAs used were found to be capable of almost complete inhibition of *P. coloratum* or *L. theobromae*, respectively (El-Tarabily et al., 1997; Seethapathy et al., 2016). Seethapathy et al. (2016) have demonstrated that dual culture technique of bacterial

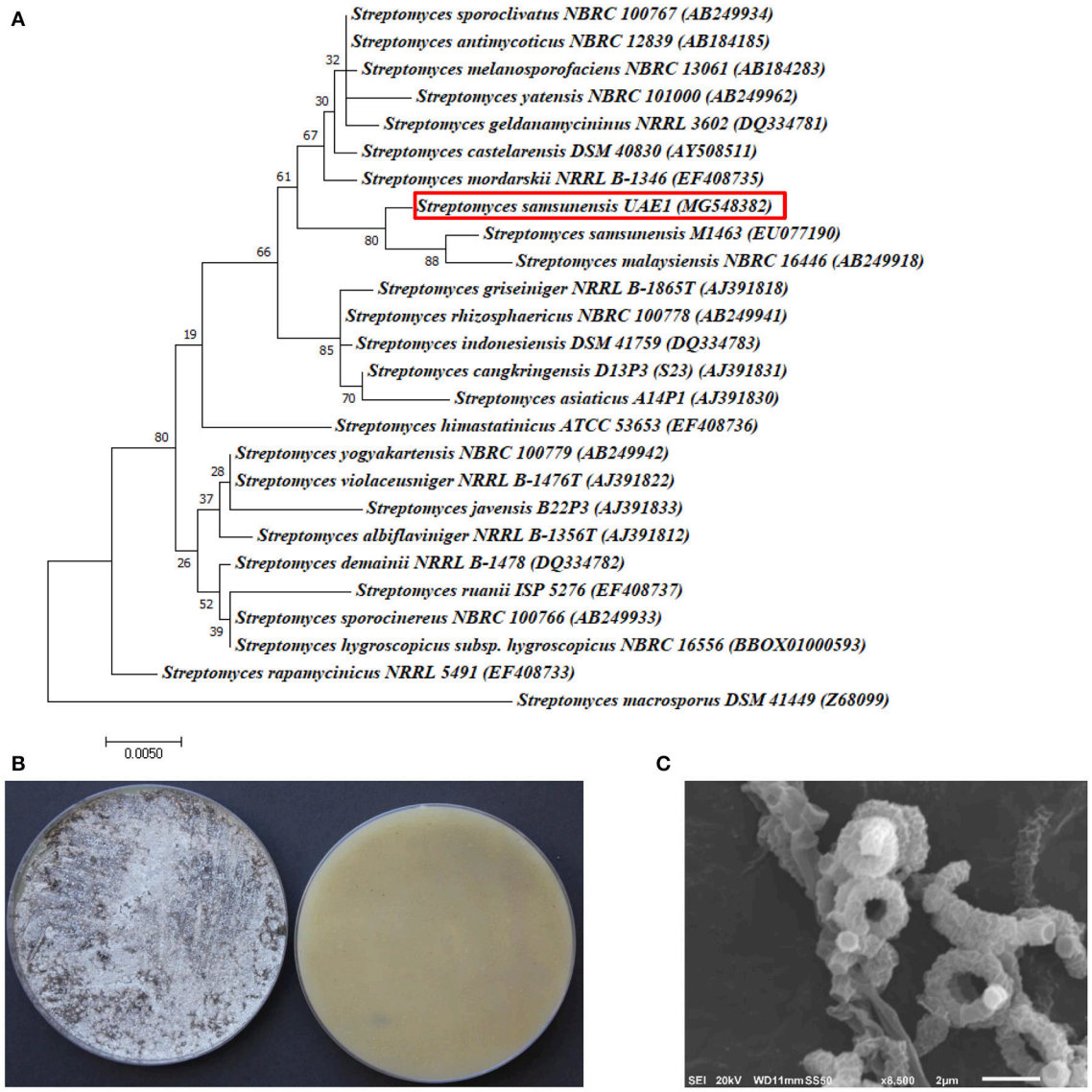


FIGURE 5 | Taxonomic determination of *Streptomyces samsunensis* UAE1, based on phylogenetic, cultural and morphological characteristics. **(A)** The tree showing the phylogenetic relationships between *S. samsunensis* UAE1 (*MG548382*; 1,475 bp) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences. **(B)** Grayish black aerial mycelia (left) and grayish yellow substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, **(C)** scanning electron micrograph (8,500X) showing the spiral chains of rugose ornamented spores of *S. samsunensis* UAE1 (BCA1; isolate #12). In **(A)** numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1,000 resampled datasets. Bar, 0.005 substitutions per site. *S. macrosporus DSM 41449* (Z69099) was used as an outgroup. GenBank accession numbers are given in parentheses.

anatogonists using *Pseudomonas fluorescens* (Pf1) and *Bacillus subtilis* (EPCO16) reduced the pathogen population *in vitro*; and further strengthened the cell-wall structures of mango fruits against *L. theobromae* infection. This is consistent with the present study that many isolates killed the pathogen *in vitro*; whereas certain isolates completely arrested development of lesions, with others reducing only the lesion size or having no effect on the disease in the mango fruit bioassay (**Table 2**). According to El-Tarabily et al. (1997), the failure of isolates to reduce lesion diameter in the mango fruit bioassay indicated that their ability to produce antifungal metabolites in agar did not necessarily address that this performance would be reproducible on plant material. Our data indicate the importance of the *in vitro* as well as the mango fruit bioassay for the selection of potential antagonists prior to their screening on plants in the greenhouse.

The efficacious isolates from the *in vitro* and *in vivo* assays were further identified. The three candidates represent 5.7% of the total isolated actinobacteria from the soil of healthy mango rhizosphere. In this study, the two identified isolates (#12 and #29) belonging to *Streptomyces* spp. confirm the findings from previous reports that SA are predominant among

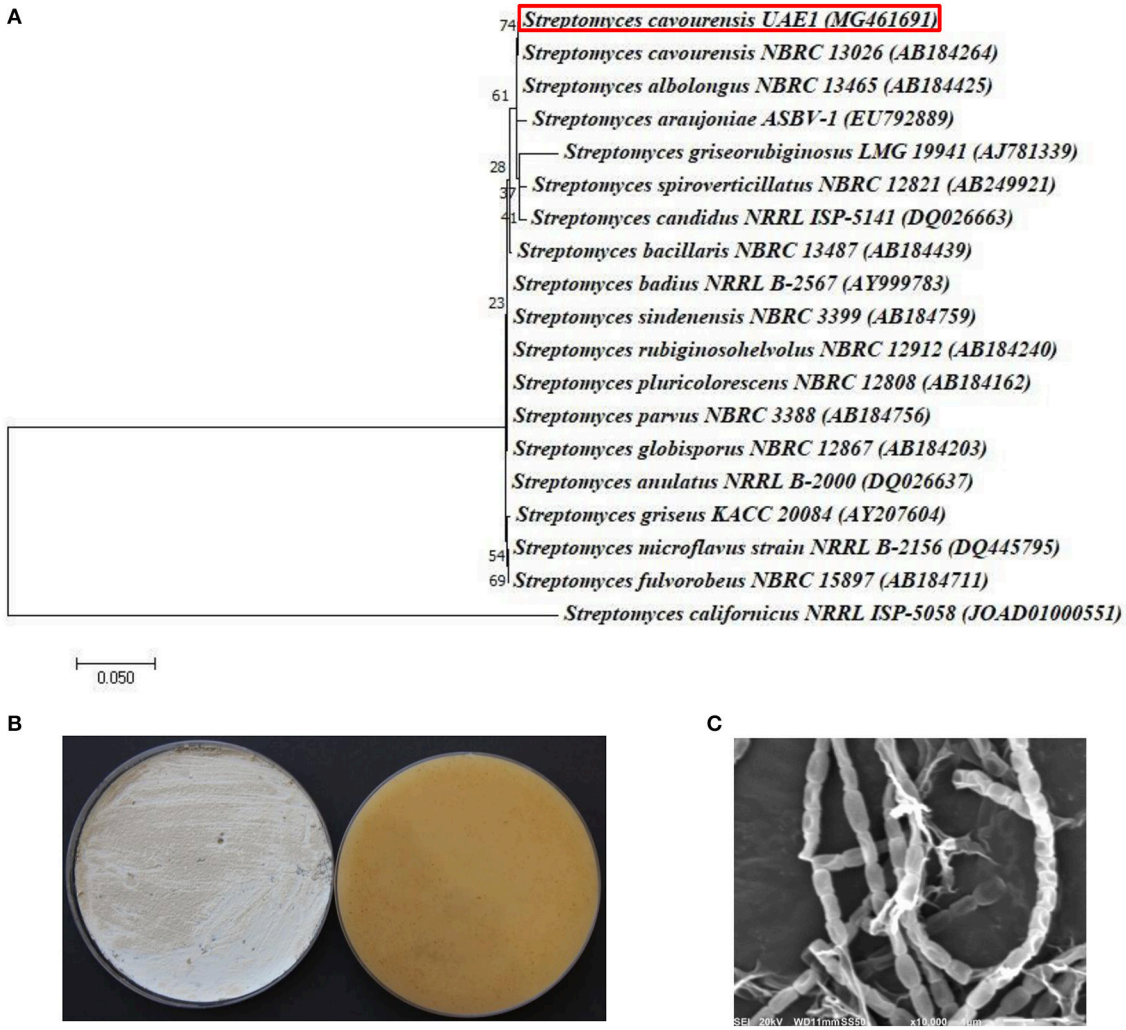


FIGURE 6 | Identification of *Streptomyces cavourensis* UAE1, based on phylogenetic, cultural and morphological characteristics. **(A)** The tree displaying the phylogenetic relationships between *S. cavourensis* UAE1 (*MG461691*; 1,484 bp) and other members of *Streptomyces* spp. on the basis of 16S rRNA sequences. **(B)** Yellow aerial mycelia (left) and yellowish-brown substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, and **(C)** scanning electron micrograph (10,000X) of the straight to flexuous (Rectiflexibiles) chains and smooth-surfaced spores of the strain of *S. cavourensis* UAE1 (BCA2; isolate #29). In **(A)** numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1000 resampled datasets. Bar, 0.05 substitutions per site. *S. californicus* NRRL ISP-5058 (JOAD0100051) was used as an outgroup. GenBank accession numbers are given in parentheses.

actinobacteria appearing in isolation plates and more often produce useful antibiotics and active secondary metabolites (Thenmozhi and Krishnan, 2011; Barka et al., 2016). Isolates #12 (BCA1) and #29 (BCA2) were identified as *S. samsunensis* and *S. cavourensis*, respectively, while BCA3 (isolate #44) was considered as *M. tulbaghiae*. The mechanisms involved in disease reduction or prevention of lesion development appeared to be antibiosis (diffusible antifungal metabolites and volatile compounds) for *S. samsunensis* and *S. cavourensis*, and the production of CWDEs such as chitinase and ß-1,3-glucanases for *S. samsunensis* and *M. tulbaghiae*. Several actinobacteria have been demonstrated to inhibit the growth of soil-borne plant pathogens such as *Pythium ultimum*, *Rhizoctonia solani* (Yuan and Crawford, 1995), *P. coloratum* (El-Tarabily et al., 1997) and *Thielaviopsis punctulata* (Saeed et al., 2017b) *in vitro* via the production of inhibitory diffusible antifungal metabolites.

The cell walls of filamentous fungi consist largely of chitin and ß-glucans (Osherov and Yarden, 2010), it is probable that ß-1,3-glucanase and chitinases produced by the antagonistic actinobacteria in this study may be involved in the pathogen suppression. The exposure of phytopathogenic fungi to CWDEs can result in the lysis and degradation of the fungal cell walls (Doumbou et al., 2001; Whipps, 2001; Berini et al., 2018). Therefore, the production of chitinase and ß-1,3-glucanase was set, in this study, as a criteria for selection of potential BCA against *L. theobromae*. Chitinase-producing actinobacteria tested previously under *in vitro* conditions have included *Streptomyces*

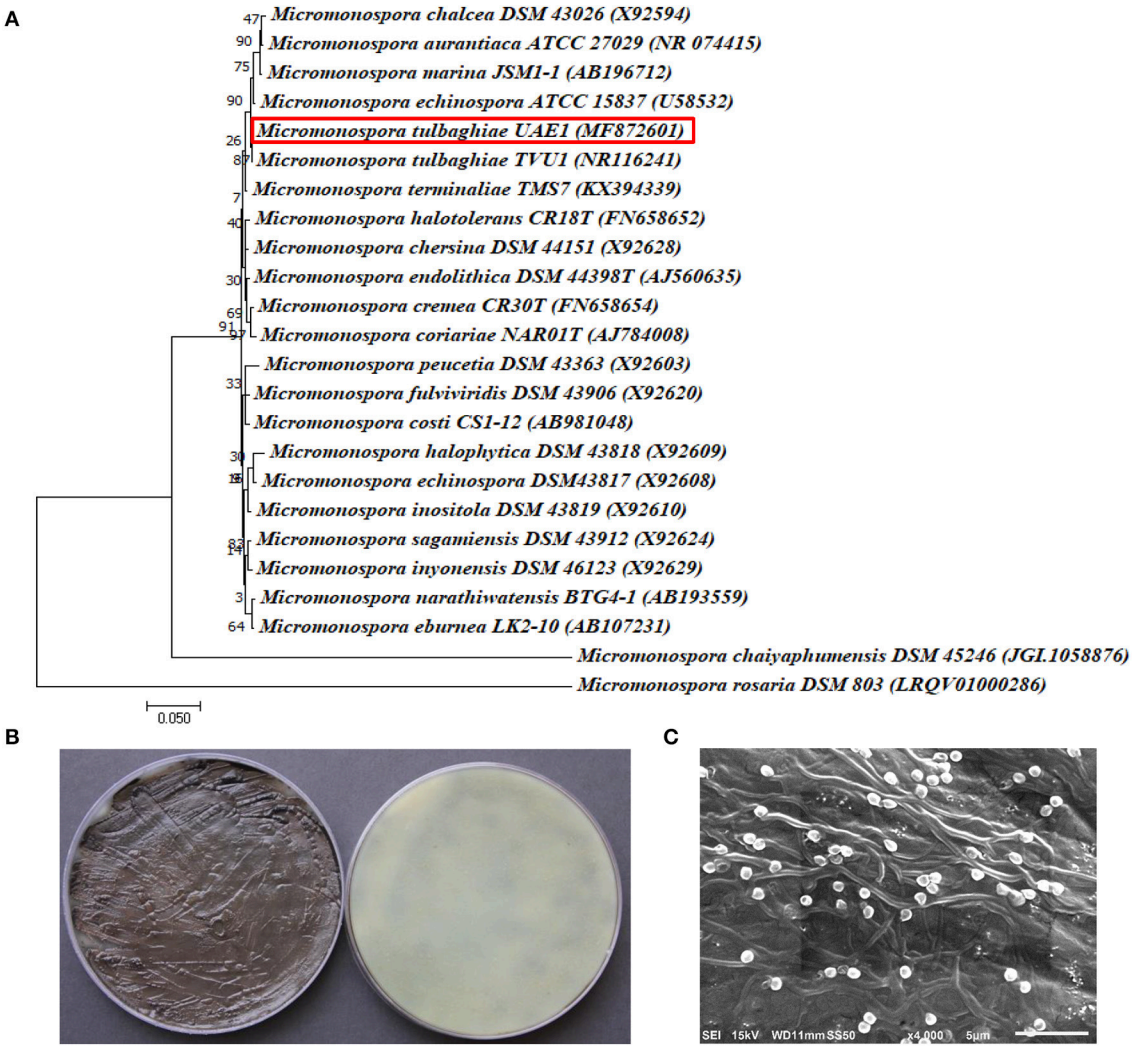


FIGURE 7 | Identification of *Micromonospora tulbaghiae* UAE1 using phylogenetic, cultural and morphological characteristics. **(A)** The tree shows the phylogenetic relationships among *M. tulbaghiae* UAE1 (*MF872601*; 1,486 bp) and other members of *Micromonospora* spp. on the basis of 16*S* rRNA sequences. **(B)** Brownish-black charcoal-like substrate mycelia without the formation of aerial mycelium (left) and the brownish-black color of the substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, and **(C)** scanning electron micrograph (4,000X) of the single oval to spherical smooth-surfaced spores of the strain of *M. tulbaghiae* UAE1 (BCA3; isolate #44). In **(A)** numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1,000 resampled datasets. Bar, 0.05 substitutions per site. *M. rosaria* DSM 803 (LRQV01000286) was used as an outgroup. GenBank and Joint Genome Institute (JGI) accession numbers are given in parentheses.

viridicans (Gupta et al., 1995), *S. viridodiasticus* (El-Tarabily et al., 2000), and *Streptomyces* spp. (Singh and Gaur, 2016). In addition, several chitinase-producing SA such as *Streptomyces* spp. (Singh et al., 1999), and NSA such as *Micromonospora carbonacea* (El-Tarabily et al., 2000) and *Actinoplanes missouriensis* (El-Tarabily, 2003) were used for the management of cucumber wilt caused by *Fusarium oxysporum* f.sp. *cucumerinum*, lettuce basal drop caused by *Sclerotinia minor*, and lupin root rot caused by *Plectosporium tabacinum*, respectively. Valois et al. (1996) have reported some ß-glucanase-producing actinobacterial isolates that hydrolyzed cell wall glucans, caused hyphal lysis and resulted in the suppression of root rot disease of raspberry caused by *Phytophthora fragariae.*

Beside its ability to produce diffusible and volatile inhibitory antifungal compounds and siderophores, *S. samsunensis* produced CWDEs. Better control of mango dieback disease by *S. samsunensis* in comparison to the other BCA candidates (*S. cavourensis* or *M. tulbaghiae*) may indicate that this response could be a result of synergetic effects of the multiple modes of action i.e., co-antagonism. Our results are in agreement with other reports that *Streptomyces spiralis* and *Actinoplanes campanulatus* which produced diffusible inhibitory antifungal metabolites and CWDEs were superior on *Micromonospora chalcea* which produced CWDEs only in controlling root rot and crown rot of cucumber caused by *P. aphanidermatum* (El-Tarabily et al., 2009).

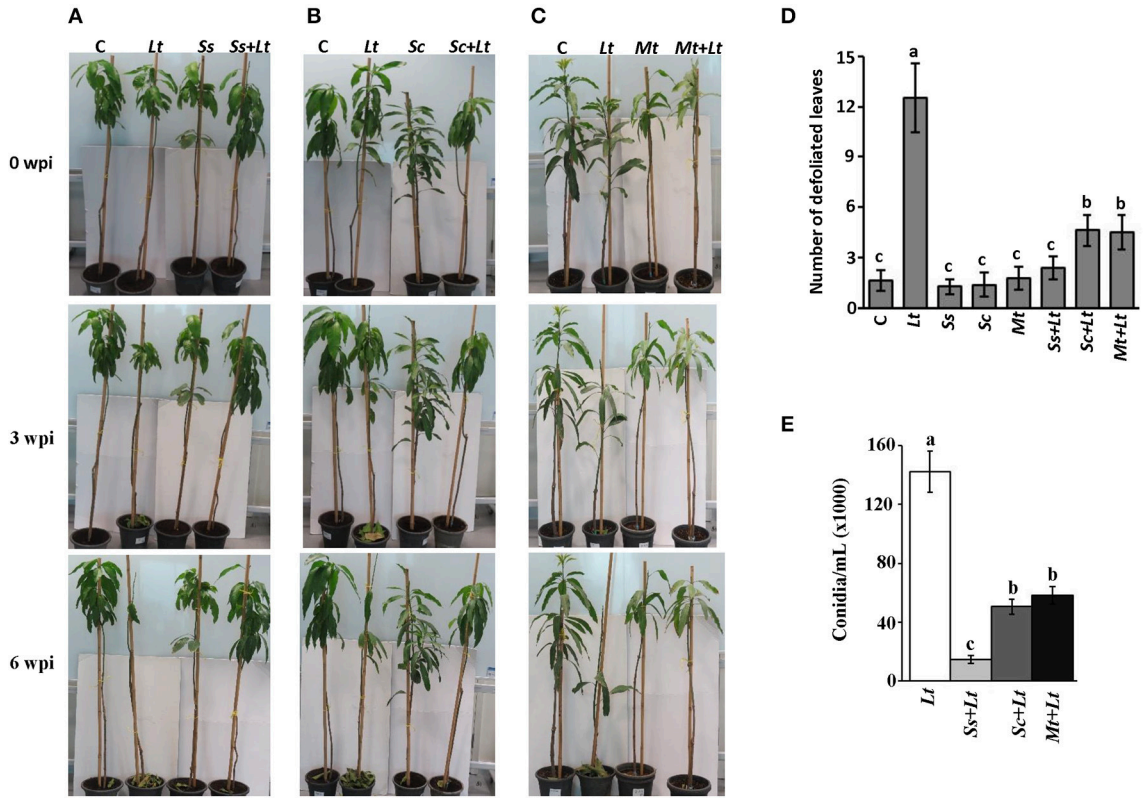


FIGURE 8 | Antagonistic effect of BCA candidates against mango dieback disease under greenhouse conditions. Effect of preventive biocontrol treatment of **(A)** *Streptomyces samsunensis* UAE1 (BCA1; isolate #12); **(B)** *S. cavourensis* UAE1 (BCA2; isolate #29); and **(C)** *Micromonospora tulbaghiae* UAE1 (BCA3; isolate #44). The number of **(D)** defoliated leaves; and **(E)** conidia after recovery of the pathogen from affected mango stem tissues (cv. Badami) at 6 and 9 wpi with *L. theobromae*, respectively. In **(D,E)** values with different letters are significantly different from each other at $P < 0.05$. C, non-inoculated control seedlings, *Lt*, seedlings inoculated with *L. theobromae* only; *Ss*, *Sc* or *Mt,* seedlings inoculated with only *S. samsunensis* UAE1, *S. cavourensis* UAE1 or *M. tulbaghiae* UAE1, respectively; *Ss+Lt*, *Sc+Lt* or *Mt+Lt*, seedlings inoculated with the individual BCA, *S. samsunensis* UAE1*, S. cavourensis* UAE1 or *M. tulbaghiae* UAE1, respectively, 1 week prior to *L. theobromae* inoculation; wpi, weeks post inoculation.

Bailey and Falk (2011) have stated that less than 1% of candidate microorganisms isolated by routine isolations make successful antiobiotic products and secondary metabolites. Among these, *Streptomyces griseoviridis* K61 (Mycostop®), *S. lydicus* strain WYEC108 (Actinovate®, Actino-iron®, or Micro108® and *S. saraceticus* KH400 (YAN TEN) are commercial BCA products that reduce spore germination and inhibit hyphal growth of plant fungal pathogens (Minuto et al., 2006; Elliott et al., 2009; Palaniyandi et al., 2013). In the UAE, *Streptomyces globosus* UAE1 has recently been reported as the first effective BCA against black scorch disease in date palm plantations (Saeed et al., 2017b). On the other hand, few NSA have been recognized as BCA and/or PGP (El-Tarabily et al., 1997; El-Tarabily and Sivasithamparam, 2006). This suggests that the SA and NSA strains isolated in our study may also serve as producers of potentially useful antifungal products active against *L. theobromae*. Therefore, efforts to be among the first to manage mango dieback by the three antagonists were aimed in greenhouse trials.

One should take cautions when assuming a correlation between *in vitro* inhibition and greenhouse or field performance (Fravel, 2005; Parnell et al., 2016). In the present study, the actinobacteria were inoculated at the apices of the mango stems 1 week before inoculation with the pathogen. The gap in the incubation periods may favor the establishment of the introduced actinobacteria prior to the exposure to the pathogen, and/or to enable them to propagate on the mango stem or to activate the mechanism(s) of antagonism (Rothrock and Gottlieb, 1984). Prevention of infection using actinobacteria is a highly recommended management strategy in plant-pathogen interaction systems (Saeed et al., 2017b). It was clear that the application of any of the BCAs prior to pathogen invasion helped to establish the required biomass of the BCA; and thus delay the systemic invasion by *L. theobromae* within the host plant (**Figure 8**). The results obtained from the DSI indicated that *S. samsunensis*, *S. cavourensis*, or *M. tulbaghiae* have good potential as BCAs of the mango dieback caused by *L. theobromae*; yet it was difficult to choose the most effective BCA. With all the successful BCA applications, a significant reduction in disease symptoms regarding the number of falling leaves at 6 wpi and the conidia counts in affected tissues at 9 wpi in BCA1-treated seedlings was found. This suggests that *S. samsunensis* is the

most efficient BCA among the tested strains, and may serve as a candidate biofungicide for the control of *L. theobromae*-affected mango orchids. To a lesser degree, *S. cavourensis* and *M. tulbaghiae* were also notably effective in reducing the pathogenicity of *L. theobromae* in the greenhouse.

Biological control can be used as an alternative method to agrochemicals if the BCA or its product survives temporarily adverse conditions and preferably improves plant performance (El-Tarabily and Sivasithamparam, 2006). The antagonistic *Streptomyces* and *Micromonospora* spp. identified in this study are safe, inexpensive, long lasting and well-suited to extreme harsh conditions, meeting the expectations previously reported in literature (Goodfellow and Williams, 1983; Ningthoujam et al., 2009). These indigenous strains of BCA are well-adapted to the local conditions of the UAE of dry soils and arid environments. In addition, actinobacteria are capable of producing spores resisting heat and drought stresses (Goodfellow and Williams, 1983), making them adequately suitable for being implemented as a prime component in IPM leading to sustainable agriculture in the future. In general, it is well-known that the CFU of actinobacteria remains high as soils dry out; while the relative incidence of bacteria is adversely affected as they lack tolerance to arid conditions (Alexander, 1977).

Together, the diffusible compounds and/or CWDEs produced by the BCA candidates were closely associated with the inhibition, suppression and destruction of *L. theobromae* within the plant host. The mechanisms identified are most likely to have significantly contributed to the relative success of these selected strains as BCAs. Other factors, such as the production of compounds capable of inducing host resistance (e.g., ISR) to the pathogen by the BCA (Martínez-Hidalgo et al., 2015) could also be attributed to the reduction of the mango dieback incidence detected. This form of resistance by the BCA candidates was not investigated in this research, but will surely be looked at in future studies. The present study provided the first record of SA and NSA as microbial antagonists to control a *Lasiodiplodia* disease. To our knowledge, the results demonstrate, for the first time, the isolation, identification and confirmation of the biocontrol potential of actinobacteria from soil native to the UAE, and likely to be naturally suited to be suppressive to *L. theobromae*. In order to make biocontrol more effective, future research focusing on development of novel formulations, broadening of host range targets, and increasing biomass production of BCAs in association with the use of biotechnology in improvement of BCA mechanisms will effectively develop schemes to widely manage the dieback disease using environmentally sustainable strategies.

AUTHOR CONTRIBUTIONS

KE-T and SA designed the research and supervised the study. FK, ES, and KE-T performed *in vitro* and *in vivo* experiments. ES, KE-T, and SA performed *in vivo* greenhouse experiments. SA developed the phylogenetic analysis. KE-T and SA analyzed the data. FK and ES assisted with experiments and/or data evaluation. KE-T and SA wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00829/full#supplementary-material

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How *Streptomyces anulatus* Primes Grapevine Defenses to Cope with Gray Mold: A Study of the Early Responses of Cell Suspensions

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Gray mold, caused by *Botrytis cinerea*, is one of the most destructive diseases of grapevine and is controlled with an intense application of fungicides. As alternatives to chemicals, beneficial microbes may promote plant health by stimulating the plant's immune system. An actinomycete, *Streptomyces anulatus* S37, has been screened from the rhizosphere microbiome of healthy *Vitis vinifera* on the basis of its ability to promote grapevine growth and to induce resistance against various phytopathogens, including *B. cinerea*. However, molecular mechanisms involved locally after direct perception of these bacteria by plant cells still remain unknown. This study focuses on local defense events induced in grapevine cells during interactions with *S. anulatus* S37 before and after pathogen challenge. We demonstrated that *S. anulatus* S37 induced early responses including oxidative burst, extracellular alkalinization, activation of protein kinases, induction of defense gene expression and phytoalexin accumulation, but not the programmed cell death. Interestingly, upon challenge with the *B. cinerea*, the *S. anulatus* S37 primed grapevine cells for enhanced defense reactions with a decline in cell death. In the presence of the EGTA, a calcium channel inhibitor, the induced oxidative burst, and the protein kinase activity were inhibited, but not the extracellular alkalinization, suggesting that Ca^{2+} may also contribute upstream to the induced defenses. Moreover, desensitization assays using extracellular pH showed that once increased by *S. anulatus* S37, cells became refractory to further stimulation by *B. cinerea*, suggesting that grapevine cells perceive distinctly beneficial and pathogenic microbes.

Keywords: ***Actinobacteria*, *Botrytis cinerea*, defense responses, early signaling, grapevine**

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INTRODUCTION

Plant innate immunity is a potential basal defense system, which provides powerful weapons to the host plants to fight against invaders. Beneficial bacteria or derived elicitors referred to as microbial-associated molecular patterns (MAMPs) have been identified as alarm/danger signals to trigger the plant innate immune responses (Jones and Dangl, 2006). In addition, they are able to activate the second line of defense mechanisms leading to induced systemic resistance (ISR)

from a pathogen. The perception of some of these beneficial microbes involves early responses including ion fluxes, MAP kinase cascade activation, extracellular medium alkalinization, and the production of reactive oxygen species (ROS) (Van Loon et al., 2008; Verhagen et al., 2010; Bordiec et al., 2011). These events are followed by an activation of various molecular and cellular host-defense responses namely, defense-related gene expression, phenolic accumulation, lignin and callose deposition as well as hypersensitive response (HR) (Jones and Dangl, 2006; Bordiec et al., 2011; Verhagen et al., 2011; Farace et al., 2015). The induced defense responses are often linked to an activation of a complex signal transduction network, which involves mainly salicylic acid (SA), jasmonic acid (JA), and/or ethylene (ET) as important regulators of plant immunity (Pieterse et al., 2012), depending on pathosystems (Thomma et al., 1998; Kunkel and Brooks, 2002). The role of these signaling pathways in the regulation of ISR was pointed out in many plant-beneficial microorganisms interaction (Pieterse et al., 2014), supporting the idea that JA and ET are dominant players in the regulation of SA-independent systemic immunity conferred by beneficial microorganisms. In response to pathogen attacks, the production of ROS is also of considerable importance in plant defense (for review, Heller and Tudzynski, 2011). ROS are considered as second messengers, inducing several resistance responses including synthesis of pathogenesis-related proteins and phytoalexins, and programmed cell death in neighboring cells (Torres et al., 2006). ROS are also directly detrimental to pathogens, which prevent further disease spread (Torres and Dangl, 2005). In the host plant, the intensity of the induced oxidative burst correlates with the aggressiveness of *Botrytis cinerea* (Tiedemann, 1997). Further, instead of suppressing the plants oxidative burst, *B. cinerea* seems to exploit and might even contribute to this defense reaction (Govrin and Levine, 2000).

The production of H_2O_2 has also been assumed to be one of the most important signal molecules that could be linked to the development of ISR in whole plants (Van Loon et al., 2008; Verhagen et al., 2010; Bordiec et al., 2011; Farace et al., 2015). H_2O_2 can diffuse freely across membranes and, therefore, has been implicated in the signal transduction and the activation of defense responses (Aziz et al., 2004). This can lead to the cell wall protein cross-linking, thereby strengthening the cell wall (Lamb and Dixon, 1997).

The rapid activation of MAP kinase cascades is also involved in various signaling and regulatory mechanisms as well as alterations in the expression of several defense genes, which lead to the plant resistance. Most research in innate immunity gravitates toward MAMPs than live beneficial bacteria. In *Arabidopsis*, flagellin perception is transduced through a MAP kinase cascade (Asai et al., 2002) resulting in enhanced resistance against *Pseudomonas syringae* pv. tomato, which was associated with a callose deposition and an activation of *PR* genes (Gómez-Gómez et al., 1999). Similarly, pretreatment of tobacco leaves with lipopolysaccharides from the non-pathogenic *Burkholderia cepacia* was associated with the phosphorylation of an ERK-like MAP kinase and enhanced protection against *Phytophthora nicotianae* (Piater et al., 2004). Some non-pathogenic rhizobacteria-derived MAMPs primed plants for enhanced defense reactions upon challenge with a pathogen without extensive transcriptional reprogramming or cell death (Pieterse et al., 2014). In grapevine, Gruau et al. (2015) have reported that *Pseudomonas fluorescens* PTA-CT2-mediated ISR was accompanied by a down-regulation of *HSR* gene, a marker of HR/cell death after the *B. cinerea* challenge.

Another important response in the grapevine is the accumulation of stilbenic phytoalexins, especially *trans*-resveratrol (3,5,4′-tryhydroxystilbene) and its oligomer, *trans*-ε-viniferin during plant–microbe interactions (Delaunois et al., 2009; Jeandet et al., 2014). These stilbenic compounds are selectively accumulated in leaves and grape cell suspensions in response to various rhizobacteria (Verhagen et al., 2010; Aziz et al., 2016), and were shown to be associated with resistance of plants to pathogens.

Research aimed at understanding induced resistance mechanisms has scarcely elucidated in grapevine plants. For instance, pretreatment of grapevine plants with *Burkholderia phytofirmans* PsJN, *Pseudomonas* sp., *Pantoea* sp., or *Acinetobacter* sp. improve the grapevine resistance to subsequent infection with *B. cinerea* by eliciting defense-responses, including a stimulation of chitinases and β-1,3-glucanases activities, accumulation of phytoalexins, and an induction of defense genes (Compant et al., 2008; Trotel-Aziz et al., 2008; Gruau et al., 2015). *Streptomyces* is another important and versatile genera of *Actinobacteria* (Ait Barka et al., 2016) that may impact plants growth promotion by affecting their metabolism (Salla et al., 2014). In addition, *Streptomyces* sp. can also induce a local and a systemic resistance in grapevine and *Arabidopsis* to pathogens (Conn et al., 2008; Loqman et al., 2009; Couillerot et al., 2014). The *Streptomyces*-induced resistance in *Arabidopsis* seems to be dependent on SA but not on JA/ET pathway (Conn et al., 2008).

Streptomyces anulatus S37 isolated from the rhizosphere of healthy wild *Vitis vinifera* have been shown as an endophytic plant growth promoting bacteria that enhances disease resistance against several pathogens including *B. cinerea* (Loqman et al., 2009). However, despite their putative importance for biocontrol and/or growth stimulation, the cellular and molecular mechanisms involved in the perception of this bacterium by plant cells still remain unknown.

Many aspects of the defense response can be observed in suspension-cultured plants treated with substances of fungal or bacterial origin, so called-elicitors. Therefore, cultured plant cells have been widely used as model systems to study the recognition and the transduction of microbial signals as well as the defense response itself (Dixon and Lamb, 1990). While the systemic induction of resistance by beneficial bacteria is well understood, little data are available on local defense events taking place during the interaction between grape cells and these bacteria. Additionally, no direct comparison has been made between defense responses prompted by actinomycete bacteria and the typical defense reactions occurred during non-host or incompatible interactions triggered by *B. cinerea*.

Herein, our objective is to understand how plants could integrate signals induced by beneficial *S. anulatus* S37 into an immune response that maximizes both profitable and protective functions against the necrotrophic fungus *B. cinerea*. This will

be accomplished by underlying the local defense events induced by *S. anulatus* S37 after their perception by grapevine cells, and after infection with *B. cinerea*. Our results indicate that *S. anulatus* S37 was perceived by grapevine cells by triggering early and late responses including oxidative burst, extracellular alkalinization, activation of protein kinases, induction of defense genes expression and phytoalexin accumulation, but not the programmed cell death. Further, upon challenge with *B. cinerea*, the *S. anulatus* S37 primed grapevine cells to enhanced defense reactions and reduced the pathogen-induced cell death. Once stimulated by the bacterium, plant cells became refractory to further stimulation by *B. cinerea*, suggesting a different mode of perception of beneficial and pathogenic microbes by grapevine cells.

MATERIALS AND METHODS

Plant Cell Culture

Concord grape (*Vitis labrusca*) cell suspensions were cultured in Murashige and Skoog (MS) medium (pH 5.8) containing vitamins (×1.5), sucrose (30 g l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.2 mg l^{-1}), 6-benzylaminopurine (BAP, 0.5 mg l^{-1}) and were propagated in the dark at 25°C under shaking at 120 rpm. They were sub-cultured every 7 days to be maintained in exponential phase. For experiments, 30 ml of cells sub-cultured for 7 days were collected and resuspended in fresh MS medium for 24 h before treatment (Aziz et al., 2004).

Microorganisms

Bacteria were collected by centrifugation (3,000 × *g* for 15 min) and washed twice with a phosphate-buffered saline (PBS) (10 mM, pH 6.5). The pellet was resuspended in the PBS and used as inoculum. The bacterium *S. anulatus* S37 concentration was estimated by the spectrophotometer (600 nm) and adjusted to 10^6 colony forming units per ml (cfu ml^{-1}) with the PBS (Loqman et al., 2009).

Botrytis cinerea strain 630 used in this study was provided by Dr. Brygoo (INRA, Versailles, Grignon, France) and maintained on the potato dextrose agar (PDA, Difco, United States). The *B. cinerea* inoculum was initiated by growing the fungus on fresh PDA medium to obtain abundant hyphal swellings. After 3 weeks, conidia collected from the PDA medium with sterile distilled water were filtered through a sterile 25 μm filter. The density of *B. cinerea* conidiospores was then determined and adjusted to 10^5 spores ml^{-1} for all bioassays.

Cell Treatments

Cells were collected during the exponential growth phase and washed by filtration in a suspension buffer containing 175 mM mannitol, 0.5 mM K2SO4, 0.5 mM CaCl2, and 2 mM MES, pH 5.5. Cells were resuspended at 0.1 g FW ml^{-1} with a suspension buffer and equilibrated for 2 h on a rotary shaker (120 rpm, 25°C). Grapevine cells were then used to analyze the extracellular pH, the H_2O_2 production, the MAP kinase assay, the cell death, the defense-related gene expression, and the phytoalexin production after a treatment with the *S. anulatus* S37 and/or the *B. cinerea*. Control cells were incubated under the same conditions without treatment. The EGTA (Sigma) was supplied at 3 mM, for 10 min, before an inoculation with the S37 and/or the *B. cinerea*.

Determination of Extracellular pH and Hydrogen Peroxide

The extracellular pH variation was analyzed according to Felix et al. (1993) in 10 ml of agitated cell culture using a glass pH electrode (Basic, Denver Instrument, Gottingen, Germany). The production of H_2O_2 was analyzed by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol using a luminometer (Lumat LB 9507, Berthold) as described previously (Aziz et al., 2004). The chemiluminescence was integrated and expressed as nmol H_2O_2 per g FW, using a standard calibration curve with H_2O_2.

The refractory state experiments on grapevine cell suspensions were analyzed by the extracellular pH change after a successive addition of the *S. anulatus* S37 and the *B. cinerea*. Cells were first treated at time 0 with the *S. anulatus* S37 at 10^6 cfu ml^{-1} or with the *B. cinerea* at 10^5 spores ml^{-1}, washed at 50, 100, and 150 min with a fresh medium, and then treated, at 150 min, a second time with the *S. anulatus* S37 or the *B. cinerea* at the same concentrations.

In-gel Protein Kinase Assay

In vivo experiments followed by in-gel kinase assays were performed as previously described (Vandelle et al., 2006) using myelin basic protein (MBP) as a MAP kinase substrate at the final concentration of 0.2 mg ml^{-1}.

Detection of Cell Death

The cell death was visualized by the FDA (fluorescein diacetate; Sigma–Aldrich) staining. Fresh cells were incubated in FDA (10 μg ml^{-1} of PBS) in the dark to maximize the formation of the fluorescein. The fluorescent FDA signals were detected with a fluorescence microscope (Olympus BX 51, Olympus, Japan). Only cells that exhibited bright green fluorescence from their cytosol were considered to be viable.

The cell death was also evaluated by analyzing caspase-like activity in grapevine cell suspensions, using the Muse MultiCaspase-7-AAD Assay as described by the manufacturer (Millipore, Molsheim, France). The assay determines simultaneously the percentage of cells showing a caspase activity and cell death fraction. Briefly, after each experimental condition, cells are resuspended at a density of 2–5 × 10^5 cells ml^{-1} in the 1X Assay Buffer. The 50 μl of cell suspension were then added to each measurement tube. Then, 5 μl of Muse™ MultiCaspase Reagent working solution were added to each tube. After vortexing at a medium speed for 3 to 5s, tubes were incubated for 30 min in the 37°C incubator with 5% CO_2. Subsequently, 150 μl of Muse™ 7-AAD working solution were added to each tube. After vortexing as indicated before, tubes were incubated at room temperature for 5 min, protected from light. After cell analysis, percentages of gated cells were calculated. Data indicate viable cells without caspase activity, cells exhibiting caspase activity without death marker,

cells in the late stages of caspase activity with death marker, and cells that have died *via* necrosis but not through the caspase pathway.

RNA Extraction and Quantitative RT-PCR Analysis

Cells were ground in the liquid nitrogen and total RNA was extracted from 50 mg of ground powder following the Concert PlantRNA reagent protocol according to the manufacturer's instructions (Life Technologies). The RNA pellets were resuspended in 30 µl of RNAse-free water and incubated 2 h at −20°C for solubilization. The genomic DNA was removed by a DNAse treatment according to the manufacturer's instruction (RQ1 RNase-Free DNase – Promega). The first-strand cDNA was synthesized from 150 ng of total RNA using the Verso cDNA Synthesis kit (Thermo Scientific). The quantitative RT-PCR was performed as described in Gruau et al. (2015), with Absolute Blue QPCR SYBR Green ROX Mix (Thermo Scientific) using a CFX96 system thermocycler (Bio-Rad). PCR reactions were carried out in duplicates in 96-well plates in a 15 µl final volume containing Absolute Blue SYBR Green ROX mix (including Taq polymerase, dNTPs, and SYBR Green dye), 280 nM forward and reverse primers and 30-fold diluted cDNA. The specificity of PCR reaction was also checked and *EF1α* and *60RSP* genes were used as internal controls for the normalization (Gruau et al., 2015). Results are expressed as the folds increase of transcript level relative to untreated cells as the control sample (1x expression level). The gene-specific primers (Supplementary Table S1) were designed based on sequences present in databases (Gruau et al., 2015).

Phytoalexin Extraction and Analysis

Stilbenic phytoalexins were extracted from 200 mg of freeze-dried cell powder with 2 ml of methanol:water 85% v/v. Tubes were placed in the shaker for 1 h at the room temperature and then centrifuged for 10 min at 8000 × *g*. The supernatant was dried under a nitrogen stream and residues were solubilized with 1 ml of methanol, filtered through 0.22 µm PTFE filters. Stilbenes were analyzed using an Acquity^{TM} UPLC system (Waters Corporation, Milford, CT, United States) as described in Hatmi et al. (2014), identified and quantified with reference to retention time and calibration with external standards.

Statistical Analyses

Each experiment was repeated at least three times, unless indicated elsewhere. For gene expression, medium alkalinization, H_2O_2 production and phytoalexin production, results are expressed as the mean ± SE of a triplicate from one representative experiment out of three independent experiments (in each experiment three different extractions were pooled). For cell death, MAP kinase and Caspase-like activity, the data have been presented as a representative of three independent experiments. The refractory state experiment, reported data mean ± SE of duplicates, representative of two independent experiments. All collected data were submitted to ANOVA and the significance of differences among treatments was recorded at $p < 0.05$.

RESULTS

Early Events Induced by *S. anulatus* S37 in Grapevine Cell Suspensions

Extracellular Alkalinization

The alkalinization of the extracellular medium is a useful tool for analyzing rapid events known to mediate MAMP-induced defense responses (Felix et al., 1998; Küpper et al., 2001). Grapevine cell suspensions treated with *S. anulatus* S37, *B. cinerea,* and S37 + *B. cinerea* showed an increase in the pH of the medium by 1.1 unit within 10 min. The pH variation was a transient and almost similar when cells were treated with *S. anulatus* S37 or *B. cinerea* (**Figure 1A**), returning to the basal value after 120 min. However, in the presence of S37 + *B. cinerea,* the medium alkalinization was maintained longer that individual treatment and came down to a basal level only after 150 min.

Production of H_2O_2

Oxidative burst is one of the early events that generally assumed to be involved in the regulation of induced plant immune responses (Aziz et al., 2004; Torres, 2010; Zipfel and Robatzek, 2010). Here, grapevine cell suspensions were inoculated with *S. anulatus* S37 and/or *B. cinerea*, and the released H_2O_2 was quantified. As shown in **Figure 1B**, grapevine cells responded to S37 and *B. cinerea* with transient and similar release of H_2O_2. For both, the oxidative burst was detected approximately after 10 min and peaked 45 min after the inoculation. Thereafter, the H_2O_2 concentration in the medium declined. When grapevine cells were inoculated with S37 + *B. cinerea*, a high burst of H_2O_2 was observed, which was almost seven-fold superior at 20 min as compared to S37 or *B. cinerea* separately. Thus, we might assume that S37 primes the oxidative burst in grapevine in the presence of *B. cinerea*.

Activation of MAP Kinases

A rapid activation of MAP kinases has been shown in different plant systems to mediate elicitor-induced defense responses (Gomez-Gomez and Boller, 2002; Zhang et al., 1998). The treatment of grapevine cells with *S. anulatus* S37, *B. cinerea,* and S37 + *B. cinerea* activated rapidly and transiently both 45 and 49 kD MAP kinases (**Figure 1C**). This activation was detected as soon as 15 min (**Figure 1C**), maintained at a high level for 30 min and reached the basal level around 60 min (data not shown).

Analyzing Cell Death and Caspase-Like Activity

During the cell and microbes interaction, cells can be damaged, thereby affecting their viability. To further elucidate cell viability during the process of inoculation with *B. cinerea* and to study the impact of the presence of *S. anulatus* S37, grapevine cells were stained using fluorescein diacetate as a vital stain. Grapevine cells with intact plasma membranes and respiration revealed a green fluorescence while those without FDA-fluorescence

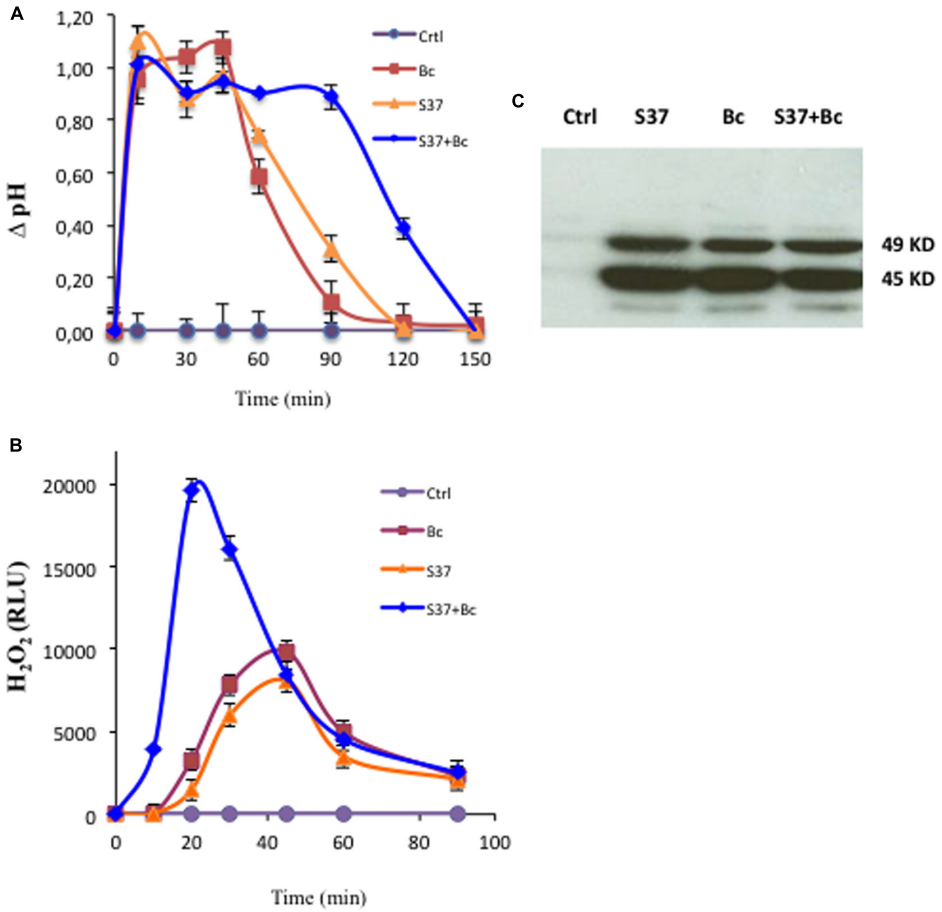


FIGURE 1 | Early events in grapevine cell suspensions. Medium alkalinization **(A)**, accumulation of H_2O_2 **(B)** or MAP kinase activity **(C)** in control (circle), cells challenged with *B. cinerea* (square), with S37 (triangle), and with S37 + *B. cinerea* (diamond). MAPK activity was performed after 15 min of treatment. Data presented are means of triplicate experiments ± SD. For MAPK, data presented are representative of triplicate experiments. Bc, *Botrytis cinerea;* S37, *S. anulatus* S37.

were considered dead. Control cells showed strong FDA signals (**Figure 2A**), with high viability levels. While the presence of *S. anulatus* S37 in contact with cells has no impact on the viability, the FDA signal was weaker in cells infected with *B. cinerea*, indicating disturbed cell membranes and an inhibited respiratory activity. However, when cells were inoculated with S37 then with *B. cinerea*, the FDA signal was weak than the control but significantly higher than in cells infected solely with *B. cinerea*.

The multi-caspase activity was measured as described in "Materials and Methods." The data in **Figure 2B** showed that this activity was not impacted significantly when grapevine cells suspension was brought into contact with S37. However, in the presence of *B. cinerea,* the percentage of cells with caspase activity increased significantly compared to the previous two terms (9.90%). When grapevine cells were previously inoculated with S37 before their infection with *B. cinerea*, this activity decreased significantly (2.55%).

Expression of Defense-Related Genes

Expression profiles of number defense-related genes were analyzed by qRT-PCR in grapevine cells suspension after their inoculation with *S. anulatus* S37 and/or *B. cinerea*. Genes used correspond to those reported to be up-regulated during grapevine-microbe/MAMP interactions (Aziz et al., 2003; Varnier et al., 2009; Bordiec et al., 2011) and include those encoding a lipoxygenase (LOX9), a phenylalanine ammonia-lyase (PAL), a stilbene synthase (STS), a basic glucanase (Gluc), and a glutathione *S*-transferase (GST). All defense genes were up-regulated when the cells were inoculated with S37 or *B. cinerea* starting after 9 h except for *LOX9* (**Figure 3**).

The level of gene expression was lower in S37-treated cells compared to those infected with *B. cinerea*. Interestingly, inoculation with S37 + *B. cinerea* results in the priming of *LOX9* gene expression, which was almost 20 times higher than control. A priming effect was also seen for *PAL*, *STS,* and *GST*. The basic glucanase (*Gluc*) was the only gene, which was induced but not primed by S37 (**Figure 3**).

Phytoalexin Production

The resveratrol (*trans*-3,4′,5-trihydroxystilbene) and its dehydrodimer viniferin have been reported as the major phytoalexins produced by grapevine in response to the microbial

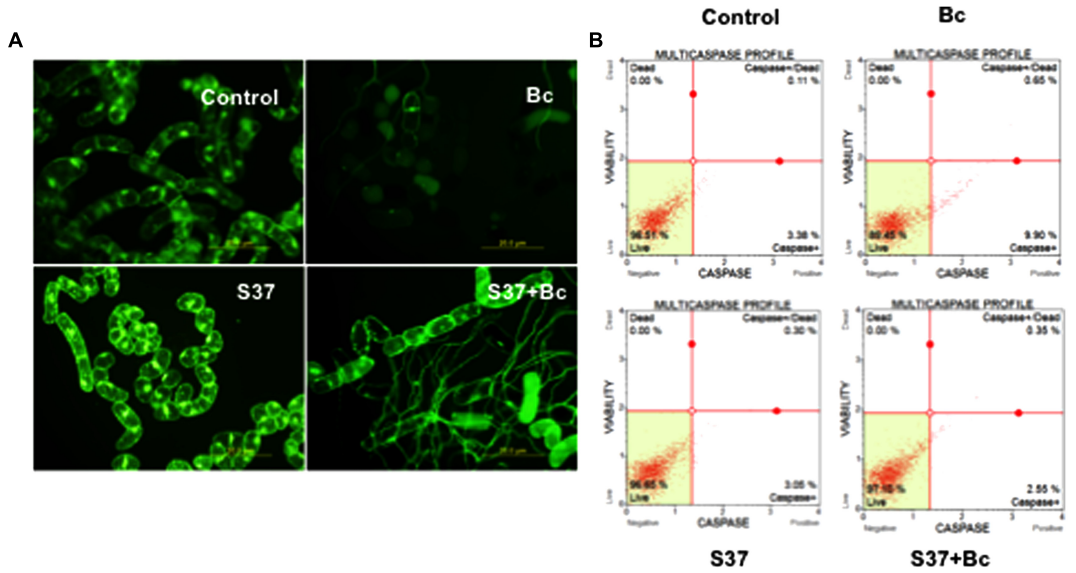


FIGURE 2 | Monitoring cell death in grapevine cell suspensions by FDA as a probe **(A)** or by multicaspase profile **(B)** in control, cells challenged with *B. cinerea,* with S37, or with S37 + *B. cinerea.* Data presented are representative of triplicate experiments. Bc, *Botrytis cinerea;* S37, *S. anulatus* S37.

infection and was associated with plant resistance to fungal pathogens (Adrian et al., 1997; Aziz et al., 2016). As shown in **Figure 4**, induction of resveratrol and *trans*-δ-viniferin was observed following treatment of grapevine cells with S37. A slight induction of both stilbenes was also observed after challenge with *B. cinerea*. Amounts of δ-viniferin accumulated were generally greater than resveratrol. However, with both microbes, the level of the glycosylated resveratrol, piceid, remained comparable to that of the control. With S37 + *B. cinerea*, the production of resveratrol and piceid showed similar levels than with S37, while δ-viniferin accumulation was primed.

Refractory State

One of the characteristics of cells is the occurrence of the refractory state, which often occurs after the perception of MAMPs/PAMPs at high-affinity protein receptors in the plasma membrane. As *S. anulatus* S37 and *B. cinerea* induce the same pattern of early events, refractory assays (i.e., the inability of the cells to react to a second application of the same elicitor), using extracellular pH were performed by successive additions of S37 and/or *B. cinerea*. Grape cells pretreated with the S37 were shown to be refractory to the second application of S37. However, they were not refractory to an application of *B. cinerea* (**Figure 5A**). Similarly, cells pretreated with *B. cinerea* were shown to be refractory to the second application of *B. cinerea*, while they were not refractory to an application of S37 (**Figure 5B**).

DISCUSSION

The *Streptomyces anulatus* S37 has been demonstrated as a protector of the grapevine against *B. cinerea* (Loqman et al., 2009), but molecular mechanisms involved in this interaction remains unknown. In this study, we attempt to decipher early events involved during the bacterial recognition and subsequent defense reactions after the *B. cinerea* challenge.

S. anulatus S37 Induces Early Events in Grapevine Cells

Our results showed that *S. anulatus* S37 triggers the oxidative burst, one of the key events, in grapevine cell signaling. ROS play a crucial role either as toxic substances to halt the growth of the pathogen or as second messengers in the defense mechanism, leading to efficient plant protection (Torres, 2010; Zipfel and Robatzek, 2010). Nevertheless, only a few examples have described the involvement of ROS as a signal during the grapevine plants-beneficial bacteria interaction. For instance, *P. fluorescens* PTA-CT2, *P. aeruginosa* 7NSK2, and *B. subtilis* PTA-271 trigger a strong oxidative burst in grapevine cell suspensions (Verhagen et al., 2010, 2011). Authors suggested that the H_2O_2 production in grape cell suspensions could contribute as a signaling molecule to induce a disease resistance by these strains *in planta*. Indeed, although the accumulation of H_2O_2 is often associated with a characteristic plant early response following perception of pathogen avirulence signals (Lamb and Dixon, 1997), it was also shown that, in the symbiotic interaction with rhizobia, bacteria are initially recognized as intruders but then prevent or overcome plant defense responses (Santos et al., 2001). However, in contrast with our results, no significant variation in H_2O_2 levels was induced in grapevine cells by *B. phytofirmans* PsJN (Bordiec et al., 2011). Further, Van Loon et al. (2008) have reported that the production of H_2O_2 induced by *P. fluorescens* WCS417 was not matched with the expression of ISR in the whole tobacco against *Erwinia carotovora*.

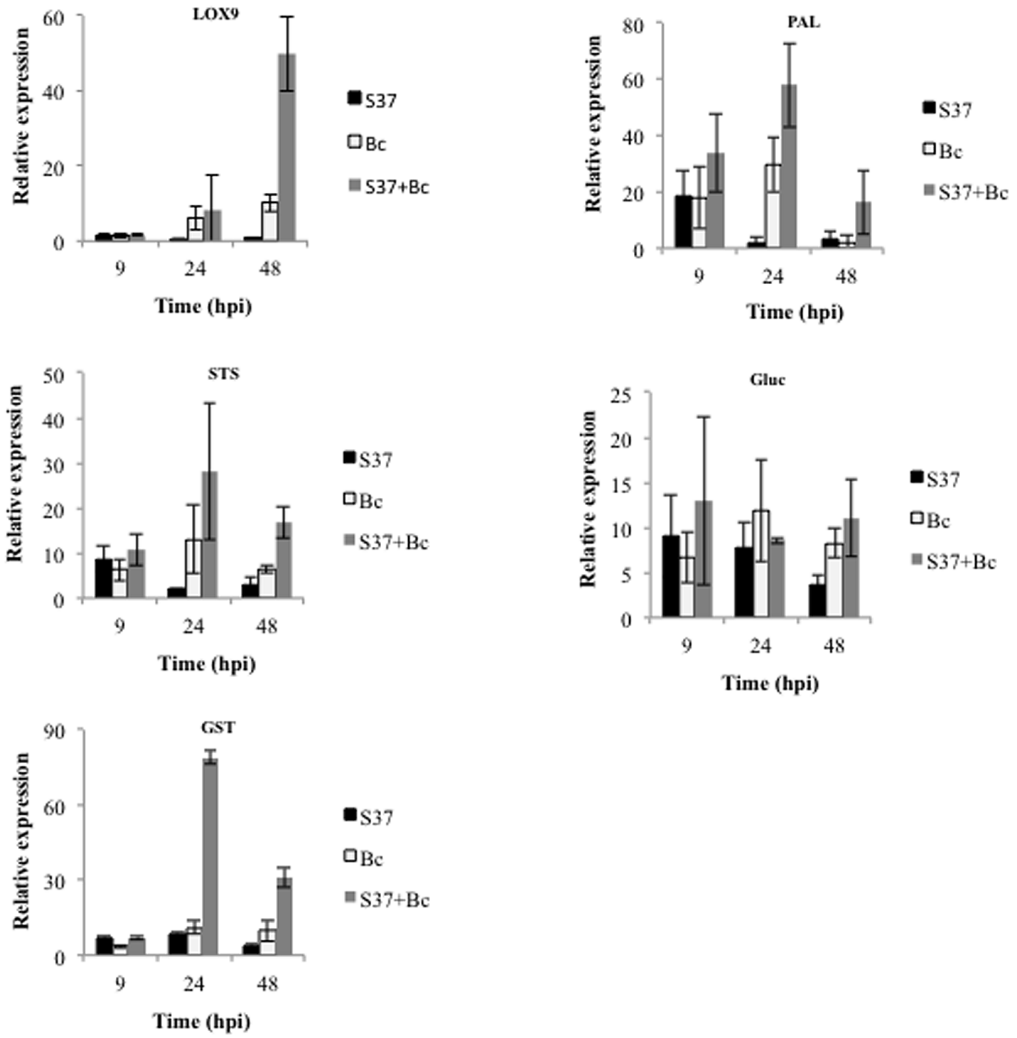


FIGURE 3 | Relative expression of defense related genes in grapevine cell suspensions. Gene expression was monitored in control, cells challenged with *B. cinerea,* with S37, or with S37 + *B. cinerea.* Data presented are means of triplicate experiments ± SD. Bc, *Botrytis cinerea;* S37, *S. anulatus* S37; LOX9, lipoxygenase; PAL, phenylalanine ammonia-lyase; STS, stilbene synthase; Glue, glucanase; GST, Glutathione-*S*-transferase.

The *S. anulatus* S37 also induces a strong extracellular alkalinization, which suggests an activation of plasma membrane H^+-ATPases, to restore the pH gradient between the apoplast and the cytosol. This typical signature has been previously demonstrated during the microbial or MAMP perception (Aziz et al., 2004; Bordiec et al., 2011; Farace et al., 2015). The perception of S37 by grapevine cells was also associated with the phosphorylation of two mitogen-activated protein (MAP) kinases with relative molecular masses of 49 and 45 kDa. These effects resembled those achieved by the MAMPs in tobacco (Zhang et al., 1998) and in grapevine (Aziz et al., 2004; Trouvelot et al., 2008). MAP kinase cascades are major pathways downstream of sensors/receptors that transduce extracellular stimuli into intracellular responses in plants (Zhang et al., 1998). Their activation is known as an early physiological response to microbial recognition and plays a pivotal role in the plant innate immune system (Asai et al., 2002; Aziz et al., 2004; Meng and Zhang, 2013). In tobacco, two MAP kinases, designated SA-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK) are activated in a disease resistance-specific manner following pathogen infection or elicitor treatment (Kishi-Kaboshi et al., 2010). Similarly, it has been shown that lack of MPK3 increases the basal susceptibility of *Arabidopsis* to *B. cinerea*, while the lack of MPK6 suppresses flg22-induced resistance to *B. cinerea* (Galletti et al., 2011). It is noteworthy that these early responses induced by the *S. anulatus* S37 were not accompanied by a programmed cell death of grapevine, as shown by the FDA signal and the multicaspase activity. This result is in line with previous reports who indicated that oxidative burst or the activation of MAP kinases induced by beneficial bacteria are independent of hypersensitive cell death (Bargabus et al., 2003; Van Loon et al., 2008; Verhagen et al., 2010).

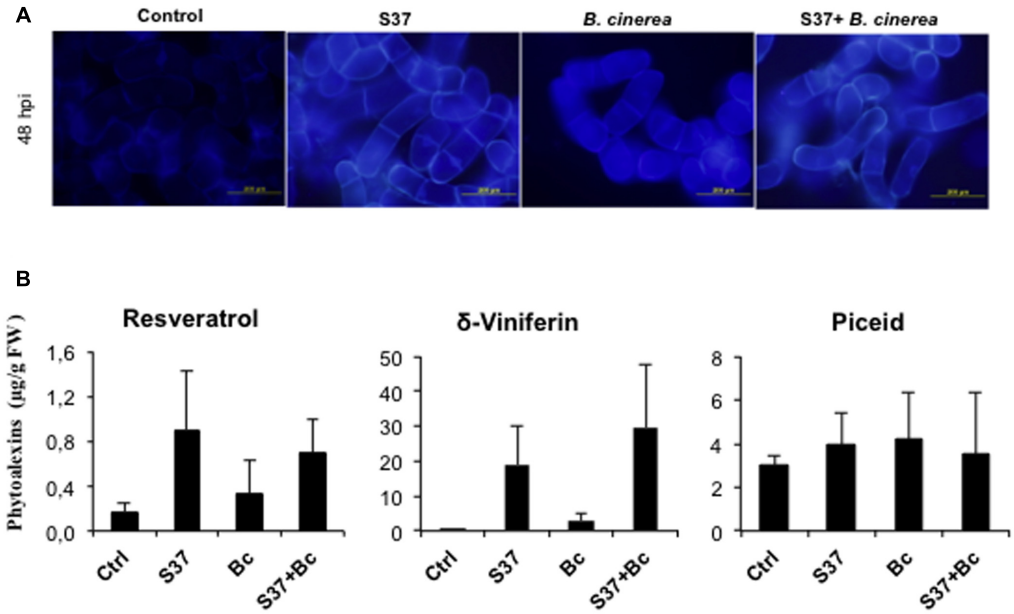


FIGURE 4 | Production of phytoalexins by grapevine cells. **(A)** Autofluorescence microscopy under UV excitation of inoculated cells. Data presented are representative of triplicate experiments. **(B)** Phytoalexins, resveratrol δ-viniferin and piceid were measured at 48 h from control, cells challenged with S37, with *B. cinerea,* or with S37 + *B. cinerea.* Data are means from three replicates ± SD. Bc, *Botrytis cinerea,* S37, *S. anulatus* S37.

The Ca^{2+} influx is also involved in the microbial signal perception and can regulate many early responses in plant cells. In this context, we investigated whether extracellular alkalinization, H_2O_2 production, and MAPK activity depend on the calcium influx in grapevine cell suspensions. Using the EGTA, a Ca^{2+} influx inhibitor, the extracellular medium alkalinization was not impacted for all treatment, suggesting that this event does not depend on Ca^{2+}-mediated pathways (Supplementary Figure S1). However, the high oxidative burst and MAPK activity induced by *S. anulatus* S37 and/or *B. cinerea* were completely abolished by EGTA in grapevine cells, suggesting that both microorganisms trigger H_2O_2 production and MAPK activity through Ca^{2+} dependent pathways. Similar results have been reported by Vandelle et al. (2006), using elicitors derived from *B. cinerea*. The possible regulation of the oxidative burst and the MAPK activation by the Ca^{2+} influx could result from a stimulation of NADPH-oxidase, the major enzymatic source of ROS, together with activation of Ca^{2+}-dependent MAPK.

S. anulatus S37 Induces the Defense-Related Genes Expression and Phytoalexin Accumulation

The treatment with S37 induced the upregulation of genes that are responsive to *B. cinerea* (Bézier et al., 2002), MAMPs (Aziz et al., 2004), and beneficial bacteria (Gruau et al., 2015). These include genes encoding secondary metabolism (PAL, STS, LOX9, GST), and the glucanase (PR2). However, we observed that the expression of all selected genes was lower in S37-treated cells compared to those infected with *B. cinerea*. This suggests that S37, as for other beneficial bacteria, can initially be perceived as a potential invader, resulting in the activation of the plant immune system (Zamioudis and Pieterse, 2012). These results are consistent with the hypothesis that plants respond weakly to beneficial microbes or derivative MAMPs to avoid a strong activation of defense responses that could be detrimental to fitness (Van der Ent et al., 2009). However, even the level of gene expression was low during the S37-grapevine cell interaction, it emphasizes the involvement of JA and SA signaling pathways upon the S37 perception. This was supported by an enhanced expression of *LOX9,* as a key element of the oxylipin synthesis, and an induced expression of *PR-2* (Gluc) and GST genes, which were found to be induced by SA in grapevine leaves (Dufour et al., 2013; Gauthier et al., 2014). The expression of these genes as JA- or SA-dependent responses was also responsive to *Pseudomonas fluorescens* PTA-CT2 and *Burkholderia phytofirmans* PsJN and as beneficial bacteria in grapevine tissues (Gruau et al., 2015; Miotto-Vilanova et al., 2016).

S. anulatus S37 Primes Cell Responses after *B. cinerea* Inoculation

Our results show that grapevine cells infected with the *B. cinerea* exhibited similar early responses to those induced by the beneficial bacterium *S. anulatus* S37, except that the pathogen triggered the programmed cell death. This suggests the existence of some similarities and differences in signaling pathways involved in the recognition of beneficial and pathogenic microbes. However, the triggered cell death by *B. cinerea* might be a capital mechanism in the infection process with this necrotrophic pathogen (Gruau et al., 2015). As S37 and *B. cinerea*

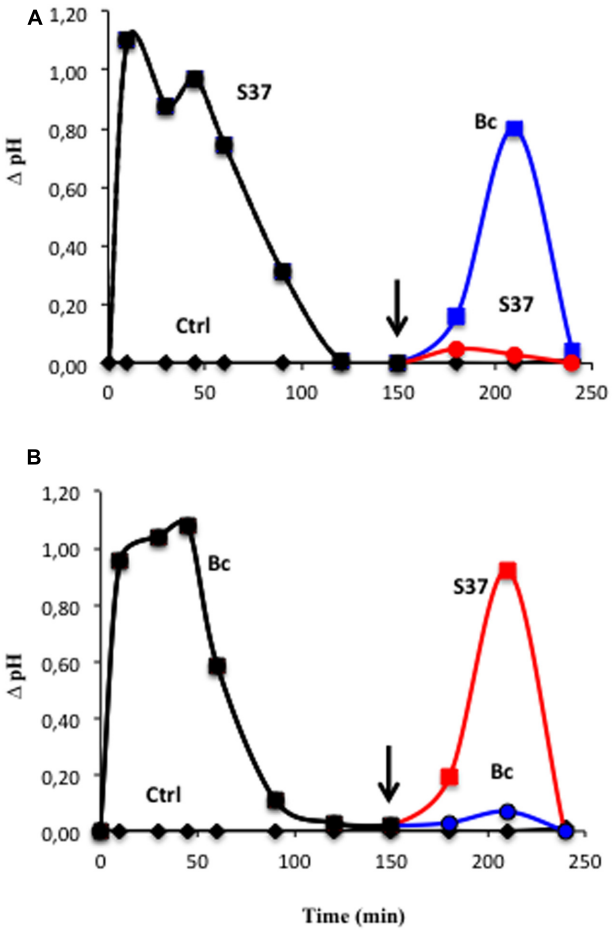


FIGURE 5 | Refractory state experiments on grapevine cell suspensions monitored by extracellular pH measurement after successive inoculation with S37 and/or *B. cinerea*. Cells were first treated at time 0 with S37 **(A)** or Bc **(B)**, washed three times with fresh medium, then treated a second time (arrow) with S37 (read line) or with Bc (blue line). Data are means ± SD of duplicates, representative of two independent experiments. Bc: *Botrytis cinerea*; S37, *S. anulatus* S37.

induced the same pattern for early events, we further performed desensitization assays using extracellular pH. We demonstrated that once increased by *S. anulatus* S37, the grapevine cells became refractory to further stimulation by *B. cinerea*, and inversely. This suggests a different mode of perception of the beneficial and pathogenic microbes by grapevine cells. Interestingly, upon a challenge with *B. cinerea, S. anulatus* S37 primed grapevine cells for enhanced defense reactions with a decline in the cell death. A higher production of H_2O_2 and an enhanced extracellular alkalinization were observed in S37-treated grapevine cells once challenged with *B. cinerea*. Since in this study S37 or *B. cinerea* do not produce H_2O_2 (data not shown), we might suggest that S37 primes the oxidative burst in grapevine cells as evident by the enhanced accumulation of H_2O_2 after pathogen challenge. *B. cinerea* itself has been shown to produce H_2O_2 in germinating conidia during the early steps of tissue infection (Heller and Tudzynski, 2011; Viefhues et al., 2014), or in response to $CaCl_2$ exposure (Marschall and Tudzynski, 2016). However, the priming state of grapevine cells to produce more H_2O_2 without direct contribution of S37 or *B. cinerea* is consistent with our previous study showing that the bacterium S37 exerts an antifungal effect on *B. cinerea* by destructing its mycelium (Couillerot et al., 2014). In meanwhile, it cannot be excluded that the magnitude of the burst of H_2O_2 could be ascribed to different PAMP/MAMP compounds released by both the bacterium (Van Loon et al., 2008) and *B. cinerea* (Vandelle et al., 2006) in the medium. Furthermore, the time course of H_2O_2 burst is more in agreement with the timeline observed in grapevine cell cultures, but not in *B. cinerea*, after treatment with different MAMPs (Aziz et al., 2007), in which this response was linked to the expression of defense-related genes and development of leaf resistance against *B. cinerea*.

Alternatively, the low contribution of *B. cinerea*, if any, to the enhanced oxidative burst, could be due the low virulence of the strain used in this study. Indeed, it has been shown that ROS production by *B. cinerea* is an important component of its virulence, and increased levels of ROS in plant cells may contribute to host cell death and favors fungal infection (Heller and Tudzynski, 2011; Marschall and Tudzynski, 2016).

The level of activation of the 45 and 49 kDa MAP kinases was also maintained at high level in S37-treated cells after *B. cinerea* challenge. We may suggest, the MAP kinase activity is already at it maximum and no more activation was observed after subsequent exposure to pathogen. In accordance with our results, it has been reported that, in *Arabidopsis*, MP3 kinase was not primed, but rather linked to direct responses to pathogen infection and MAMP signaling (Nakagami et al., 2005). This is in line with the fact that both oxidative burst and activation of MAPK were considered as possible regulators of defense responses (Aziz et al., 2004; Beckers et al., 2009).

Several reports have suggested that most beneficial bacteria primed plants for the activation of various cellular defense responses upon the pathogen attack (Conrath et al., 2006; Van der Ent et al., 2009; Gruau et al., 2015). In this study, we further showed that the relative expression of some defense-related genes was upregulated by S37 after *B. cinerea* challenge. The expression of *LOX9* gene was almost 20 times higher in primed cells than in *B. cinerea*-infected ones. A priming effect was also seen with the expression of *PAL*, *STS*, and *GST* genes, while the basic glucanase (PR2) was not primed by the S37. Interestingly, the primed *PAL* and *STS* expressions were correlated with the S37-enhanced phytoalexin accumulation after *B. cinerea* challenge. These results are consistent with other previous researches showing that beneficial bacteria prime grapevine cells and leaves for an accelerated and an enhanced capacity to activate defense responses (Verhagen et al., 2011; Gruau et al., 2015), such as the rapid accumulation of hydrogen peroxide and phytoalexins as well as the activation of some defense-related genes upon the *B. cinerea* infection. Engineering the STS into plants of interest resulted in resveratrol accumulation and elevated pathogen resistance (Jeandet et al., 2013). A slight

induction of stilbenic phytoalexins, resveratrol and its metabolic products, the glycoside piceid and the oxidized dimer δ-viniferin was observed after perception of S37, while only amount of δ-viniferin accumulated was greater after *B. cinerea* challenge. Similar results were observed in grapevine plants treated with *P. fluorescens* PTA-CT2 and *Pantoea agglomerans* PTA-AF2, which showed enhanced resistance to *B. cinerea* (Aziz et al., 2016). The accumulation of δ-viniferin in S37-treated cells indicated that this oligomer could be a possible marker for induced resistance to gray mold.

Overall, this study demonstrated for the first time that *S. anulatus* S37 induced a rapid and transient generation of H_2O_2, extracellular alkalinization and an activation of two MAPKs followed by a differential expression of some defense-related genes and a phytoalexin accumulation to lesser amounts, but not the programmed cell death. Interestingly, most of these defense responses were primed by the S37 after the pathogen challenge, with a decline in the cell death. Desensitization assays using the extracellular pH showed that once increased by the *S. anulatus* S37, cells became refractory to further stimulation by the *B. cinerea*, suggesting that grapevine cells distinctly perceived beneficial and pathogenic microbes.

AUTHOR CONTRIBUTIONS

PV-P and EAB designed the research. PV-P, EAB, AA, MR, SV, and HM carried out the experiments and analysis/interpretation of data. PV-P, AA, MR, and EAB wrote the manuscript with contributions and discussion from all of the co-authors. All authors have given approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01043/full#supplementary-material

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Biocontrol Rhizobacterium *Pseudomonas sp.* 23S Induces Systemic Resistance in Tomato (*Solanum lycopersicum* L.) Against Bacterial Canker *Clavibacter michiganensis* subsp. *michiganensis*

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Tomato bacterial canker disease, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is a destructive disease and has been a serious concern for tomato industries worldwide. Previously, a rhizosphere isolated strain of *Pseudomonas sp.* 23S showed antagonistic activity toward *Cmm in vitro*. This *Pseudomonas sp*. 23S was characterized to explore the potential of this bacterium for its use in agriculture. *Pseudomonas sp*. 23S possesses ability to solubilize inorganic phosphorus, and to produce siderophores, indole acetic acid, and hydrogen cyanide. The strain also showed antagonistic activity against *Pseudomonas syringae* pv. *tomato* DC 3000. A plant assay indicated that *Pseudomonas sp*. 23S could promote growth of tomato seedlings. The potential of treating tomato plants with *Pseudomonas sp.* 23S to reduce the severity of tomato bacterial canker by inducing systemic resistance (ISR) was investigated using well characterized marker genes such as *PR1a* [salicylic acid (SA)], *PI2* [jasmonic acid (JA)], and *ACO* [ethylene (ET)]. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S by soil drench, and *Cmm* was inoculated into the stem by needle injection on 3, 5, or 7 days post drench. The results indicated that plants treated with *Pseudomonas sp*. 23S, 5 days prior to *Cmm* inoculation significantly delayed the progression of the disease. These plants, after 3 weeks from the date of *Cmm* inoculation, had significantly higher dry shoot and root weight, higher levels of carbon, nitrogen, phosphorus, and potassium in the leaf tissue, and the number of *Cmm* population in the stem was significantly lower for the plants treated with *Pseudomonas sp.* 23S. From the real-time quantitative PCR (qRT-PCR) analysis, the treatment with *Pseudomonas sp.* 23S alone was found to trigger a significant increase in the level of *PR1a* transcripts in tomato plants. When the plants were treated with *Pseudomonas sp.* 23S and inoculated with *Cmm*, the level of *PR1a* and *ACO* transcripts were increased, and this response was faster and greater as compared to plants inoculated with *Cmm* but not treated with *Pseudomonas sp.* 23S. Overall, the results suggested the involvement of SA signaling pathways for ISR induced by *Pseudomonas sp.* 23S.

Keywords: tomato, *Pseudomonas*, *Clavibacter michiganensis* subsp. *michiganensis*, PGPR, biocontrol, induced systemic resistance

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INTRODUCTION

Bacterial canker disease, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is one of the most destructive diseases in tomato (Gleason et al., 1993; de León et al., 2011). It has been reported in both greenhouse and field tomato production worldwide, and has caused substantial crop losses (Chang et al., 1992a; Hausbeck et al., 2000; de León et al., 2011). Once plants are infected by *Cmm*, initial marginal leaf necrosis symptoms widen and lead to wilting of all leaves while canker develops on the stem, and the whole plants can be stunted and severely wilted leading to death (de León et al., 2011; Sen et al., 2015). *Cmm* inoculum can originate from infected soils, seeds, transplants, tomato debris in soil, and operating tools and equipment. The bacteria can enter the plants through wounds and natural openings such as stomata and hydathodes after which they move to the xylem and multiply rapidly (Carlton et al., 1998; Gartemann et al., 2003; Sharabani et al., 2013). Farming practices such as tying, pruning, clipping, spraying and harvesting can cause a high level of secondary infection spread to nearby healthy plants via workers' fingers and tools (Ark, 1944; Gleason et al., 1993). Despite the seriousness of this disease, no control methods have been found to be completely effective. As no *Cmm*-resistant seeds are commercially available, current control primarily relies on the use of pathogen-free certified seeds and transplants, good hygiene, disinfection of all tools, and crop rotations (Xu et al., 2015). Hence an effective control method for bacterial canker is urgently needed.

Use of plant growth-promoting rhizobacteria (PGPR) as biocontrol agents offers an ecological means to manage disease problems in agriculture. PGPR are rhizosphere free-living bacteria that colonize plant roots and have beneficial effects on plant growth (Kloepper and Schroth, 1978; Kloepper et al., 1989; Bouizgarne, 2013). The biocontrol ability of PGPR can be attributed to two general mechanisms: direct antagonism against pathogens or induction of systemic resistance throughout the plant. Production of antimicrobial compounds, such as antibiotic metabolites, and bacteriocin has been observed from many PGPR, and their inhibitory actions against pathogens contribute to reduction of plant diseases (Subramanian and Smith, 2015). In addition to direct suppressive effects on the pathogens, PGPR can trigger systemic resistance throughout the plant. PGPR-mediated induced systemic resistance (ISR) is often achieved by priming (Pieterse et al., 2014). Priming is characterized as potentiated activation of defense responses, which are subsequently induced upon pathogen attack, resulting in enhanced plant defense capacity (Conrath et al., 2006).

Although many ISR-inducing PGPR have been discovered, signaling and activation mechanisms of the ISR are not completely understood. The involvement of three plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have been well documented. Conventionally, SA is believed to be involved in systemic acquired resistance (SAR), which is induced by pathogens attack and follows induction of PR proteins. PGPR-mediated ISR is known to be dependent on JA and ET signaling; it is a SA-independent process and does not lead to induction of PR proteins (Van Loon and Bakker, 2005; Van Wees et al., 2008; Pieterse et al., 2014). As more research on ISR has been conducted, however, evidence of SA-dependent ISR has been observed for some PGPR (De Meyer et al., 1999; Tjamos et al., 2005; Schuhegger et al., 2006; Conn et al., 2008; Rudrappa et al., 2008; Niu et al., 2012).

Regarding tomato bacterial canker, several PGPR having antagonistic activities toward *Cmm* have been isolated and studied (Amkraz et al., 2010; Lanteigne et al., 2012; Deng et al., 2015; Aksoy et al., 2017). Among them, the induction of ISR was only reported for *Pseudomonas putida* (CKPp9; Aksoy et al., 2017). This ISR was accompanied by induction of significant amounts of phenolic compounds, which contributed to the disease reduction.

Our laboratory has been working to develop PGPR-based technologies for agriculture. As a part of our work on identifying new PGPR (Jung et al., 2014), we isolated a rhizobacterium that inhibited the growth of *Cmm in vitro* (**Supplementary Figure S1**). According to the 16S rRNA sequencing, this bacterium was identified as a strain of *Pseudomonas*, and is thus referred to here as *Pseudomonas sp*. 23S.

This study was conducted to achieve three objectives. The first objective was to characterize the newly isolated *Pseudomonas sp*. 23S for important PGPR traits. Our second objective was then to determine whether *Pseudomonas sp*. 23S induces ISR in tomato plants and reduces the disease severity specifically, bacterial canker, caused by *Cmm*. Not all PGPR possess ISR-inducing ability, and the question as to whether *Pseudomonas sp*. 23S, which has direct antagonistic activity against *Cmm*, is also able to induce ISR is meaningful to answer, because this could greatly enhance the use of this bacterium as a biocontol agent. Given that initial work reported in this paper did show that the bacterium was an ISR inducer, our final objective was then to determine whether treatment with *Pseudomonas sp*. 23S causes changes in the transcript levels of defense-related genes, specifically *PR1a*, *PI2* and *ACO*. Investigating the transcript levels of these three genes could help to determine the possible involvement of SA, JA and/or ET in the ISR response, and to understand the ISR signaling pathway used by *Pseudomonas sp*. 23S specific to this biotic stress.

MATERIALS AND METHODS

Bacterial Growth Condition

Pseudomonas sp. 23S was grown in Nutrient Broth (NB, Difco; 8 g L^{-1}) media at 28°C, at 100 rpm. *C. michiganensis* subsp. *michiganensis* strain 930 (*Cmm*) was provided by Agriculture, Pecheries et Alimentation, Quebec. *Cmm* was grown in NB media at 28°C, at 150 rpm. Both bacteria were maintained as a glycerol stock in −80°C.

In vitro Assay for General PGPR Traits

Pseudomonas sp. 23S was assessed for important PGPR traits: (i) phosphorous solubilization; (ii) siderophore production; (iii) hydrogen cyanide production; (iv) indole acetic acid production, and; (v) antagonistic activity against an important phytopathogenic bacterium. The abilities to

solubilize phosphorous and to produce indole acetic acid were investigated since these traits improve plant growth. Production of siderophores and hydrogen cyanide, both of which can suppress phytopathogenic bacteria, was studied for the biocontrol traits. Phosphorous solubilization was studied using Pikovskaya medium (PVK; Pikovskaya, 1948) and the National Botanical Research Institute's phosphate growth medium (NBRIP; Nautiyal, 1999). The two types of plates were used to corroborate the results since the PVK plate could sometime give variable results (Nautiyal, 1999). The Fiske and Subbarow method (Fiske and Subbarow, 1925) was applied for quantitative evaluation. Siderophore production was studied using the chrome azurol S (CAS) assay developed by Alexander and Zuberer (1991). For quantitative assessment, percent siderophore production was calculated by using the following formula:

$$\% \text{ siderophore production} = \text{Ar} - \text{As}/\text{Ar} \times 100$$

Where, Ar represents the absorbance of reference (CAS assay solution plus growth medium) at 630 nm and As represents the sample (CAS assay solution plus bacterial supernatant) at 630 nm (Schwyn and Neilands, 1987; Alexander and Zuberer, 1991; Ghosh et al., 2015).

For the hydrogen cyanide, *Pseudomonas sp*. 23S was grown in Kings B medium (per L of distilled H_2O: proteose peptone No.3 20 g, glycerol 10 mL, K_2HPO_4 1.5 g, $MgSO_4$ 1.5 g), in which glycerin serves as a precursor molecule (Knowles, 1976; Askeland and Morrison, 1983; Schippers et al., 1990). Indole acetic acid production was evaluated as described by Deaker et al. (2011). In the NB medium where *Pseudomonas sp.* 23S was grown, DL-tryptophan (TM 7425 Sigma) was added to serve as a precursor of IAA, at two concentrations 0.5 g L^{-1} or 1.0 g L^{-1}. Antagonistic activity of *Pseudomonas sp*. 23S was assessed against *Pseudomonas syringae* pv. *tomato* DC3000 (provided by Dr. Diane Cuppels, AAFC, London). *Pseudomonas syringe* pv. *tomato* DC3000 was grown in Kings B media and 100 μL of 2-day-old culture was spread on Kings B agar plates. A sterile filter-paper disk, with 10 μL of *Pseudomonas sp*. 23S culture was placed on each pathogen inoculated plate, and the plates was sealed with parafilm and incubated for at least 2 days at 28°C, to observe development of inhibition zones.

Seedling Assay for Plant Growth Promotion

Tomato seeds (Bush Beefsteak 351; Stroke Seeds Inc., Thorold, ON, Canada) were surface-sterilized by soaking in 3% (v/v) hypochlorite solution for 3 min, washing thoroughly with water, and drying overnight. The seeds were sown in pots (7.5 mm diameter; 2 seeds pot^{-1}) filled with a mix of sand and turface (5:5). The pots were washed with bleach, and a mix of sand and turface was autoclaved prior to use. The seedlings were thinned to leave 1 plant pot^{-1} after emergence. After 7 days from the day of seeding, 50 mL suspension of *Pseudomonas sp*. 23S (10^8 cfu mL^{-1} in 10 mM $MgSO_4$) was applied to each pot as a soil drench. For the control treatment, 50 mL of 10 mM $MgSO_4$ was applied to each pot. The seedlings were grown in a growth chamber with a 14/10 h photoperiod and a 25/23°C day/night temperature. Sterilized water was applied as needed. After 3 and 7 days from *Pseudomonas sp.* 23S treatment (10 and 14-day-old seedlings), the population of *Pseudomonas sp.* 23S around the roots was enumerated. For this, the seedling was removed from the soil, shaken to dislodge the soil as much as possible, and the root was cut from the plant. The root was then ground with a mortar and pestle in 500 μL of 10 mM $MgSO_4$. The solution was centrifuged (10,000 rpm, 1 min) to remove the debris, and 100 μL of its serial dilution was plated on *Pseudomonas* Isolation Agar plates (PIA; Difco). The plates were incubated overnight at 28°C, after which the number of colonies formed was counted. Eleven days after *Pseudomonas sp.* 23S treatment (18-day-old seedlings), the whole plants were harvested. The shoots were dried in an incubator for 2 days at 60°C and shoot dry weight was determined. The roots were first scanned (Modified Epson Expression 10000XL, Regent Instruments Inc., Quebec, QC, Canada) at 400 dots per inch (dpi) resolution and then, images were analyzed by using WinRHIZO software (Reagent Instruments Inc.) to study morphological features and later used for determination of root dry weight. There were five biological replicates per treatment at each time point for the population study, and there were seven biological replicates for the dry weight study. The experiment was conducted twice. A student's *t*-test was applied to determine significant differences between control and bacterial treatments.

Effects of *Pseudomonas* sp. 23S on Bacterial Canker

Tomato seeds were surface-sterilized as described above. The seeds were sown into pots (13 mm diameter) filled with agromix (G10). The plants were grown in a plant growth chamber under the following conditions: 16/8 h of photoperiod, 25/20°C of day/night temperature, and 65% of relative humidity; they were watered daily. Once true leaves emerged, half-strength Hoagland solution was provided once a week (Hoagland and Arnon, 1950; PlantMedia #30630037-5). The experiment was organized following a factorial design with two levels of *Pseudomonas sp.* 23S treatments (+ and −), and two levels of *Cmm* inoculation (+ and −). Treatments consisted of: (1) *Cont*, without *Pseudomonas sp.* 23S treatment, and *Cmm* inoculation (negative control); (2) *Pse,* treated with *Pseudomonas sp.* 23S; (3) *Cmm,* without *Pseudomonas sp.* 23S treatment, and inoculated with *Cmm*; and, (4) *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm*. After 2 weeks from sowing, the plants were treated with *Pseudomonas sp.* 23S. Each plant received 100 mL of the *Pseudomonas sp.* 23S cells suspended in 10 mM $MgSO_4$ (approximately 10^8 cfu mL^{-1}) for *Pse* and *P+C* treatments and 100 mL of 10 mM $MgSO_4$ for *Cont* and *Cmm* treatments. The *Cmm* inoculation was conducted on one of the 4 days, after 1, 3, 5, and 7 days from the date of *Pseudomonas sp.* 23S treatment (corresponding to 15, 17, 19, and 21 day-old plants); 10 μL of *Cmm* cells suspended in 10 mM $MgSO_4$ (approximately 10^8 cfu mL^{-1}) for *Cmm* and *P+C,* or 10 μL of 10 mM $MgSO_4$ (approximately 10^8 cfu mL^{-1}) was inoculated by injecting into the main stem, where the cotyledon emerged, in each plant using a syringe (31 gauge needle, Thermo Scientific #3170513). The number of wilted leaves was counted for monitoring the

disease progression. Area under disease progress curve (AUDPC) was calculated based on the formula $\Sigma[(d_{i+1} + d_i)/2](t_{i+1} - t_i)$, where d_{i+1} and d_i are the percentage of wilted leaves and t_{i+1} and t_i are the days after *Cmm* inoculation (Xu et al., 2012). After 3 weeks from the date of *Cmm* inoculation, plants were harvested, and shoots (leaves and stems) and root dry weight, plant height, and leaf areas were measured. For each plant, a stem piece (approximately 1 cm) was sampled from 2-cm above the *Cmm* inoculation site in order to evaluate the *Cmm* population (number of cells per gram of tissue). The stem piece was weighed and ground with 1 mL of 10 mM $MgSO_4$ using mortar and pestle. The extract was centrifuged at 10,000 rpm for 1 min, and 10-fold serial dilutions of the supernatant were plated on *Cmm*-selective agar plates (Ftayeh et al., 2011). The number of *Cmm* colonies were enumerated after 3–4 days after incubation at 28°C. Dried shoot tissues were ground to fine powder with mortar and pestle and used to analyze carbon (C), nitrogen (N), phosphorus (P), and potassium (K) contents. An elemental analyzer (Model NC2500; CE Instruments, Milan, Italy) was used for the C and N analysis. For the P and K analysis, a flow injection analyzer and atomic absorption spectrophotometer were used, respectively (Parkinson and Allen, 1975). First, the tissues were digested in sulfuric acid and peroxide with the addition of catalysts (lithium and selenium) at 340°C for approximately 3 h. The content was diluted to 100 mL and used for the flow injection analyzer. Phosphorous was measured colorimetrically at 880 nm following a complexation with ammonium molybdate (Lachat Instruments QuikChem Method 13-115-01-1-B, 6645 West Mill Road, Milwaukee, WI, United States). Potassium was read on a 10-fold diluted subsample (from the same diluted sample used for the P analysis) by emission on a Varian 220FS (now part of Agilent) atomic absorption spectrophotometer. Seven plants were sampled for each inoculation time (1, 3, 5, and 7 days) per treatment, and the experiment was repeated twice. Since the results from these two experiments were comparable, they were combined and presented in this paper.

Quantitative Real-time PCR (qRT-PCR) Analysis

Two analyses were performed to study: (1) the effects of *Pseudomonas sp.* 23S on defense-related genes of tomato plants, and (2) the effects of *Pseudomonas sp.* 23S on defense-related genes of tomato plants that are infected with bacterial canker by *Cmm*. For the first analysis, the plants were treated with *Pseudomonas sp.* 23S with cells suspended in 10 mM $MgSO_4$ (approximately 10^8 cfu mL^{-1}), and applied as a soil drench, 2 weeks after sowing. For control plants, 100 mL of 10 mM $MgSO_4$ was applied to each plant in the same manner. At 1, 3, 5, and 7 days after the date of *Pseudomonas sp.* 23S application, the shoot was harvested (biomass pooled for each four replicate plants), flash-frozen with liquid nitrogen, and stored in −80°C for subsequent real-time quantitative PCR (qRT-PCR) analysis. For the second analysis, tomato plants were treated in the same manner as described for the first analysis, with a 5 day-time interval between *Pseudomonas sp.* 23S application and *Cmm* inoculation. At 1, 3, 5, and 7 days after the date of *Cmm* inoculation, the shoot was harvested (pooled for the four replicate plants), flash-frozen with liquid nitrogen, and stored at −80°C for subsequent qRT-PCR analysis. For both situations, the plants were grown under growth chamber condition as described above, and the experiment was conducted three times with independent biological replicates. For the qRT-PCR-analysis, total RNA from tomato leaves was extracted using TRIzol Reagent (Thermo Fisher Scientific catalog#: 15596026), and the RNAs were treated with DNase I (Ambion™ DNaseI, Thermo Fisher Scientific catalog#: AM2222) according to the manufacturer's instructions. The integrity of the extracted RNA was checked on agarose gel electrophoresis, and its purity and concentration were assessed by a ND-1000 spectrophotometer (NanoDrop). Complementary DNA (cDNA) was synthesized using an iScript Advanced cDNA Synthesis Kit (Bio-Rad, catalog#: 1725037), following the manufacturer's instructions. The cDNA was diluted to 400 ng uL^{-1} and stored in −20°C for qPCR. Primer sequences, linear equations, correlation coefficients (R^2), and reaction efficiencies for each gene used in this study are provided in **Table 1**. The qPCR was conducted on a CFX Connect Real Time System (Bio-Rad) with Green-2-Go qPCR Mastermix (Biobasic, catalog#: QPCR004-S), using the cycling program of: 95°C for 10 min for the enzyme activation step, 95°C for 15 s for the initial denaturation step, 60°C for 60 s for annealing and extension, repeated for 40 cycles (*PR1*: 52°C; *PI2*: 56°C; *ACO*: 52°C: *GAPDH*: 56.4°C). Each plate consisted of three technical replicates from the three independent biological replicates. The Ct value obtained was normalized against the housekeeping gene

TABLE 1 | List of tomato genes used for the gene expression study.

ID	Target gene	Primer sequence (5′ to 3′)	Linear equation	Correlation coefficient (R^2)	PCR efficiency
M69247	*Pathogenesis related protein (PR1a)*[1]	GTGGGATCGGATTGATATCCT CCTAAGCCACGATACCATGAA	$Y = 3.394X + 27.172$	0.991	98.3
X94946	*Proteinase inhibitor (PI2)*[2]	AATTATCCATCATGGCTGTTCAC CCTTTTTGGATCAGATTCTCCTT	$Y = 3.471X + 25.992$	0.997	97.1
AB013101	*1-aminocyclopropane-1-carboxylix acid oxidase (ACO)*[3]	AAGATGGCACTAGGATGTCAATAG TCCTCTTCTGTCTTCTCAATCAAC	$Y = 3.545X + 18.479$	0.985	94.1
U97257	*GAPDH*[4]	CTGGTGCTGACTTCGTTGTTG GCTCTGGCTTGTATTCATTCTCG	$Y = 3.362X + 17.064$	0.993	91.5

[1]*López-Ráez et al. (2010) and Martínez-Medina et al. (2013);* [2]*Song et al. (2015);* [3]*Yim et al. (2014);* [4]*Chalupowicz et al. (2010).*

GAPDH, and the relative gene expression (fold change) was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

RESULTS

Pseudomonas sp. 23S Showed Characteristic PGPR Traits

To explore its potential, *Pseudomonas sp.* 23S was studied for general PGPR traits: phosphorous (P) solubilization, production of siderophores, of hydrogen cyanide, of indole acetic acid, and antagonistic activity against phytopathogens. Pikovskaya (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP) were used in P solubilization assay. In both PVK and NBRIP plates, the *Pseudomonas sp.* 23S inoculation resulted in a halo around the disk (**Supplementary Figure S2.1**; approximately 1 mm for each plate). The quantitative assay showed that *Pseudomonas sp.* 23S solubilized 5.84 μg mL^{-1} ($\pm$0.85) of inorganic phosphorus. The chrome azurol S (CAS) assay was performed to assess siderophore production. The *Pseudomonas sp.* 23S inoculation changed the blue color of CAS plates to orange (**Supplementary Figure S2.2**; size of halo was 4 mm). The quantitative assay showed that its production is 50.7% ($\pm$3.38). Hydrogen cyanide (HCN) production was examined by change in color soaked in picric acid. The negative control plate, where media had been applied, was bright yellow (**Supplementary Figure S2.3a**) whereas the positive control plate, where HCN-positive bacterium had been applied, was bright orange (**Supplementary Figure S2.3c**). *Pseudomonas sp.* 23S containing plate was neither this bright yellow nor bright orange, but rather a light orange color (**Supplementary Figure S2.3b**). To determine whether *Pseudomonas sp.* 23S could produce indole acetic acid (IAA), two concentrations of tryptophan, 0.5 g mL^{-1} and 1.0 g L^{-1}, were used as a precursor for production of IAA. The quantitative assay indicated that *Pseudomonas sp.* 23S produced 1.96 μg IAA mL^{-1} ($\pm$0.09) at 0.5 g L^{-1} tryptophan and 2.72 μg IAA mL^{-1} ($\pm$0.07) at 1.0 g L^{-1} tryptophan. Antagonistic activity of *Pseudomonas sp.* 23S against *Pseudomonas syringe* pv. *tomato* DC 3000 was assessed. *Pseudomonas sp.* 23S inhibited the growth of the *Pseudomonas syringe* pv. *tomato* DC 3000, as indicated by inhibition zones around the disk (**Supplementary Figure S2.4**; the size of the inhibition zone was 4 mm).

Pseudomonas sp. 23S Promoted Growth of Tomato Seedlings

Pseudomonas sp. 23S was applied as a soil drench and its effect on growth was examined for tomato seedlings. As **Figure 1** shows, *Pseudomonas sp*. 23S treated seedlings were visually bigger, and the dry weights of their shoots and roots were significantly higher (approximately 47% increase; **Figures 1d–f**) than those of control seedlings (**Figures 1a–c**). Roots of *Pseudomonas sp.* 23S treated seedlings appeared finer and longer (**Figures 1j–l**) than the roots of control seedlings (**Figures 1g–i**). Based on the root scanning analysis, root length, volume, and surface area of the *Pseudomonas* sp. 23S treated seedlings were significantly greater than those of control seedlings (**Table 2**). When the viable number of *Pseudomonas sp.* 23S cells around the root was enumerated, it was $10^{5.08}$ ($\pm$0.12) and $10^{5.49}$ ($\pm$0.14) colony forming units per seedling after 3 and 7 days, respectively.

Pseudomonas sp. 23S Alleviated Bacterial Canker by ISR

The effects of *Pseudomonas sp.* 23S on the disease progression were studied for tomato plants infected with *Cmm* when the time interval between *Pseudomonas sp.* 23S application and *Cmm* inoculation dates were 3, 5, and 7 days (**Figures 2A–C**). The percentage of wilted leaves increased over time under 3-day-interval and reached more than 80% at 21 days post-*Cmm* inoculation (**Figure 2A**). Most of these plants were dead; the main stems were broken at the site where the *Cmm* was inoculated. When the interval was 5 days, the *Cmm* treatment resulted in a disease progression similar to the *Cmm* treatment for the 3-day-interval. However, the disease progression for the *P+C* treatment was significantly slower, and the percentage of wilted leaves was about 60% at 21 days post-*Cmm* inoculation (**Figure 2B**). For these plants, the main stems were not broken, the symptom observed in most severely infected plants following the *Cmm* treatment (**Supplementary Figure S3**). Under the 7-day-interval, the disease progression was slower than the disease progression at 3- and 5-day intervals. The percentage of wilted leaves was also smaller at 21 days post-*Cmm* inoculation, less than 60% for the *Cmm* treatment (**Figure 2C**). Under the 5-day interval, the AUDPC from the *P+C* treatment was significantly lower than that from the *Cmm* treatment (239 for *P+C* treatment and 576 for *Cmm* treatment; **Supplementary Figure S4**). There were no disease symptoms observed for the *Cont* and *Pse* treatments at any time.

To determine whether *Pseudomonas sp*. 23S treatment results in improvement of plant biomass, the dry weights of shoots and roots for the tomato plants grown under our experimental conditions were measured (**Figures 3A,B**). The shoot dry weights of the *Cont* and *Pse* treatments were not significantly different for any of the intervals (**Figure 3A**). With an interval of 3 and 5 days, the shoot dry weights of the *Cont* and *Pse* treatments were significantly different from those of the *Cmm* and *P+C* treatments while they were not significantly different with an interval of 7 days. Under the 3 and 7-days intervals, the shoot dry weights of the *Cmm* and *P+C* treatments were not significantly different. On the other hand, the shoot dry weight was greater for the *P+C* treatments than *Cmm* treatments under the 5-day-interval. Similar trends were found for the root dry weight, plant height, and leaf areas (**Figure 3B** and **Supplementary Figure S5**).

At the harvest (21 days post-*Cmm* inoculation), a 1-cm-length stem piece above the inoculation site was taken and used for counting the colony forming units (cfu) of *Cmm* presence (**Table 3**). The number of cfu for the *P+C* treatment was significantly lower than that for the *Cmm* treatment when the interval was 5 days, while no difference was detected when the intervals were 3 and 7 days.

Nutrient levels of shoots, specifically nitrogen (N), phosphorus (P), potassium (K), and carbon (C) were measured to study the effects of the *Pseudomonas sp.* 23S treatment

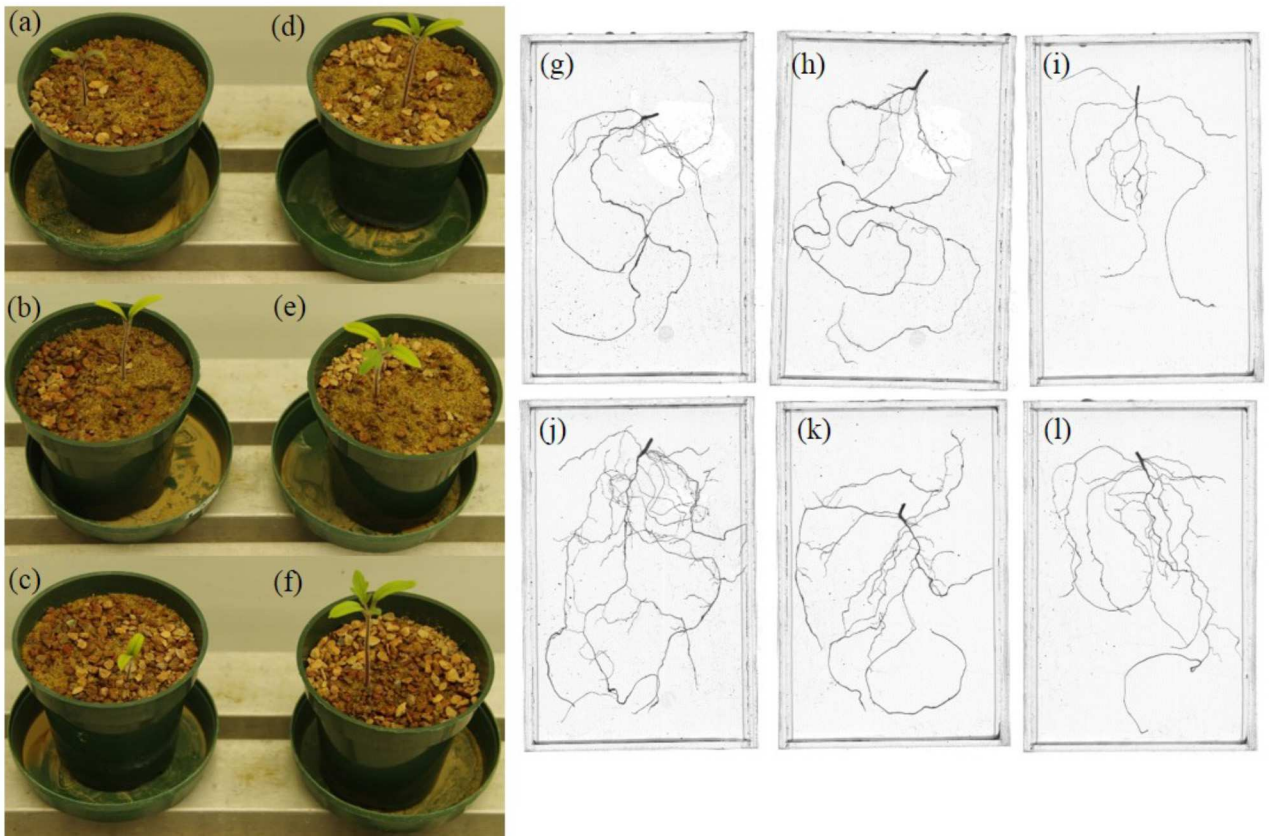


FIGURE 1 | *Pseudomonas sp.* 23S promoted the growth of tomato seedlings. Seedlings from control **(a–c)** and from *Pseudomonas sp.* 23S treatment **(d–f)**, and roots from control **(g–i)** and from *Pseudomonas sp.* 23S treatment **(j–l)**.

TABLE 2 | *Pseudomonas sp.* 23S treatment increased dry weight of shoots and roots, and improved root length, volume and surface area.

	Control	*Pseudomonas sp.* 23S
Dry shoot weight (mg)	8.81 ± 0.58	13.0 ± 0.78*
Dry root weight (mg)	4.91 ± 0.48	7.18 ± 0.70*
Root length (cm)	92.14 ± 5.83	123.18 ± 8.91*
Root volume (mm^3)	72.40 ± 4.59	97.27 ± 6.01*
Root diameter (mm)	0.32 ± 0.0054	0.32 ± 0.0032
Root surface area (cm^2)	9.13 ± 0.55	12.26 ± 0.82*

Data represented as mean ± SE (n = 15). An asterisk indicates significant difference from the control after the student's t-test (p = 0.05).

(**Figures 4A–D**). For all the nutrients that were measured, the levels were not significantly different between the *Cont* and *Pse* treatments under any of the interval times. On the other hand, the levels of the nutrients from the *Cmm* and *P+C* treatments were significantly lower than those of the *Cont* and *Pse* treatments. With an interval of 5 days, the levels of N, P, K, and C for the *P+C* treatment were significantly higher than those of the *Cmm* treatment. In addition, the nutrient levels of P and K for the *P+C* treatment were not different from those of the *Cont* treatment (**Figures 4B,C**). Under the 7-day-interval, the levels of the *Cmm* treatment tended to be lower but overall, the levels for all the nutrients were not very different among the treatments (**Figures 4A–D**).

Pseudomonas sp. 23S Treatment Increases the Transcript Level of *PR1a*

The transcript levels of *PR1a*, *PI2*, and *ACO* were studied in tomato plants 1, 3, 5, and 7 days after soil drench treatment with *Pseudomonas sp.* 23S (**Figures 5A–C**). The transcript levels of *PR1a*, a marker gene of salicylic acid activity were higher for the *Pseudomonas sp.* 23S treatment as compared with those of the Control treatment at all the time points (**Figure 5A**). Its transcript abundance reached highest at day 3 (10-fold), then diminished at days 5 (fourfold) and 7 (fivefold). The transcript levels of *PI2* and *ACO* were not different between Control and *Pseudomonas sp.* 23S treatments at any of the time points (**Figures 5B,C**).

Pseudomonas sp. 23S Prior to *Cmm* Inoculation Caused Faster and Greater Accumulation of *PR1a* and *ACO* Transcripts

Transcript levels of *PR1a*, *PI2*, and *ACO* were also examined when tomato plants treated with *Pseudomonas sp.* 23S by soil drench and inoculation of *Cmm* into the main stem by needle

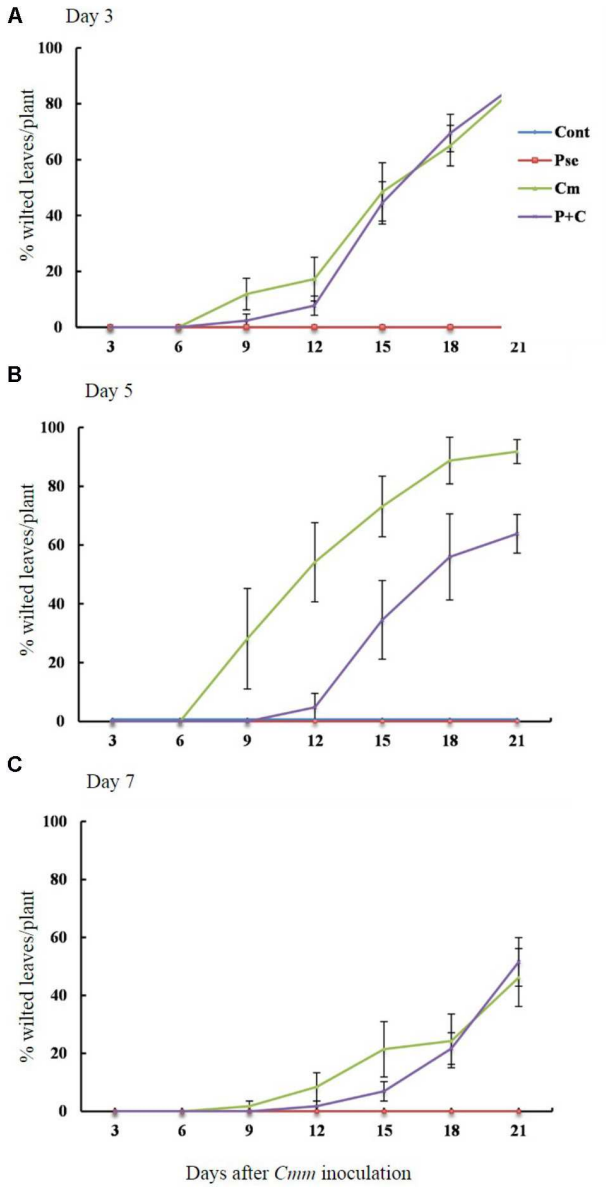


FIGURE 2 | *Pseudomonas sp.* 23S treatment, 5 days prior to *Cmm* inoculation, delayed progression of bacterial canker. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S by soil drench, and after 3 days **(A)**, 5 days **(B)**, or 7 days **(C)**, *Cmm* was inoculated in the main stem by needle injection. The number of wilted leaves was counted every 3 days for 3 weeks. Treatments are: *Cont,* control; *Pse,* treated with *Pseudomonas sp.* 23S; *Cmm,* inoculated with *Cmm*; and, *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm*. *Cont* and *Pse* treatments showed no disease symptom throughout the experiment (n = 14, p = 0.05).

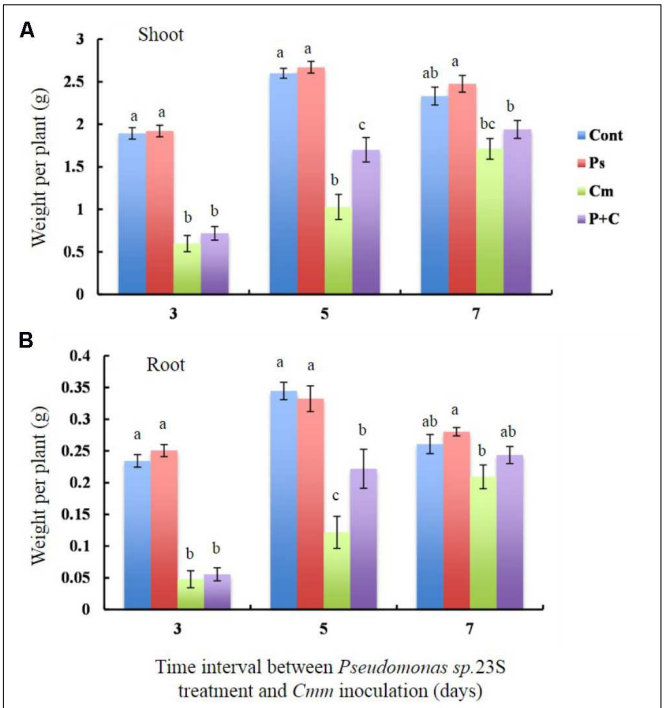


FIGURE 3 | *Pseudomonas sp.* 23S treatment, 5 days prior to *Cmm* inoculation, increased the weights of shoots and roots. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S (or 10 mM $MgSO_4$) by soil drench, and after 3, 5, or 7 days, *Cmm* (or 10 mM $MgSO_4$) was inoculated into the main stem by needle injection. The plants were harvested after 3 weeks and sampled for: **(A)** weight of dry shoots and **(B)** weight of dry roots. Error bars indicate standard error of the mean. Association with different letters indicates statistical significance based on ANOVA followed by Tukey's multiple comparison test. Treatments are: *Cont,* control; *Pse,* treated with *Pseudomonas sp.* 23S; *Cmm,* inoculated with *Cmm*; and, *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm* (n = 14, p = 0.05).

injection after 5 days (**Figures 6A–C**). Day 5 was chosen because the previous physiological experiment, described above, indicated that the disease severity was smallest when *Cmm* was inoculated after 5 days, rather than 3 or 7 days. The transcript level of *PR1a* was not different among treatments at day 1; however, at days 3 and 5, the *P+C* treatment resulted in significantly higher transcript levels than other treatments (54 fold at day 3 and 58 fold at day 5; **Figure 6A**). At day 7, its transcript level was still higher (55 fold), and the transcript levels for the *Pse* and *Cmm* treatments were also as high as that of the *P+C* treatment (34 fold for the *Pse* and 75 fold for *Cmm* treatment; **Figure 6A**). The transcript levels of *PI2* were relatively high at day 1 for all of the treatments (87, 25, 48, and 68 fold for the *Cont*, *Pse*, *Cmm* and *P+C* treatments, respectively) as compared with those at day 3, 5, 7 (**Figure 6B**). In addition, they were variable among biological replicates, resulting in large standard errors. No differences were detected among treatments at days 1, 3 and 5. At day 7, the transcript level of the *Cmm* treatment (ninefold greater than the control) was significantly higher than other treatments. The transcript level of *ACO* gradually increased going from days 1 to 7 (**Figure 6C**). For every time point: the transcript level of the *Cont* treatment was the lowest; the level of the *Pse* and *Cmm* treatments were similar to or slightly higher than those of the *Cont* treatment; and, no difference was detected between the *Pse* and *Cmm* treatments. The *P+C* treatment was always the highest among the treatments (1.5, 1.75, 2.5, and 4.5 fold relative to the *Cont*, *Pse*, *Cmm* and *P+C* treatments, respectively; **Figure 6C**).

TABLE 3 | *Pseudomonas sp.* 23S treatment, 5-day prior to *Cmm* inoculation, reduced the *Cmm* population in the stem.

	Day 3	Day 5	Day 7
Cmm[a]	6.84 ± 0.17	6.57 ± 0.15	6.11 ± 0.06
P+C[b]	7.19 ± 0.08	6.16 ± 0.16*	6.21 ± 0.16

Two-week-old tomato plants were treated with Pseudomonas sp. 23S (or 10 mM $MgSO_4$) by soil drench, and after 3, 5, or 7 days of Cmm (or 10 mM $MgSO_4$) inoculation into the main stem by needle injection. After 3 weeks, 1-cm piece of stem (2 cm above the inoculation site) was sampled for enumeration of Cmm cells. The values represent log_{10} of the number of Cmm colony forming units (cfu).
[a]*Cmm, inoculated with Cmm.*
[b]*P+C, treated with Pseudomonas sp. 23S, and inoculated with Cmm.*
Asterisk indicates statistical significance between the Cmm and P+C treatments based on the student's t-test (n = 14, p = 0.05).

DISCUSSION

Pseudomonas sp. 23S was shown to possess key PGPR traits, but its degree differed among traits. Specifically, production of siderophores and indole acetic acids were clearly demonstrated. Since siderophores facilitate iron acquisition, especially under iron-limited conditions, siderophore-production represents a biocontrol mechanism for suppression of root diseases by rhizobacteria (Schippers et al., 1987; O'Sullivan and O'Gara, 1992; Shanmugaiah et al., 2015). Similar to past findings (Idris et al., 2007), the amount of IAA produced by *Pseudomonas sp*. 23S was dependent on the concentration of the IAA precursor tryptophan. Bacteria-produced IAA is known to alter root architecture and support plant development (Dodd et al., 2010; Bhattacharyya and Jha, 2012). In addition, *Pseudomonas sp*. 23S inhibited the growth of *Pseudomonas syringae* pv. *tomato* DC 3000 that causes bacterial speck disease of tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana* (Whalen et al., 1991), suggesting potential for *Pseudomonas sp*. 23S as a biocontrol agent for this disease. On the other hand, *Pseudomonas sp*. 23S may not be a very good phosphorous (P) solubilizer, as the amount of P solubilized was relatively low as compared to other phosphorus-solubilizing PGPR (Rahi et al., 2010). *Pseudomonas sp*. 23S was shown to be a moderate producer of hydrogen cyanide (HCN) a volatile, antibiotic, secondary metabolite, which can contribute to disease suppression by some biocontrol bacteria (Voisard et al., 1989; Defago and Haas, 1990; Haas and Keel, 2003). Since HCN is known to inhibit *Cmm* growth (Lanteigne et al., 2012), production of HCN may partly explain the antagonistic activity that *Pseudomonas sp*. 23S has against *Cmm*.

While *in vitro* assay revealed the potential of *Pseudomonas sp*. 23S as a PGPR, the plant assay demonstrated that *Pseudomonas sp*. 23S has plant-growth promoting effects on tomato seedlings. In this experiment, the substrate was probably not the source

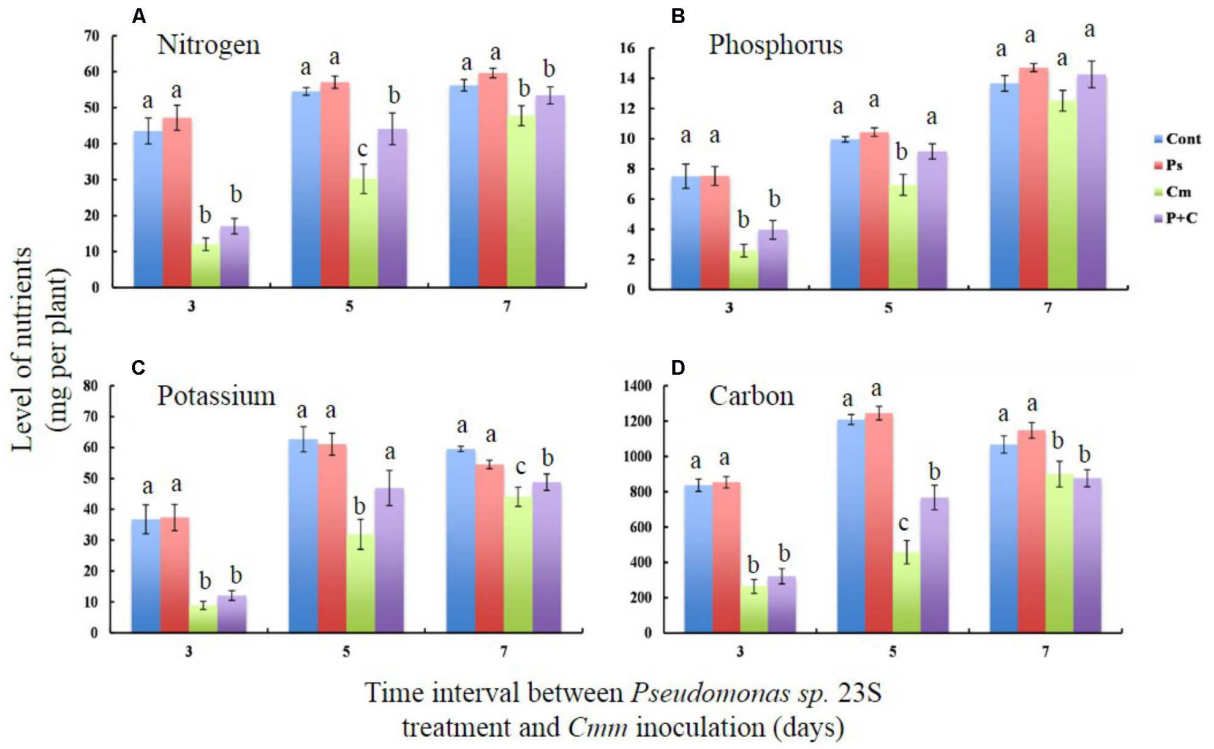


FIGURE 4 | *Pseudomonas sp.* 23S treatment, 5 days prior to *Cmm* inoculation, improved nutrient levels of leaf tissue. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S (or 10 mM $MgSO_4$) by soil drench, and after 3, 5, or 7 days, *Cmm* (or 10 mM $MgSO_4$) was inoculated into the main stem by needle injection. The plants were harvested after 3 weeks. Nutrients analyzed are **(A)** Nitrogen, **(B)** Phosphorus, **(C)** Potassium, and **(D)** Carbon. Association with different letters indicates statistical significance based on ANOVA followed by Tukey's multiple comparison test. Treatments are: *Cont,* control; *Pse,* treated with *Pseudomonas sp.* 23S; *Cmm,* inoculated with *Cmm*; and, *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm* ($n = 14$, $p = 0.05$).

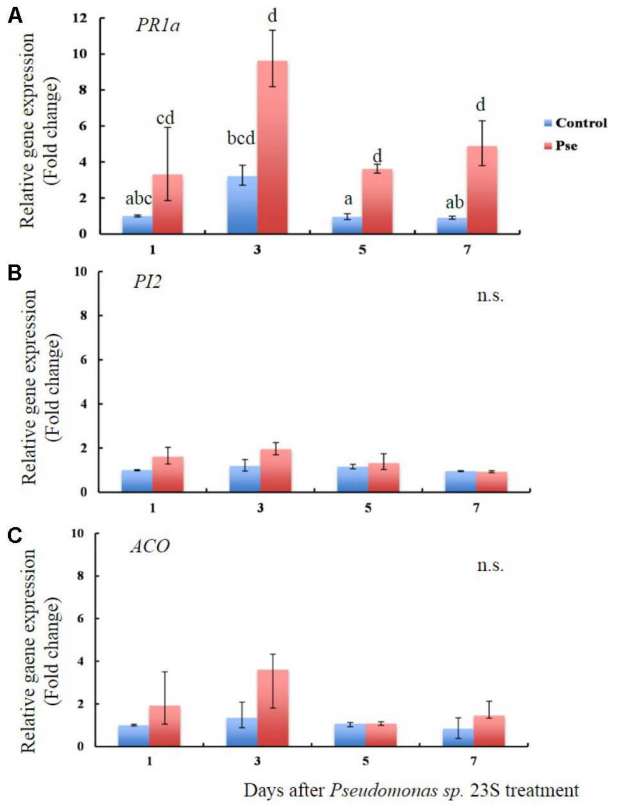


FIGURE 5 | *Pseudomonas sp.* 23S treatment induced an increase in the transcript level of *PR1a*. Two-week old tomato plants were treated with *Pseudomonas sp.* 23S (Pse) or 10 mM $MgSO_4$ (Control) and after 1, 3, 5, and 7 days, the shoots were harvested and used for RNA extraction and real-time qPCR. Genes analyzed are **(A)** *PR1a*, **(B)** *PI2*, and **(C)** *ACO*. Error bars indicate standard error of the mean. Association with different letters indicates statistical significance based on ANOVA followed by Tukey's multiple comparison test ($p = 0.05$).

of nutrients for seedlings, or for *Pseudomonas sp*. 23S, because it was composed of a mix of sand and turface, and the seedlings were supplied with water only. *Pseudomonas sp*. 23S might have utilized the nutrients from root exudates and synthesized chemicals that might have growth-promoting effects on the tomato seedlings. Many rhizobacteria are known to convert the root exudate tryptophan to IAA (Frankenberger and Muhammad, 1995), and as the *in vitro* assay suggested that *Pseudomonas sp.* 23S was an IAA producer, this mechanism could explain the enhanced root development of the plants treated with *Pseudomonas sp.* 23S. *Pseudomonas sp*. 23S was also shown to be a good colonizer of tomato roots, as it colonized the roots and established in good numbers within 3 days, similar to previous reports (Lugtenberg et al., 1999; Yan et al., 2003). We also tried to isolate *Pseudomonas sp*. 23S from inside the root tissue by sterilizing the root surface, but we were not able to do so (data not shown). Hence, *Pseudomonas* sp. 23S seems to reside only on the root surface (rhizoplane) and in the soil around the root (rhizosphere), not the inside the root, and causes plant growth promotion.

Pseudomonas sp. 23S triggered ISR, which probably contributed to the reduction of bacterial canker severity by *Cmm* in tomato plants when *Cmm* was inoculated 5 days after the *Pseudomonas sp.* 23S application. In this study, *Pseudomonas sp.* 23S was applied before *Cmm* inoculation, and the two bacteria were spatially separated since the *Pseudomonas sp.* 23S was applied as soil drench and *Cmm* was injected to the main stem by syringe needle. We tested for the presence of *Pseudomonas sp*. 23S in the stem samples by using a selective plate assay, but the bacteria were never detected (data not shown). Thus, direct contact between *Cmm* of the *Pseudomonas sp.* 23S was not likely to occur, and the bacterial canker reduction that was observed must have been a result of ISR effects. *Cmm* can survive as an endophyte in tomato plants, but induction of disease symptoms requires a certain minimum population level, generally 10^8 cfu g^{-1} of plant tissue (Gartemann et al., 2003). Compared to this number, as well as to the reports from past studies (Balaji et al., 2008; Sharabani et al., 2013), the *Cmm* population in the stem samples in our study was relatively small, although leaf wilting was clearly observed. This could be because the tomato plants used in our study were younger than those used in other studies (17–21 vs. 28 days). Since we observed the disease progression over 3 weeks, we cannot elaborate on the effects of *Pseudomonas sp.* 23S application on older plants or at later growth stages. However, most plants inoculated with *Cmm*, but without the *Pseudomonas sp.* 23S treatment, were severely infected and would not be able to recover as the main stems were completely broken down. Treatment with *Pseudomonas sp*. 23S reduced the *Cmm* population and limited the disease severity as indicated by the fact that these plants stood straight and their leaves were unwilted or less wilted. This effect is certainly important considering that the protection of plants at this stage is more critical than that of older plants that are more resistant to the disease.

In our experimental system, the age and/or size of the plants at the time of the *Cmm*-inoculation might have influenced the disease progression within plants. The date of visual symptom appearance, and the severity of bacterial canker are affected by temperature, plant age, inoculum concentration, and cultivar (Chang et al., 1992b). In young tomato plants, the disease symptoms caused by *Cmm* are known to appear earlier and they are more susceptible to infection than mature plants (Chang et al., 1992b; Sharabani et al., 2013). This may explain our observation that disease progression was relatively slow for the plants at the 7-day-interval. Nevertheless, at the plants at the 5-day interval the plants are younger than those at the 7-day interval and thus, the slower disease progression observed at this interval is most probably due to ISR, and not to an age-related resistance.

In our study, 5 days was the optimal interval between *Pseudomonas sp.* 23S application and *Cmm* inoculation, in terms of alleviating bacterial canker. Past studies showed that several days are required for ISR to develop and to deliver resistance against various phytopathogens (Babu et al., 2015). The different time interval between PGPR treatment and pathogen inoculation could be related to the population size of the PGPR. The protection by PGPR-mediated ISR is said to be apparent only when the roots were colonized by PGPR at a specific threshold population size (Raaijmakers et al., 1995). Also, Zhang et al.

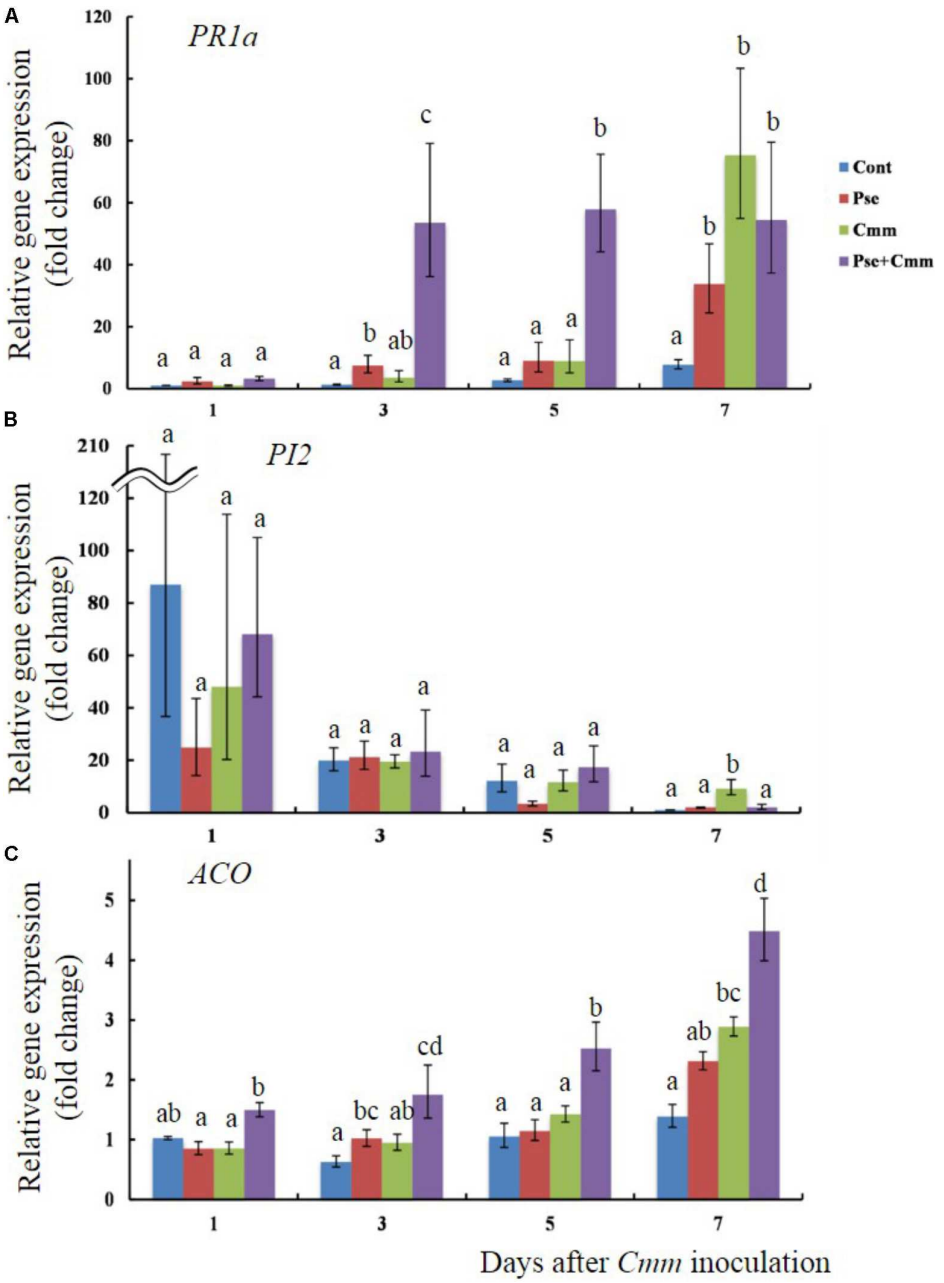


FIGURE 6 | *Pseudomonas sp.* 23S treatment, 5 days prior to *Cmm* inoculation, induced faster and greater response in transcript levels of *PR1a* and *ACO*. Two-week old tomato plants were treated with *Pseudomonas sp.* 23S (or 10 mM $MgSO_4$) and after 5 days, *Cmm* (or 10 mM $MgSO_4$) was inoculated into the stem by needle injection: *Cont,* control; *Pse,* treated with *Pseudomonas sp.* 23S; *Cmm,* inoculated with *Cmm;* and, *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm*. After 1, 3, 5, and 7 days, the shoots were harvested and used for RNA extraction, and real-time qPCR. Genes analyzed are **(A)** *PR1a*, **(B)** *PI2*, and **(C)** *ACO*. Error bars indicate standard error of the mean. Association with different letters indicates statistical significance based on ANOVA followed by Bonferroni multiple comparison ($p = 0.05$).

(2004) indicate "quorum sensing effects," where a certain bacterial population density is essential to produce a signal molecule that is involved in provoking ISR.

The results showed that the *Pseudomonas sp.* 23S increased the transcript level of the *PR1a*, but not of *PI2* and *ACO*, suggesting that the ISR induced by the *Pseudomonas sp.* 23S may involve the salicylic acid (SA) pathway, rather than jasmonic acid (JA) or ethylene (ET). The *PR1a* gene codes for a pathogenesis-related protein, and has been used as a marker gene for salicylic acid resistance induction (Park et al., 2001; Block et al., 2005; Niu et al., 2012; Martínez-Medina et al., 2013). *PI2* gene codes for a proteinase inhibitor and is induced by wounding and jasmonic

acid (JA; Peña-Cortés et al., 1995; Peiffer et al., 2009; Niu et al., 2012; Martínez-Medina et al., 2013). *ACO* is a gene coding for 1-aminocyclopropane-1-carboxylix acid (ACC) oxidase, the level of which is related to ethylene (ET) production since ACO is an enzyme that converts ACC into ethylene (Stearns and Glick, 2003; Yim et al., 2014). Although JA/ET are generally considered to be key hormone(s) for ISR response, which is mediated by non-pathogenic plant growth promoting bacteria (PGPR), different results have been reported from more recent studies (De Vleesschauwer and Höfte, 2009), and the ISR by the *Pseudomonas sp.* 23S seems to fall into this new trend. The signaling pathway for ISR seems species specific, that is specific to the rhizobacterium, and pathogen involved (Ryu et al., 2003; Djavaheri, 2007; Conn et al., 2008). Researchers agree that hormone crosstalk plays an important role in regulating ISR. Regarding SA-and JA/ET pathways, antagonistic interaction has been documented from many studies (Koornneef and Pieterse, 2008), and this might apply to our case, where *Pseudomonas sp.* 23S induced SA response but not JA and ET. The antagonistic interaction between SA and JA may be the outcome of resource allocation, costs of induction, or a means for the plant to adaptively tailor its responses to different enemies and a target for manipulation by enemies (Thaler et al., 2012). Generally, SA-dependent defense response is said to be effective against biotrophic pathogens, while JA/ET-dependent defense response is effective against necrotrophic pathogens (Sorokan et al., 2013). In this respect, *Cmm* would be a suitable target for the SA-dependent ISR because *Cmm* is considered as a biotrophic pathogen (Eichenlaub and Gartemann, 2011). We cannot exclude the possibility that JA and ET are not involved in the ISR provoked by the *Pseudomonas sp.* 23S. Mutant plants impaired in SA, JA, and ET pathways could be utilized to confirm whether these hormones are required for ISR.

Furthermore, the results demonstrated priming effect of the *Pseudomonas sp.* 23S treatment. Faster and/or greater response of defense-genes - priming has been observed for many ISR-inducing PGPR (Pieterse et al., 2014). Accumulation response of *PR1a* after *Cmm* inoculation was faster and quantitatively greater with the plant pre-treated with the *Pseudomonas sp.* 23S than the plants without the pre-treatment. Since the *Pseudomonas sp.* 23S treatment alone also induces accumulation response of *PR1a*, the prior- *Pseudomonas sp.* 23S treatment probably prepares the plants for the pathogen attack, by making this response faster and greater and enhancing the defense capacity of the plants. Priming may explain the disease reduction observed under the 5-day interval in our study. To understand the effects of the *Pseudomonas sp.* 23S treatment for disease reduction, studying the responses of other defense-related genes (e.g., other PR proteins, defense-related enzymes) and whether they do also show priming effects would be helpful.

Faster and greater accumulation response was also observed for the *ACO*, but the situation may be different from the *PR1*, because the *ACO* transcript abundance was not affected by *Pseudomonas sp.* 23S treatment alone. From past studies, ethylene is known to play a critical role in bacterial canker of tomato. Plants with reduced ethylene production or impairment of ethylene perception results in decrease in the disease severity (Balaji et al., 2008; Savidor et al., 2011), and thus host-derived ethylene is suggested to be a requirement for the disease development by *Cmm*. Our results that the *Pseudomonas sp.* 23S treatment alone did not significantly affect the *ACO* abundance but the same treatment did after *Cmm* inoculation supports the past studies in that ethylene is involved in the disease infection by *Cmm*. At the same time, the fact that *Pseudomonas sp.* 23S treatment can make this response faster and greater in quantitatively and alleviate the disease may suggest that the *Pseudomonas sp.* 23S might have modulated the role of ethylene. This consequently could influence the disease development by *Cmm* and thus might have contributed to the disease reduction as observed in our study.

The transcript level of *PI2* showed different trend from that of *PR1a* or *ACO*, elevated on day 1, especially for the control treatment. This may be due to the damaging nature of applying the mock (needle injection) as the transcript level decreased at later time points. For *Cmm* inoculated plants, however, its level remained high at day 7. Treatment with *Pseudomonas sp.* 23S might have explained this: while the *Cmm* inoculated plants must combat *Cmm* invasion, the same plants were less affected by the prior *Pseudomonas sp.* 23S treatment due to ISR effects.

In this study, we investigated *Pseudomonas sp.* 23S, which was previously isolated based on the *in vitro* antagonistic activity against *Cmm*. The characterization study of *Pseudomonas sp.* 23S revealed great potential of this strain in agriculture, both for plant growth promotion and as a biocontrol agent. Future study could investigate its effectiveness in field condition. This study also demonstrated that *Pseudomonas sp.* 23S could induce ISR in tomato plants and reduce the severity of tomato bacterial canker disease that is caused by *Cmm*. The best time interval between the *Pseudomonas sp.* 23S treatment and *Cmm* inoculation for reducing the severity of bacterial canker was 5-days in our experimental system, which used drench application of *Pseudomonas sp.*, stem inoculation of *Cmm*, and young tomato plants; this interval, as well as the effectiveness of ISR, could change with different methods, timing of bacterial application and of pathogen inoculation, and plant ages. Such information would be useful, especially for the commercial use of this bacterium in the future. Our study also suggested that the ISR by *Pseudomonas sp.* 23S may involve SA in its signaling pathway. However, the possibility of JA and/or ET involvement should not be ignored. Mutant plants with impaired hormonal pathways could be studied in the context of a *Cmm* infection to confirm their involvement. In addition, our results provided new insights on the role of ethylene in disease development of *Cmm*. Further studies to elucidate the signaling pathways of *Pseudomonas sp.* 23S ISR would certainly add knowledge for understanding molecular mechanism of ISR induced by PGPR but it would provide useful information regarding the disease strategies taken by *Cmm*.

AUTHOR CONTRIBUTIONS

YT wrote the manuscript, conducted the growth chamber experiments, gene expression studies, and analysis of the data. J-BC and DS guided and supervised the overall study.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02119/full#supplementary-material

FIGURE S1 | Anti-*Cmm* activity of *Pseudomonas sp*. 23S *in vitro*. One hundred micro liter of *Cmm* culture was spread on Nutrient Broth Yeast Extract (NBYE) agar. A sterile filter-paper-disk (6 mm diameter) was placed on the agar surface. Five micro liter of the newly isolated bacterium culture (on the right), and Nutrient Broth (on the left) were applied on the respective disks. The plate was sealed with parafilm and incubated at 28°C for 2 days. Zone of inhibition = 5 mm.

FIGURE S2 | Characterization of *Pseudomonas sp.* 23S for PGPR traits. **1 (a)** Phosphorus solubilization on a PVK, NB media (top), *Pseudomonas sp.* 23S (left and right), and positive control bacterium (bottom). **1 (b)** Phosphorus solubilization on a PBRIP agar plate, NB media (left), and of *Pseudomonas sp.* 23S (right). **2** Siderophore production, NB media (left), and *Pseudomonas sp.* 23S culture (right). **3** Hydrogen cyanide production, **(a)** Kings B media, **(b)** *Pseudomonas sp*. 23S, and **(c)** a positive control bacterium. **4** Antagonistic activities against *Pseudomonas syringae* pv. *tomato* DC3000.

FIGURE S3 | Representive plant photos showing the effects of *Pseudomonas sp*. 23S treatment 5-day prior to *Cmm* inoculation. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S by soil drench, and after 5 days, *Cmm* was inoculated in the main stem by needle injection: **(A)** *Cont,* control; **(B)** *Pse,* treated with *Pseudomonas sp.* 23S; **(C)** *Cmm,* inoculated with *Cmm*; and **(D)** *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm.*

FIGURE S4 | Area under disease progression curve (AUDPC). Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S by soil drench, and after 5 days, *Cmm* was inoculated in the main stem by needle injection. The AUDPC was based on the percentage of wilted leaves during 3 weeks after the date of *Cmm* inoculation (presented by **Figure 2** in the main text): *Cmm,* inoculated with *Cmm*; and *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm*. Error bars indicate standard error of the mean. An asterisk indicates significant difference from the *Cmm* treatment after ANOVA followed by Tukey's multiple comparison test (n = 14, p = 0.05).

FIGURE S5 | Effects of *Pseudomonas sp.* 23S treatment 5 days prior to *Cmm* inoculation on **(A)** plant height, and **(B)** leaf area. Two-week-old tomato plants were treated with *Pseudomonas sp.* 23S (or 10 mM $MgSO_4$) by soil drench, and after 3, 5, or 7 days, *Cmm* (or 10 mM $MgSO_4$) was inoculated into the main stem by needle injection. The plant height and leaf areas were measured. Error bars indicate standard error of the mean. Association with different letters indicate statistical significance based on ANOVA followed by Tukey's multiple comparison test. Treatments are: *Cont,* control; *Pse,* treated with *Pseudomonas sp.* 23S; *Cmm,* inoculated with *Cmm*; and, *P+C,* treated with *Pseudomonas sp.* 23S, and inoculated with *Cmm* (n = 14, p = 0.05).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Biocontrol of Bacterial Leaf Blight of Rice and Profiling of Secondary Metabolites Produced by Rhizospheric *Pseudomonas aeruginosa* BRp3

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Xanthomonas oryzae pv. *oryzae* (Xoo) is widely prevalent and causes Bacterial Leaf Blight (BLB) in Basmati rice grown in different areas of Pakistan. There is a need to use environmentally safe approaches to overcome the loss of grain yield in rice due to this disease. The present study aimed to develop inocula, based on native antagonistic bacteria for biocontrol of BLB and to increase the yield of Super Basmati rice variety. Out of 512 bacteria isolated from the rice rhizosphere and screened for plant growth promoting determinants, the isolate BRp3 was found to be the best as it solubilized 97 μg/ mL phosphorus, produced 30 μg/mL phytohormone indole acetic acid and 15 mg/ L siderophores *in vitro*. The isolate BRp3 was found to be a *Pseudomonas aeruginosa* based on 16S rRNA gene sequencing (accession no. HQ840693). This bacterium showed antagonism *in vitro* against different phytopathogens including Xoo and *Fusarium* spp. Strain BRp3 showed consistent pathogen suppression of different strains of BLB pathogen in rice. Mass spectrometric analysis detected the production of siderophores (1-hydroxy-phenazine, pyocyanin, and pyochellin), rhamnolipids and a series of already characterized 4-hydroxy-2-alkylquinolines (HAQs) as well as novel 2,3,4-trihydroxy-2-alkylquinolines and 1,2,3,4-tetrahydroxy-2-alkylquinolines in crude extract of BRp3. These secondary metabolites might be responsible for the profound antibacterial activity of BRp3 against Xoo pathogen. Another contributing factor toward the suppression of the pathogen was the induction of defense related enzymes in the rice plant by the inoculated strain BRp3. When used as an inoculant in a field trial, this strain enhanced the grain and straw yields by 51 and 55%, respectively, over non-inoculated control. Confocal Laser Scanning Microscopy (CLSM) used in combination with immunofluorescence marker confirmed *P. aeruginosa* BRp3 in the rice rhizosphere under sterilized as well as field conditions. The results provide evidence that novel secondary metabolites produced by BRp3 may contribute to its activity as a biological control agent against Xoo and its potential to promote the growth and yield of Super Basmati rice.

Keywords: ***Xanthomonas oryzae*, super basmati, mass spectroscopy, HAQ, CLSM, BLB**

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INTRODUCTION

Rice is an important staple food crop. The global production of rice paddy was 746.9 million tons and 496 million tons milled rice (http://www.fao.org/economic/est/publications/rice-publications/rice-market-monitor-rmm/en/). The crop is widespread all over the world due to its wider adaptability under different environmental conditions. For this reason, Food and Agriculture Organization (FAO) regarded it as a strategic crop for food security in the world (Montano et al., 2014). Rice is used as a staple food in different areas of world especially in Asia (http://www.britannica.com/plant/rice). In Pakistan, beside wheat, rice is the 2nd major cereal crop, which is widely cultivated, consumed, and exported. Among various cultivated varieties, Super Basmati rice variety is the most sought variety, as it is very popular among consumers, farmers, traders, and exporters due to its high quality grain and aroma (http://reap.com.pk/news/news_detail.asp?newsid=3905). However, susceptibility of this variety to different diseases is a major problem. Among these, *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight (BLB) is considered to cause severe yield losses (Arshad et al., 2015). This disease is widely prevalent among various rice varieties worldwide (Singh et al., 2015). Historically, BLB was initially reported in Japan during 1884–1885 and then reported in other rice growing countries (Gnanamanickam, 2009). Rice crop was severely affected by this disease in areas of tropica and Asia with heavy rainfall in monsoon. Initially, its occurrence in Pakistan was reported by Mew and Majid (1977) and Arshad et al. (2015). Later studies indicated an alarming increase in BLB incidence in Basmati rice growing areas of the country (Shah et al., 2009).

BLB occurs at different growth stages of rice and is manifested by either leaf blight or "Kresek" (acute wilting of young plants) symptoms. Xoo invades the plant through wounds or water pores. Lesions with wavy margins start from the tip of the leaf as the water pores are located at the margins of upper parts of the leaf. These water soaked lesions enlarge in size, turn yellow and ultimately lead to the death of plant (Nino-Liu et al., 2006).

In the past, various disease management strategies have been employed to reduce the yield losses and to avoid disease epidemics but use of chemicals has not been successful due to variation in sensitivity of pathogenic races toward applied chemicals. Development of mutation in pathogenic races is a major hindrance in developing a durable control (George et al., 1997). Moreover, due to toxic residues, usage of antibiotics and chemicals against rice BLB, has limitations (MacManus et al., 2002). Although, the use of host resistance genes seems to be practicable, single gene (Xa4) based breeding for BLB management has been shown to be ineffective due to evolution of sub-populations that overcome these resistance genes (Shanti et al., 2010). As a result, biological control seems to be a cost effective and environmentally friendly way to manage this serious threat (Gnanamanickam, 2009).

Most of the rhizospheric antagonistic bacteria such as *Pseudomonas* spp. can indirectly increase plant resistance by improving the plant growth. Responses of the host plant are due to root colonization of a plant by antagonistic rhizobacteria that play an important role in disease suppression. *Lysobacter antibioticus* have been documented as biocontrol agents against Xoo due to their rapid growth, easy application and effective leaf colonization (Ji et al., 2008). Plant growth promoting *Bacillus* spp. were found to suppress BLB in rice under greenhouse conditions (Chithrashree et al., 2011). Li et al. (2011) reported *Streptomyces globisporus* for the suppression of rice blast caused by *Magnaporthe oryzae*. The incidence of sheath blight was reduced by some biofilm forming and surfactant producing strains of *Bacillus subtilis* (Mousivand et al., 2012). *Streptomyces philanthi* and a commercial formulation of *B. subtilis* were found to be biologically active against rice sheath blight when integrated with chemical fungicides (Boukaew et al., 2013). Hydrogen cyanide (HCN) producing *Pseudomonas chlororaphis* significantly inhibited the growth of *M. oryzae*, showing its biocontrol properties against the causal agent of rice blast (Spence et al., 2014).

These antagonistic bacteria can directly suppress plant pathogens by producing antibiotics, enzymes like chitinases, glucanases, proteases, and siderophores or indirect mechanisms in which the antagonistic bacteria compete with the pathogen for a niche or nutrient sites (Bardin et al., 2015). These bacteria have been reported to reduce the disease incidence significantly under controlled as well as under natural field conditions. *Bacillus* and *Pseudomonas* spp. control the diseases caused by rice pathogens i.e., *X. oryzae* pv. *oryzae*, *Rhizoctonia solani,* and *M. oryzae* up to 90% depending on the bacteria used, pathogen and the rice variety (Montano et al., 2014). Induced systemic resistance (ISR) is an environmentally attractive option for disease control whereby plant defenses are enhanced as a result of their interaction with certain rhizobacteria. ISR contributes positively toward the biological control of plant pathogens and the defense related enzymes induced by the inoculated bacteria protect the host plants (Chithrashree et al., 2011). Systemic resistance was induced by *Serratia* sp. causing resistance against necrotrophic leaf pathogens in rice (Vleesschauwer et al., 2009).

However, most of the previous studies investigated either plant growth promoting activities or biocontrol activities of the bacterial isolates, exclusively. Previously, Kumar et al. (2005) and Fang et al. (2013) reported broad spectrum antifungal and biofertilizer activity of *Pseudomonas aeroginosa* and *Pseudomonas aurantiaca.* Similarly, different endophytic strains of *B. subtilis* have been reported to have plant growth promoting activity on cacao and biocontrol activity against phytopathogens like *Moniliophthora perniciosa* and *Colletotrichum* spp. but the antifungal metabolites were not investigated (Kumar et al., 2012; Nain et al., 2012; Falcao et al., 2014). The use of *Pseudomonas* and *Bacillus* strains have been reported for the biocontrol of rice pathogens such as Xoo, *M. oryzae,* and *R. solani* (Ji et al., 2008; Helene et al., 2011; Spence et al., 2014). However, again the major emphasis of these previous studies was on the biocontrol activity. Rhizospheric antagonistic *Pseudomonas aeruginosa* have been documented as beneficial biocontrol agents against Xoo (Yasmin et al., 2016) but the role of diverse secondary metabolites produced by different strains of *P. aeruginosa* in the suppression of BLB pathogen has not been reported earlier.

Members of the *Pseudomonas* spp., including pathogenic as well as non-pathogenic strains, are capable of producing various extracellular secondary metabolites. These metabolites exhibit diverse properties i.e., function as virulence factors, siderophores (having high-affinity of iron ions), biosurfactants, and antimicrobial agents as well as in cell-to-cell signaling etc. These metabolites enable *Pseudomonas* spp. to adapt in different environments, colonize different hosts and compete with other species. *Pseudomonas* metabolites constitute chemical entities such as N-acylhomoserine lactone (Cataldi et al., 2009), phenazine (Price-Whelan et al., 2006; Kumar et al., 2011; Jain and Pandey, 2016), pyochellin (Youard et al., 2007; Jayaseelan et al., 2014), phloroglucinol (Kidarsa et al., 2011), lahorenoic acid, diketopiperazines (Mehnaz et al., 2013), 4-hydroxy-2-alkylquinolines (Lepine et al., 2004), rhamnolipids (Soberon-Chavez et al., 2005; Grosso-Becerra et al., 2016), and cyclic lipopeptides (Mehnaz et al., 2013). Knowing the chemistries of these metabolites can help with characterization of their biological and physiological activities. However, most of the existing studies have been confined to the analysis of individual metabolite class analysis and its biological properties (Kumar et al., 2005). There are few studies where more than one class of metabolites have been analyzed simultaneously. Even during the study of one physiological aspect like bacterial quorum-sensing, analysis of multiclass metabolites [N-acyl-L-homoserine lactone and 2-alkyl 4-(1H)-quinolone] can enhance the scope of study (Ortori et al., 2011). In this study, detailed chemical characterization of the BRp3 supernatant has led to identification of a variety of previously known as well as novel metabolites of several chemical classes.

Despite the economic importance of BLB, complete resistance to this disease has not been reported. Furthermore, no local resistant varieties are commercially available in Pakistan. Therefore, to select bacteria with multiple beneficial applications for the improvement of rice crop, 512 rhizobacteria were isolated from different field sites of Punjab (Pakistan). After screening their plant growth promoting as well as biocontrol activities, the bacterium termed as BRp3, demonstrated the best results in rice yield improvement and biocontrol activity against the prevalent rice pathogen Xoo. This bacterium was subjected to detailed characterization of the secondary metabolites with ultimate aim to develop inocula of functionally well-characterized native antagonistic bacteria, having a "dual-purpose inoculum" with strong plant growth promoting and biocontrol aspects, for yield enhancement of "Super Basmati" rice variety.

Mass spectrometric analysis revealed the production of siderophores, rhamnolipids, a series of previously known and novel HAQs in the crude extract of BRp3. These results high-lighted the facts that *P. aeroginosa* BRp3, owing to its capability to produce a number of secondary metabolites in HAQ, rhamnolipids, and siderophores (phenazines and pyochellin) series, exhibited intense antimicrobial activity against Xoo pathogens. Hence, this bacterium could be used as an effective biocontrol agent against the rice pathogen Xoo.

MATERIALS AND METHODS

Bacterial Pathogens Used

Six *X. oryzae* pv. *oryzae* (Xoo) strains i.e., Xoo1, Xoo2, Xoo4, Xoo5, Xoo6, and Xoo7 were obtained from NIBGE Biotech Resource Centre (NBRC), Faisalabad (Yasmin et al., 2016). The pathogenicity of Xoo strains was tested by clip inoculation (Kauffman et al., 1973) on three susceptible rice varieties i.e., Super Basmati, Basmati 385, and Basmati 2000. Clip inoculation of Xoo1, Xoo2, Xoo4, Xoo5, and Xoo6 to rice showed typical symptoms of BLB on the inoculated leaves i.e., white to gray lesions starting from leaf tip to downward along leaf veins and margins. Bacterial Xoo pathogens were stored in PSA agar (Ou, 1985) slants at 4°C and in 20% glycerol at −80°C for further use.

Isolation of Rhizobacteria with Biocontrol Activity

Root samples of healthy rice plants in the vicinity of BLB infected plants were collected from fields of different rice growing sites. These root samples with adhering soil were used to isolate bacteria from rhizosphere, rhizoplane, and endosphere on Nutrient Agar (Norris and Ribbons, 1970), King's B (King et al., 1954), and Gould's S1 medium (Gould et al., 1985) using serial dilution method as mentioned by Somasegaran and Hoben (1994). Purified bacterial strains were stored in LB agar slants at 4°C and in 20% glycerol at −80°C.

The rhizobacteria were screened *in vitro* for inhibition of Xoo using plate diffusion method (Hewitt and Vincent, 1989). A fresh culture (100 μL) of Xoo grown in PSA broth was spread onto LB plates. The liquid culture of bacterial strains grown in LB broth to be tested for antagonistic activity was spotted on LB plates already spread with Xoo strain. These plates were kept in incubator at 30 ± 2°C for 48 h and the antibiosis was observed by measuring the zone of inhibition of pathogen's growth (Velusamy et al., 2006).

Antagonistic activity of *P. aeruginosa* BRp3 was tested against phytopathogenic fungi like *Fusarium oxysporum, Fusarium monoliforme,* and *Fusarium solani* by dual culture assay on Potato Dextrose agar (PDA; Ji et al., 2008) and the percent inhibition was calculated (Yasmin et al., 2014).

Primary Selection of Antagonistic Bacteria for Growth Promotion

Primary selection of antagonist was carried out based on its antagonism as well as its effect on rice (Super Basmati) seedlings in a growth room experiment. The seeds were disinfected with sodium hypochlorite (1%) for 5 min and then washed thrice with sterilized water. Twenty seeds soaked in over-night grown bacterial broth culture of antagonistic strain (log phase containing 10^9 CFU mL^{-1}), were grown aseptically on sterile wet filter paper each kept in each sterile Petri plate (14 cm diameter). Twenty-four bacteria were selected and there were three replicates for each treatment. Un-inoculated seedlings were used as control. The plates were kept in a growth room and maintained at a day/night temperature of 30 ± 2°C/25 ± 2°C and 16 h day length with 20,000 Lux light intensity. The plates were watered with sterilized distilled water in laminar flow. The number of germinated seedlings,

radical length and hypocotyl length were measured after 10 days.

Identification by 16S rRNA Gene Sequencing and Phylogenetic Analysis

CTAB method (Ausubel et al., 1992) was used to extract total genomic DNA of strain BRp3. Universal primers P1 and P6 were used to amplify 16S rRNA gene (Tan et al., 1997). QIAquick Gel Extraction Kit (QIAGEN Sciences, Maryland 20874, USA) was used to clean the amplified PCR product about 1.5 Kb of 16S rRNA gene. Amplified PCR products were commercially sequenced by Macrogen, Inc. (Seoul, South Korea). Available sequences of bacterial lineage in NCBI were used to align and compare the sequence data using BLAST. Accession numbers were allocated after submitting the sequences to NCBI GenBank database (Yasmin et al., 2016).

For calculating phylogenetic tree of strain BRp3, closely related sequences were downloaded and aligned using CLUSTAL X and MacClade 4.05 (Thompson et al., 1997; Maddison and Maddison, 1999). Maximum Parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) methods were used for sequences analysis as mentioned by Mirza et al. (2009).

Detection of Growth Promoting and Biocontrol Determinants

Bacterial nitrogen fixation was estimated by using acetylene reduction assay (ARA; Hardy et al., 1968). Phosphate solubilization by the bacterium was measured on Pikovskaya agar medium added with insoluble tricalcium phosphate and quantified by Phospho-molybdate blue color method (Murphy and Riley, 1962). Quantification of produced Indole Acetic Acid (IAA) was carried out following the method of Tien et al. (1979). 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity was assessed in vials added with 3 μL of ACC (0.5 M) as a sole N source in 5 mL DF salt minimal medium (Penrose and Glick, 2003).

Production of siderophores was observed on universal chrome azurol "S" (CAS) agar medium (Schwyn and Neilands, 1987) and quantified as described by Rachid and Bensoltane (2005). Production of HCN was detected following the method as described by Lork (1948). Protease and chitinase activities were detected on skim milk agar (Denizci et al., 2004) and chitin agar (Brien and Colwall, 1987) media. Glucanolytic activity was detected on minimal medium supplemented with glucan source i.e., Lamimarin containing 0.5% yeast extract (Qing et al., 2002). Starch hydrolyzing ability was detected on nutrient agar added with 2% starch (Marten et al., 2000). There were three biological replicates for all the tested growth promoting and biocontrol determinants.

Antibiotic resistance of the bacterial strain BRp3 was determined on antibiotic sensitivity sulfonamide (ASS) agar medium by disc diffusion method (Valverde et al., 2005) using commercial antibiotic susceptibility discs (Bioanalyse, Turkey; Imran et al., 2015).

Mass Spectrometric Analysis of Culture Supernatant

For the detection and identification of secondary metabolites, *P. aeruginosa* BRp3 was inoculated to LB medium (500 mL) and incubated with shaking at 30 ± 2°C for 24 and 48 h. To remove the bacterial cell pellet, the bacterial culture was centrifuged at 4°C for 15 min at 10,000 rpm. pH of the supernatant was reduced to 3.0 using 3M HCl solution before its extraction with 500 mL ethyl acetate. This extraction step was repeated using 300 mL ethyl acetate. The combined organic layers were obtained and evaporated under reduced pressure by rotary evaporation. Residues were dissolved in 5 mL LCMS grade methanol and subjected to LCMS/MS analysis using mass spectrometer (LTQ XL Linear Ion Trap Mass Spectrophotometer, Thermo Scientific, USA), equipped with an ESI source. The samples were filter sterilized and were injected through direct syringe pump with a flow rate of 8 μL min^{-1}. Samples were scanned at both positive and negative total ion full scan modes (mass scan range m/z 50–2,000) with source voltage and capillary voltage of 4.8 kV and 23 V, respectively. Capillary temperature and sheath glass flow (N_2) were 350°C and 30 arbitrary units, in both scan modes. The selected analytes were fragmented at positive and negative ion modes by employing collision induced dissociation (CID) energy of 35 (percentage of 5 V) or otherwise stated.

The supernatant of the bacterial culture was studied for its antimicrobial activity against Xoo in plate diffusion assay at different time intervals (24–144 h) as described in section Isolation of Rhizobacteria with Biocontrol Activity. The supernatant of the bacterial culture with higher antimicrobial activity (after 24 and 48 h) was further subjected to LCMS analysis for the detection of secondary metabolites.

In Planta Evaluation for the Suppression of BLB

Net House Experiment

Antagonistic bacteria BRp3 was evaluated *in vivo* for the suppression of BLB against reference strain Xoo2 (selected on the basis of its virulence) under natural light and temperature conditions at NIBGE net house during the rice growing season. The seeds were treated with the antagonistic bacteria (10^9 CFU mL^{-1}) for 2 h and sown in small plastic pots of 12 × 7 cm^2 diameter filled with 3 kg soil/ pot (texture clay loam, organic matter 0.4%, pH 8.5, EC 4.1 mS, viable cell count 1.8 × 10^6 cell g^{-1} soil). The experiment was carried out using completely randomized block design (CRD). There were four replicates for each treatment with 15 plants per pot. After 21 days, the plants were given foliar spray treatment with their respective antagonistic bacteria (10^9 CFU mL^{-1}) made in sterilized distilled water. Control plants (healthy control) were sprayed with sterilized water. Antibiotic streptocyclin applied @ 5 mg/pot was used as positive control. Sterilized water was used to clip inoculate the leaves of control plants whereas leaves of inoculated treatments and infected control (IC) were clip inoculated with broth culture of Xoo (10^6 CFU mL^{-1}) on 23rd day of sowing as per the method of Kauffman et al. (1973). Lesion length was measured on 15th day of the inoculation and data for

one treatment was obtained from 40 inoculated leaves. The plant dry weight obtained in the inoculated treatments was compared with the non-inoculated healthy control. Suppression of BLB was measured in terms of reduction in the mean bacterial blight lesion length on treated leaves compared to those of non-inoculated control using the following formula:

$$\% \text{ Diseased leaf area (\%DLA)} = \frac{\text{Total lesion length of the test sample}}{\text{Total leaf length of the test sample}} \times 100$$

Antagonistic bacterium BRp3 was re-evaluated against another reference strain Xoo1 in large earthen pots of 24 cm diameter containing 10 kg soil with same characteristics as mentioned earlier in this section.

Induction of Defense Related Enzymes

Rice plants grown under net house condition, applied with foliar spray of BRp3 and clip inoculated with Xoo2 pathogen as described in section Net House Experiment, were also used to study the induction of defense related enzymes (Chithrashree et al., 2011).

Leaves were harvested at 24, 48, 72, and 144 h after application of foliar spray of strain BRp3 on rice plants. There were 15 plants per pot and five leaves were taken randomly from replicates of each treatment. These leaves were mixed thoroughly after cutting them and then 0.1 g sample was used immediately for analysis of different enzymes. Among different defense related enzymes, peroxidase (POD) activity was assayed using guaicol as a substrate at 470 nm wavelength (Hammerschmidt et al., 1982; Liang et al., 2011). Polyphenol oxidase (PPO) was determined at 280 nm using L-tyrosine as the substrate (Worthington, 1988). Decrease in the amount of hydrogen per oxide was determined at 240 nm wavelength to study the catalase (CAT) activity as described previously (Weisany et al., 2012). Phenylalanine ammonia-lyase (PAL) activity was analyzed at 290 nm wavelength by measuring the conversion of L-phenylalanine into trans-cinnamic acid (Benkeblia, 2000).

Field Experiment

Bacterial strain BRp3 was evaluated under field conditions against BLB using rice variety Super Basmati during crop season June-Oct 2013 in a BLB nursery at Plant Protection Division, NIAB, Faisalabad (a hot spot for BLB incidence). The soil sample was homogenized and used for further analysis (pH 8.2, EC 1.8 mS, 1.3 mg total N g^{-1} soil, 1 μg available P g^{-1} soil, 0.03% organic matter and population density of indigenous bacteria i.e. 1×10^6 cells g^{-1} soil). Seeds were dipped in broth culture of antagonistic bacteria (1×10^9 CFU mL^{-1}) for half an hour. Inoculated/un-inoculated seeds were directly sown in seedbeds of 1 m row plots. Each bed was irrigated independently without allowing the water flow from one bed to irrigate another. The beds were separated by a distance of 0.5 m with mud bunds. Each treated row was bordered with a row as a buffer zone. Seeds treated with sterilized LB broth medium were considered as negative controls. Each treatment had three replicates arranged in a Randomized Complete Block Design (RCBD). Plant to plant distance was 22 cm. Application of biocontrol agent and antibiotic i.e., streptocyclin (500 g ha^{-1}) was carried out as mentioned earlier for pot experiment. The leaves (60 leaves per treatment) were clip inoculated with Xoo7 at maximum tillering stage. Xoo7 was the prevalent strain isolated in the same season from BLB infected area of Sheikhupura, Pakistan. In addition to disease severity, straw weight, and grain weight were also recorded.

Field Evaluation for Yield Increase of Rice

Another field experiment was conducted during crop season June–Oct 2014 to compare the effects of BRp3 with that of commercial biofertilizer (*BioPower)* on the yield of rice variety Super Basmati with different levels of fertilization. The soil was collected from 10 sites of field (0–200 mm depth) before sowing and after application of fertilizer. The soil sample were homogenized and used for further analysis i.e., pH (8.6), EC (4 mS), total N (2 mg g^{-1} soil), available P (8 μg g^{-1} soil), organic matter (0.1%), and population density of indigenous bacteria (1×10^6 cells g^{-1} soil) by viable cell count. At the time of transplanting, *BioPower* and the bacterial strain BRp3 were inoculated by root dip method (Tariq et al., 2007). Non-inoculated treatments with recommended N and P (140–80 kg NP $acre^{-1}$) and with 80% of the recommended N and P doses were considered as controls of experiment. Each treatment had four replicates in RCBD. Nutrient inputs of N and P were applied as urea and di-ammonium phosphate (DAP), respectively. Complete dose of DAP was applied before sowing and urea was applied in three split doses i.e., first one was added at the time of field preparation, second and third dose after 25 and 50 days of transplantation, respectively. The plot size was 4 × 7 m. Planting distance and row spacing was 22 cm. Plots were weeded by hand after water removal at each fertilizer application. The plants were harvested at maturity. Whole plot grain yield and straw yield was recorded after threshing. Plants were manually harvested and weighed after sun drying. Rice yield was expressed as weight of paddy at 14% water content (Wimberly, 1983).

Rhizosphere Colonization Studies

Viable count was used to monitor variations in the population of *P. aeruginosa* BRp3 associated with rice variety Super Basmati. The morphology of the colonies of the antagonistic strain was used as a monitoring tool as it produced green pigments in LB agar plates. The inoculated bacterium was further identified on the basis of its antibiotic resistance pattern, antagonism against Xoo strains, production of IAA and siderophores and P solubilization.

Three root and shoot samples from each replicate field plot were collected 14, 21, 40, and 60 days after treatment. Root and shoot samples were washed with sterile distilled water for few minutes. The shoot parts were crushed in sterilized phosphate buffer (10 mL). The suspension was used for preparing serial dilutions followed by plating on LB agar plates. Population level of strain was measured as $\log_{10}$ CFU g^{-1} fresh weight of roots and shoots.

Fluorescent antibodies (FA) against *P. aeruginosa* BRp3 were raised for colonization studies. Immunoglobins were separated

and then conjugated with fluorescein isothiocynate (FITC). Unconjugated dye was separated with a Sephadex G-25 column chromatography following the method of Bilal et al. (1993). The quality and specificity of the FA was determined by staining a number of known cultures of *P. aeruginosa* (Somasegaran and Hoben, 1994).

In a pot experiment, surface sterilized seeds of rice variety Super Basmati were sown in small plastic pots having 50 g air-dried, sieved sterilized sand. The non-inoculated pots were treated independently as control. *P. aeruginosa* BRp3 was seed inoculated (10^9 CFU mL^{-1}). All pots were kept in net house during rice growing season. Plants were harvested 21 days after seed germination. Nonspecific adsorption of stain was suppressed using RhITC conjugate. Specific FA was used to stain the roots (Yasmin et al., 2014). Confocal laser scanning microscope (Olympus FV1000, Japan) facilitated with an Argon-ion laser and FV10-ASW 1.7 imaging software was used to observe the fluorescent bacteria at 488 and 525 nm for absorption and emission of FITC, respectively.

Statistical Analysis

Data obtained from *in vitro* and *in vivo* seed/ plant inoculation experiments was subjected to Analysis of Variance (ANOVA). The treatment means were separated by Duncan's multiple range test (DMRT) for plate/pot experiments at 1% ($P \leq 0.01$) and field evaluation at 5% ($P \leq 0.05$) significance level, respectively using "MSTATC" program (Duncan, 1995).

RESULTS

Isolation and Identification of Rhizobacteria with Biocontrol Activity

Out of 512 bacterial isolates obtained from different host plants, 79 isolates showed antagonistic activity against Xoo (Tables S1, S2). Isolate BRp3 showed maximum growth inhibition of all the tested Xoo strains with zone of inhibition ranged from 10 to 24 mm (**Table 1**). There were three replicates each time and the experiment was repeated thrice. On PDA medium, BRp3 also showed the inhibition of *F. moniliforme* and *F. solani* up to 43.3 and 75%, respectively (Table S1).

16S rRNA gene sequencing identified the strain BRp3 as *Pseudomonas* sp. Results of Blast showed 99% homology with 16S rRNA gene of *P. aeruginosa* isolate PM-007 (Accession no. KY908465.1). 16S rRNA gene sequence of selected strain was submitted to GenBank data base and accession number was allocated (HQ840693). 16S rRNA sequence based phylogenetic analysis showed that the bacterium BRp3 had maximum sequence similarity with *P. aeruginosa*. It occupied the same phylogenetic branch as the *P. aeruginosa* group (Figure S1).

Primary Selection of Antagonistic Bacteria for Growth Promotion

To study the effect of antagonistic bacteria on seedlings, rice seeds were pretreated with different bacteria. Antagonistic bacteria showed variable effects on germination of rice seeds, radical, and hypocotyl length (Figure S2). Bacterial isolates showing less effect

TABLE 1 | Growth promoting and biocontrol determinants of rice rhizosphere associated *Pseudomonas aeruginosa*.

Strain	IAA[a] Sμg mL^{-1}	P[b] Sμg mL^{-1}	Proteases[c]	HCN[d]	Starch hydrolysis[e]	Siderophores production[f] mg L^{-1}	Diameter of inhibition of Xoo strains[g] (mm)					Effect of bacterial inoculation on rice seedlings in a plate assay (% increase compared to non-inoculated control)[h]		
							Xoo1	Xoo2	Xoo4	Xoo5	Xoo6	Xoo7	Germination	Radical length	Hypocotyl length
Pseudomonas aeruginosa BRp3	30 ± 2	97 ± 4	+++	+++	++	15 ± 1.5	20 ± 1	24 ± 2	10 ± 1	10 ± 1	20 ± 1	12 ± 1	19%	65%	56%

BRp3[a]. Indole acetic acid detected qualitatively by HPLC.

[b]Phosphate solubilization was quantified was quantified using spectrophotometer.

[c]Protease production was carried out on skim milk agar medium. ++ Represent 5–10 mm halo zone, +++ Represents >10 mm halo zone.

[d]Hydrogen cyanide (HCN) production.

[e]Starch hydrolysis was detected by plate assay, ++ Represents hydrolysis of starch in more than half plate, +++ Represents complete hydrolysis of starch in plate.

[f]Siderophore production was detected on CAS agar and quantified using spectrophotometer.

[g]Inhibition of Xanthomonas oryzae pv. oryzae (Xoo) strains was determined by "Diffusion plate assay."

[h]Seed germination assay was carried out in a growth room and the data was recorded after 10 days. There were 20 seeds per plate. Seedlings without inoculation were used as control. All values are an average of three biological replicates, ± standard deviation.

on these growth parameters of the rice variety Super Basmati as compared to the control were excluded from the further study.

Detection of Growth Promoting and Biocontrol Determinants

The bacterial strain BRp3 produced 30 ± 2 μg mL^{-1} IAA when supplemented with tryptophan and solubilized P (97 ± 4 μg mL^{-1}) as quantified by spectrophotometer (**Table 1**).The bacterium did not show the nitrogenase activity but used ACC as carbon source in DF salt minimal medium. BRp3 produced volatile compounds such as HCN as observed by a color change of filter paper from yellow to brown in the plate assay. BRp3 showed the activity of proteases but did not produce chitinases and glucanases. This strain was able to hydrolyze starch and showed siderophores production on CAS blue agar medium with a color change from blue to orange. Quantification of siderophores using spectrophotometer showed the production of 15 ± 1.5 mg L^{-1} siderophores by the strain BRp3 (**Table 1**; Figure S3).

The tests on antibiotic resistance showed that BRp3 was intrinsically resistant to 12 antibiotics i.e., Amikacin, Aztrreonum, Ampicillin, Carbenicillin, Cephradine, Chloramphenicol, Doxycycline, Erythromycin, Gentamicin, Neomycin, Nalidixic acid, and Streptomycin (Table S3).

Mass Spectrometric Analysis of Culture Supernatant

Filter sterilized cell free extract of *P. aeruginosa* BRp3 was studied by ESI-MS/MS technique. Samples were injected using direct syringe pump and analyzed at both positive as well as negative scan mode. At 24 h growth (after getting the bacteria from culture stock), analysis of crude extract demonstrated the presence of predominant metabolites such as 1-hydroxy-phenazine having *m/z* at 197 $[M+H]^+$, 219 $[M+Na]^+$; pyocyanin *m/z* at 211 $[M+H]^+$, 233 $[M+Na]^+$ and possibly lahorenoic acid at *m/z* 245 $[M+H]^+$, 267 $[M+Na]^+$ (**Figure 1**). However, this trend altered after the 48 h growth and prominent peaks of 4-hydroxy-2-alkylquinolines (HAQs) exhibiting *m/z* from 214 to 340 $[M-H]^-$, siderophore (pyochellin) *m/z* 325 $[M-H]^-$, and rhamnolipids *m/z* 503, 529, 539, 635, 649 $[M-H]^-$ (**Figure 2**). The presence of these metabolites was confirmed using tandem mass spectrometry as well as through comparing the values with the literature data (**Table 2**).

The bacterium demonstrated the capability to produce large variety of HAQs. Molecular ion peaks of 17 HAQs, representing seven analogous series, were identified (**Table 2**). These HAQs were classified into various groups on the basis of hydrogen, alkyl and hydroxyl groups at the two and three positions of heterocyclic ring, as well as *N*-oxide group at the position of quinoline nitrogen (Serial No. 1–7, **Table 2**). Serial No. 1 HAQs represent the eight analogs, having hydrogen at 3-position with *m/z* $[M+H]^+$ values 216, 230, 242, 244, 258, 270, 298, and 326. These HAQs are relatively simpler 4-hydroxy-2-alkylquinolines, which only varied from each other on the basis of saturated or unsaturated alkyl side chain length. The molecular ions peaks $[M+H]^+$ and $[M-H]^-$ of these HAQs were further analyzed by collision induced dissociation (CID) and compared with literature data. In Serial No. 2 (**Table 2**), an HAQ having *m/z* at 258 $[M-H]^-$, exhibited the highest peak intensity in full scan MS negative ion mode (**Figure 3**), representing a 3,4-dihydroxy-2-heptylquinoline (HHAQ), in which hydrogen at 3-position is substituted with hydroxyl group (Deziel et al., 2004). The structure of this molecule has been thoroughly investigated through collision induced dissociation at positive and negative ion modes to unambiguously profile the fragmentation data. Assigning the three daughter peaks can confirm the structure of HHAQ (**Table 2**, **Figure 3**).

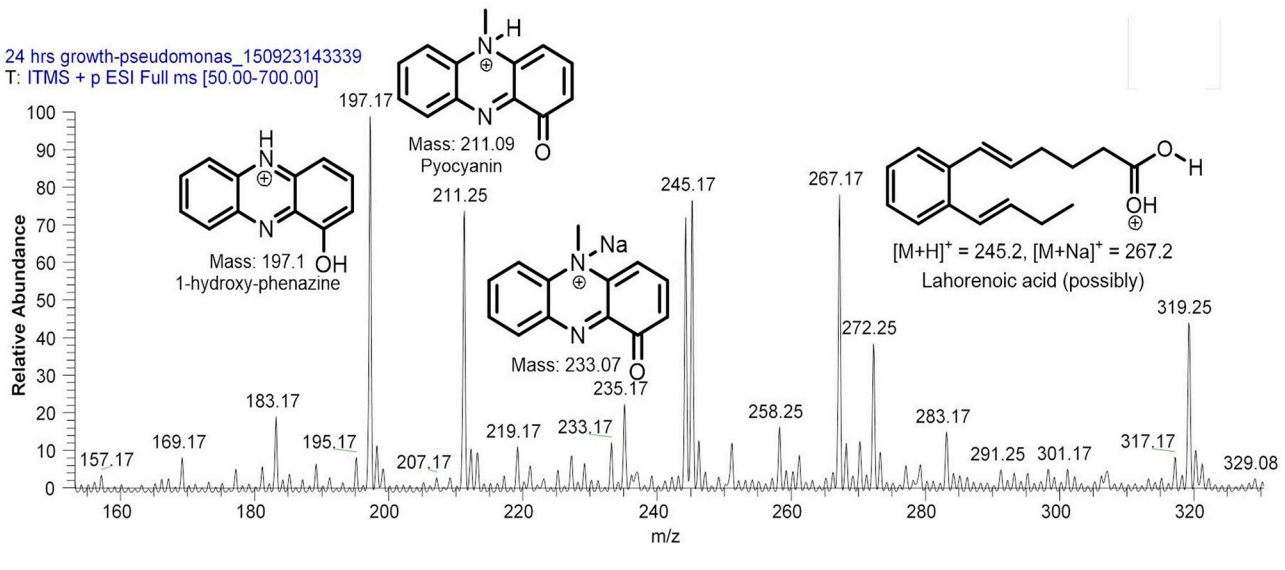


FIGURE 1 | LC/MS chromatogram of *Pseudomonas aeruginosa* BRp3 extract (24 h growth) demonstrating the presence of (predominantly) 1-hydroxy-phenazine, pyocyanin, and possibly lahorenoic acid analyzed at positive ion mode.

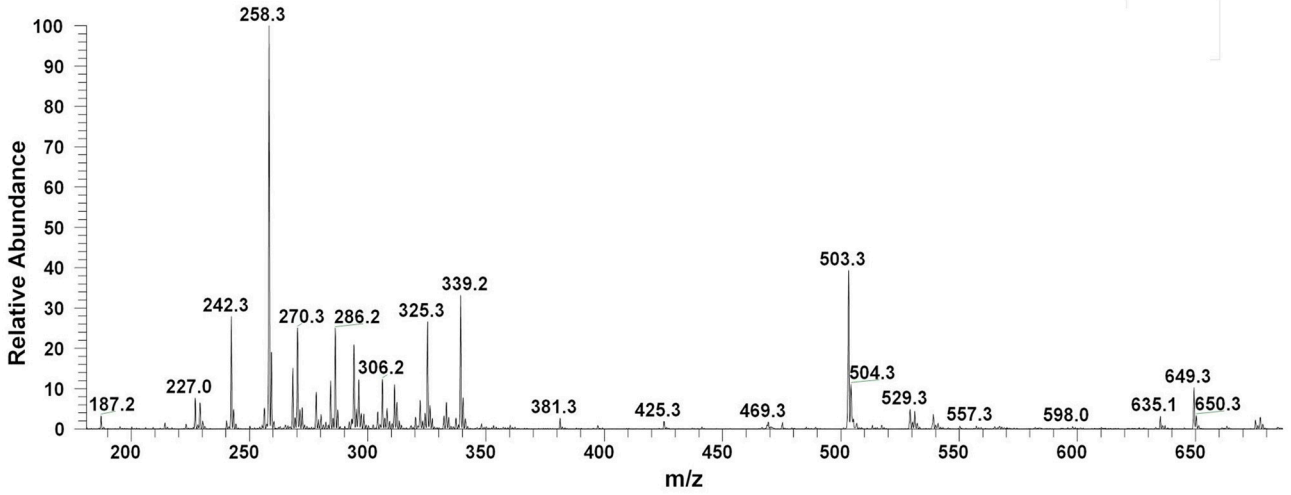


FIGURE 2 | LC/MS chromatograms of *Pseudomonas* extract (48 h growth) indicating the presence of 4-hydroxy-2-alkylquinolines (HAQs) molecular ions species, siderophore (pyochellin, *m/z* 325), and rhamnolipids (*m/z* 500–650), at negative ion mode analysis.

HAQ representing *m/z* at 270 $[M+H]^+$ & 268 $[M-H]^-$ (**Table 2**, Serial No. 3) exhibited the structure of 4-hydroxy-3-methyl-2-alkylquinolines (HMAQ). Fragmentation of the parent peaks through CID produced the daughter ions, which correlated well with the proposed structure. Two analogs of 4-hydroxy-3-methyl-2-alkylquinolines *N*-oxide (HMAQ *N*-oxide) *m/z* at 286 and 288 $[M+H]^+$, were also spotted (**Table 2**, Serial No. 4), exhibiting octaene and octane side chains, respectively.

However, to our surprise, a poly-hydroxy HAQ analog having *m/z* at 278 $[M-H]^-$, demonstrating an interesting putative structure of 2,3,4-trihydroxy-2-alkylquinoline (HHHAQ), was identified at negative ion mode (**Table 2**, Serial No. 5). On collision induced dissociation, this molecule spontaneously lost two moles of water, leaving a stable ion species at *m/z* 242 (4-hydroxy-2-alkylquinoline), which on further fragmentation yielded the expected daughter ions at *m/z* values of 198, 184, 170, 158, 157, and 144 (**Figure 4**). This hydroxylation trend was further enhanced in Serial No. 6 compounds (**Table 2**), whose putative structures were assigned as 2,3,4-trihydroxy-2-alkylquinolines *N*-oxide (HHHAQ *N*-oxide). Two analogs in this tetrahydroxyl series were identified with *m/z* 294 and 306 $[M-H]^-$, representing 2-heptyl and 2-octaenyl side chains, respectively. Collision induced dissociation of *m/z* 294 yielded the major daughter ion at *m/z* 258 after losing 2 moles of H_2O (**Figure 5**). The other fragmentation products having *m/z* values of 196, 194, 183, 170, 158, and 144 were also structurally assigned, which supported the putative structure of *m/z* 294. Similarly, collision induced dissociation of *m/z* 306 ion, produced a stable daughter ion at *m/z* 270 after the loss of 2 moles of water, which on further fragmentation yielded daughter ions at *m/z* values of 252, 242, 181, 170, 158, 157, and 144 (Figure S4). Finally, 4-hydroxy-2-alkylquinoline *N*-oxide (HAQ *N*-oxide) representing *m/z* at 340 $[M-H]^-$ (**Table 2**, Serial No. 7) was detected. Fragmentation of *m/z* 340 through CID demonstrated the daughter ions at *m/z* values of 322, 312, 297, 291, 277, 260, 198, 184, 183, 170, and 144, which correlated with the expected fragments.

The supernatant of strain BRp3 harvested at different time intervals showed Xoo suppression in plate diffusion assay with an inhibition zone of 8–15 mm. The maximum antibacterial activity against Xoo was observed for the supernatant harvested after 24–48 h growth of bacterial culture (Figure S7).

In Planta Evaluation for the Suppression of BLB

Net House Experiment

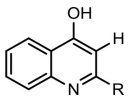
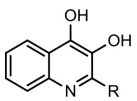
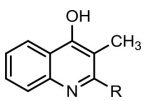
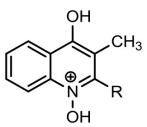
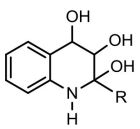
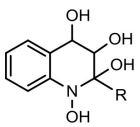
The bacterial strain BRp3 was studied for *in vivo* suppression of BLB in a pot experiment under net house conditions. Bacterial inoculation resulted in significant disease suppression shown by percent diseased leaf area (3.7% DLA) compared to that of the infected control (37.2%) against Xoo. Besides reducing the disease incidence, strain BRp3 improved the plant dry weight as compared to control plants without inoculation.

Another experiment conducted in large earthen pots showed that the effect of strain was statistically significant for disease suppression and growth promotion compared to infected and healthy control plants. DLA (%) of plants inoculated with BRp3 was 4.7% as compared to infected (23.3%) and streptocyclin treated plants (7.1%; **Figure 6**).

Induction of Defense Related Enzymes

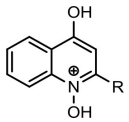
The net house experiment to study the accumulation of defense related enzymes showed that upon Xoo clip inoculation of rice plants inoculated with *P. aeruginosa* strain BRp3, higher activities of defense related enzymes were observed at 24 and 48 h post-inoculation. Peroxidase was observed to be maximum as compared to healthy and infected control after 24 h of Xoo inoculation. The higher activity of Catalase was observed after 48 h of Xoo inoculation. The inoculation of BRp3 resulted in higher induction of Poly Phenol Oxidase (PPO) activity at 24,

TABLE 2 | Metabolites produced by *Pseudomonas aeruginosa* BRp3 detected by ESI-MS/MS.

S. no.	Structures of distinct series of HAQ compounds	Side chains	Observed peaks (*m/z*)			MS/MS (verified)*	MS/MS (reported)	References
			$[M+H]^-$		$[M-H]^+$			
			m/z	Abundance**				
1		$C_{5:0}-\mathbf{C_5H_{11}}$	214	2	216	(+) 198, 194, 186, 172, 159, 146.	(+) 198, 184, 172, 159	Lepine et al., 2004
		$C_{6:0}-\mathbf{C_6H_{13}}$	228	1	230	(+) 212, 202, 194, 186, 172, 159, 146.		Deziel et al., 2004
		$C_{7:1}-\mathbf{C_7H_{13}}$	240	2	242	(+) 224, 213, 200, 194, 186, 185, 184, 172, 159, 146. (–) 198, 184, 172, 170, 158, 157, 144, 143.	(+) 198, 184, 172, 159	Deziel et al., 2004; Lepine et al., 2004
		$C_{7:0}-\mathbf{C_7H_{15}}$	242	28	244	(+) 200, 188, 186, 172, 159, 146. (–) 228, 212, 198, 184, 170, 158, 157, 144, 143.	(+)186, 172, 159, 146.	Vial et al., 2008
		$C_{8:0}-\mathbf{C_8H_{17}}$	256	6	258	(+) 240, 224, 198, 188, 186, 172, 159, 146.		Deziel et al., 2004
		$C_{9:1}-\mathbf{C_9H_{17}}$	268	14	270	(+) 228, 185, 172, 159, 146.	(+) 184, 172, 159	Lepine et al., 2004
		$C_{9:0}-\mathbf{C_9H_{19}}$	270	24	272	(+) 186, 172, 159, 146. (–) 184, 170, 158, 157, 144.	(+) 184, 172, 159	Lepine et al., 2004
		$C_{11:1}-\mathbf{C_{11}H_{21}}$	296	15	298	(+) 298, 270, 256, 242, 228, 214, 200, 186, 172, 160, 159.	(+) 160, 174	Vial et al., 2008
		$C_{13:1}-\mathbf{C_{13}H_{25}}$	324	4	326	(+) 308, 293, 284, 270, 256, 242, 228, 214, 200, 186, 172, 159. (–) 306, 296, 290, 286, 280, 260, 246, 244, 242, 223, 205, 184, 170, 158.		Lepine et al., 2004
2		$\mathbf{C_7H_{15}}$	258	100	260	(+) 242, 186, 175, 172, 162, 159. (–) 241, 240, 230, 214, 187, 173, 172, 159, 144.	(+) 188, 175	Lepine et al., 2004
3		$\mathbf{C_8H_{17}}$	268	14	270	(+) 228, 200, 186, 185, 184, 173, 172, 160, 159. (–) 226, 198, 184, 173, 170, 158, 157, 144, 143.	(+) 186, 173	Deziel et al., 2004; Vial et al., 2008
4		$C_{8:1}-\mathbf{C_8H_{15}}$	284	11	286	(+) 286, 268, 258, 240, 226, 216, 212, 202, 198, 188, 186, 184, 174, 172, 162, 160, 159, 146, 132.		Vial et al., 2008
		$C_{8:0}-\mathbf{C_8H_{17}}$	286	25	288	(+) 272, 186, 172, 159, 146. (–) 269, 268, 258, 242, 186, 174, 170, 159, 158, 157, 144.	(+) 188, 186, 172, 159, 144	Lepine et al., 2004; Vial et al., 2008
5		$\mathbf{C_7H_{15}}$	278	12	280	(–) 278 → 242 → 170, 158, 157, 144		Current study
6		$\mathbf{C_7H_{15}}$	294	30	296	(–) 279, 266, 258, 249, 248, 236, 223, 208, 196, 194, 184, 183, 170, 158.		Current study
		$C_{8:1}-\mathbf{C_8H_{15}}$	306	19	308	(–) 306 → 270 → 252, 236, 226, 198, 183, 170, 158, 157, 144.		Current study

(Continued)

TABLE 2 | Continued

S. no.	Structures of distinct series of HAQ compounds	Side chains	Observed peaks (*m/z*)			MS/MS (verified)*	MS/MS (reported)	References
			$[M+H]^-$		$[M-H]^+$			
			m/z	Abundance**				
7		$C_{12:1}-\mathbf{C_{12}H_{23}}$	340	7	342	(–) 322, 312, 297, 291, 277, 260, 198, 184, 183, 170, 144.		Lepine et al., 2004

MS/MS verified result are 2% normalized [Only those fragments (m/z) are mentioned whose abundance is >2%]. **Percent relative abundance of peaks with respect to the base peak at m/z 258 (derived from negative ionization mode, **Figure 2, Table-entry 2).*

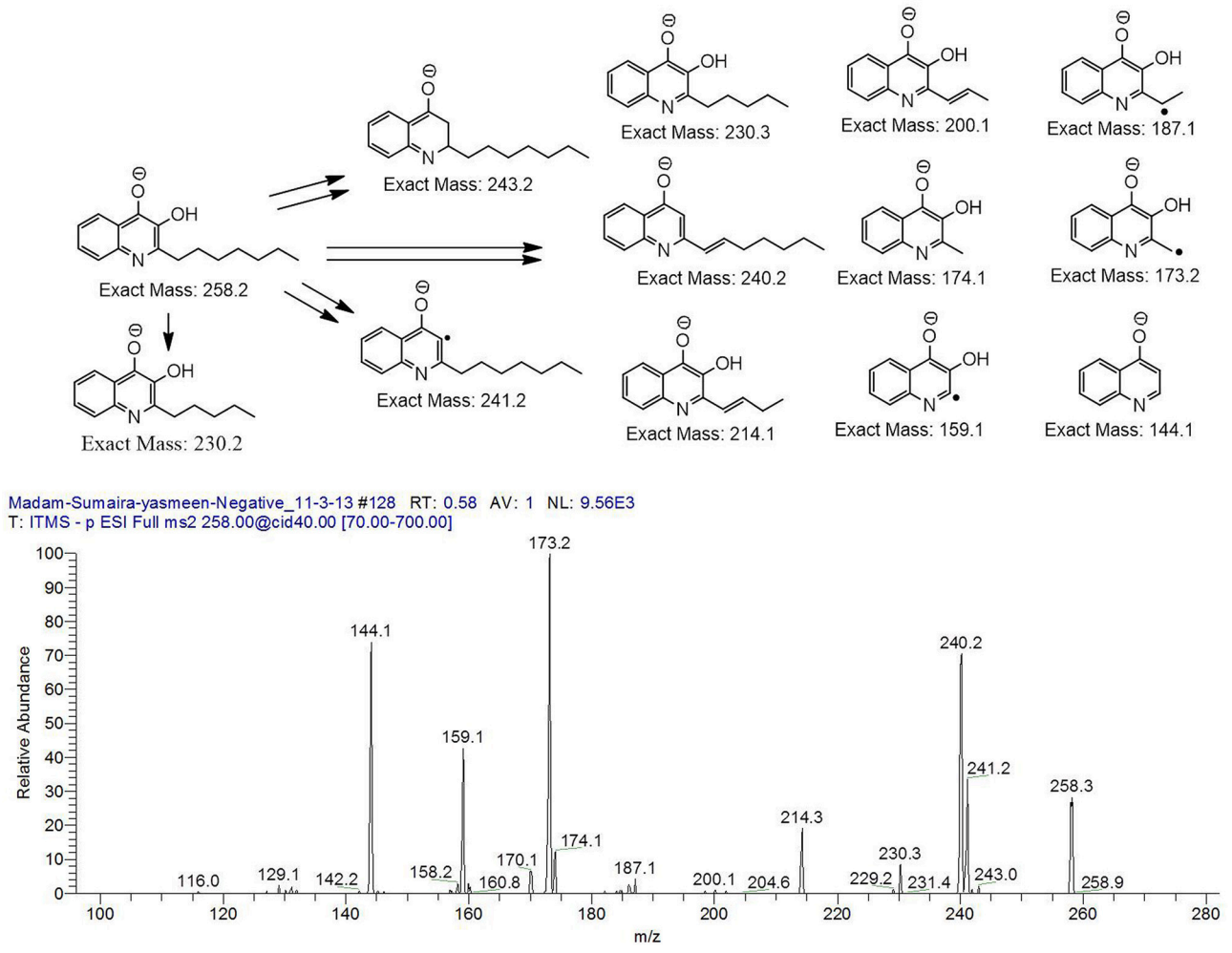


FIGURE 3 | Profiling of the fragmentation data generated through the MS/MS of *m/z* 258 using CID (energy 40.0) at negative ion mode.

72, and 144 h post-inoculation. Maximum activity of PAL was observed after 24 and 48 h of Xoo inoculation (**Figure 7**).

Field Experiment

Assessment of antagonistic strain BRp3 under field conditions was done at booting stage of rice variety Super Basmati. A prevalent virulent strain Xoo7 isolated in the present study was used for clip inoculation at booting stage. Application of *P. aeruginosa* strain BRp3 significantly reduced DLA i.e., 43% as compared with infected control i.e., 83%. Maximum reduction in disease severity was recorded by foliar application of streptocyclin with 39% DLA but it was non-significantly different from that of the strain BRp3 (**Figure 8**). The lesion length was 6.2–8.6, 5.6–9.9, and 8.5–17.9 cm in treatments of BRp3, streptocyclin and infected control plants, respectively. BRp3 inoculated rice plants showed a significant increase in straw

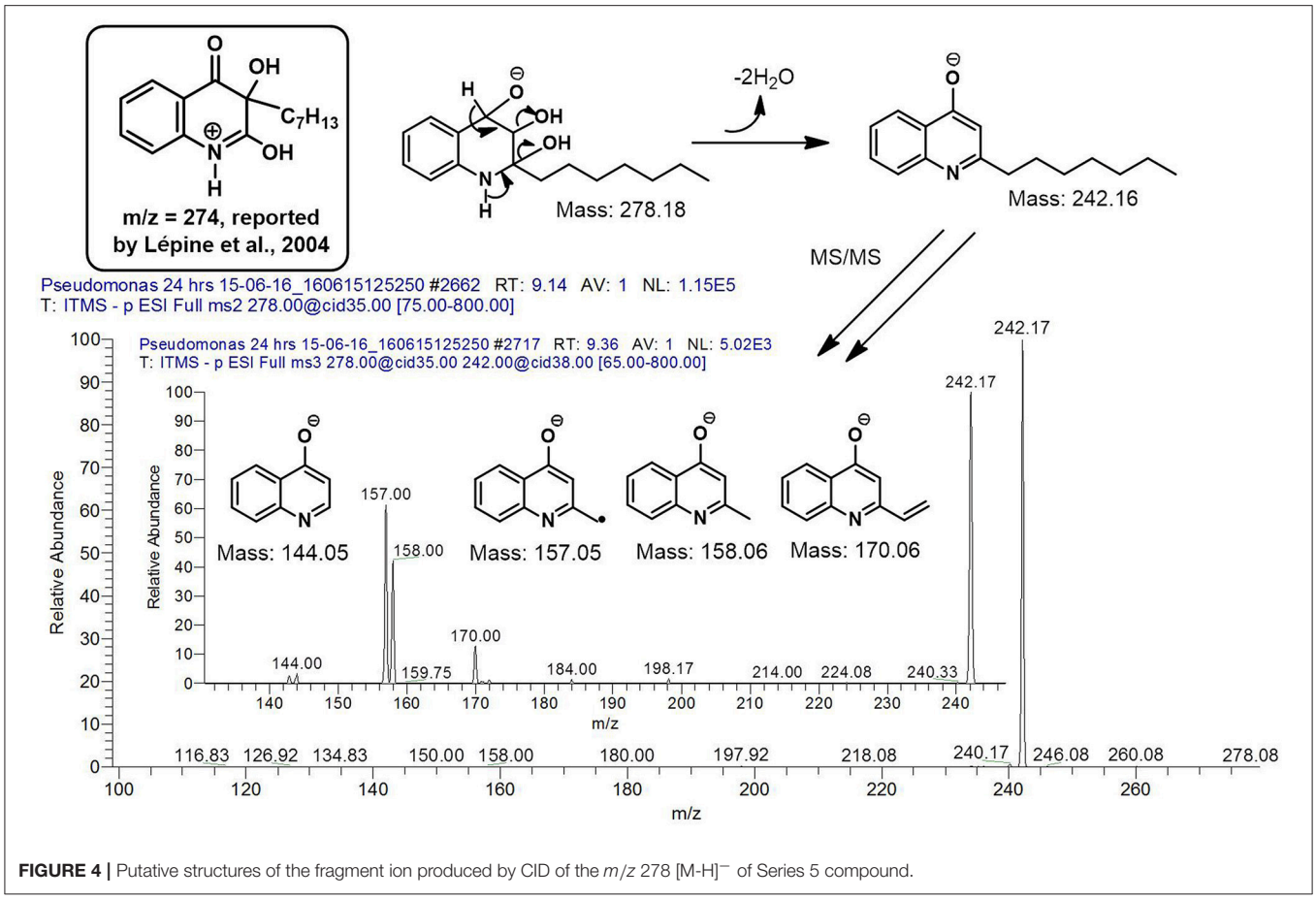


FIGURE 4 | Putative structures of the fragment ion produced by CID of the *m/z* 278 $[M\text{-}H]^-$ of Series 5 compound.

yield. Grain weight per plant was maximum with the strain BRp3 i.e., 34.2 g $plant^{-1}$ followed by healthy and streptocyclin treated plants. Minimum grain yield (19.8 g $plant^{-1}$) was observed for infected control plants (**Figure 9**).

Field Evaluation for Yield Increase of Rice

A field experiment was conducted to evaluate the inoculation effect of strain BRp3 on growth and yield of rice variety Super Basmati with different levels of fertilization. Application of the strain BRp3 either with 80% of the recommended doses or at full recommended doses of N and P, significantly increased the growth parameters in rice compared to respective non-inoculated control plants. The inoculation with the strain BRp3 showed 55% increase in straw and 51% in grain compared to non-inoculated control (with 80% N and P; **Figure 10**).

Rhizosphere Colonization Studies

Colonization studies under field conditions showed that total culturable indigenous bacterial population on rice roots as $\log_{10}$ 8.9–7.3 CFU g^{-1} root (**Figure 11**, Figure S8). The survival level of BRp3 however, declined gradually after seed treatment from $\log_{10}$ 8 to 0.7 CFU g^{-1} root (**Figure 12A**). Total culturable indigenous bacterial counts on rice shoots were $\log_{10}$ 5.3–6.9 CFU g^{-1} shoot up to 40 days after sowing. The bacterial counts of strain BRp3 was $\log_{10}$ 3.9–1.1 CFU g^{-1} shoot when enumerated DPI while no colony of strain BRp3 was detected from surface-sterilized shoots of rice plant at 60 DPI (**Figure 12B**). Antibiotic resistance pattern (Table S3), *in vitro* suppression of Xoo growth (20 mm inhibition zone) and the other plant growth promoting traits like P solubilization (94 $\pm$ 3 $\mu g\ mL^{-1}$), production of IAA (28 $\pm$ 2 $\mu g\ mL^{-1}$), and siderophores 12 $\pm$ 2 mg L^{-1}) of the inoculated bacterium were found comparable to those observed earlier for its pure culture.

Counts of total culturable bacteria on rice roots increased with plant growth. In the rhizosphere, the higher number ($\log_{10}$10.3 CFU g^{-1} root) of bacteria was found on BRp3-inoculated plants, followed by healthy control plants ($\log_{10}$ 9 CFU g^{-1}root) determined at 21 DPI. A decreasing pattern in the counts was more for infected (inoculated with Xoo only) and positive controls (streptocyclin) as compared to that of the strain BRp3 inoculated plots.

Colonization of *P. aeruginosa* BRp3 on rice roots was studied with the help of immunofluorescence (IF) assay and confocal laser scanning microscopy (CLSM). The primary antiserum raised against strain BRp3 was found to be strain specific (Figure S9). Strain BRp3 was found to colonize all over the rice roots at 100X resolution. No fluorescence was observed on the roots of un-inoculated plants (**Figure 13A**). Colonization was observed in intercellular spaces and in micro-colonies as well (**Figures 13B–D**). However, the number of BRp3 cells detected by confocal microscopy decreased gradually after 21 days of seed treatment.

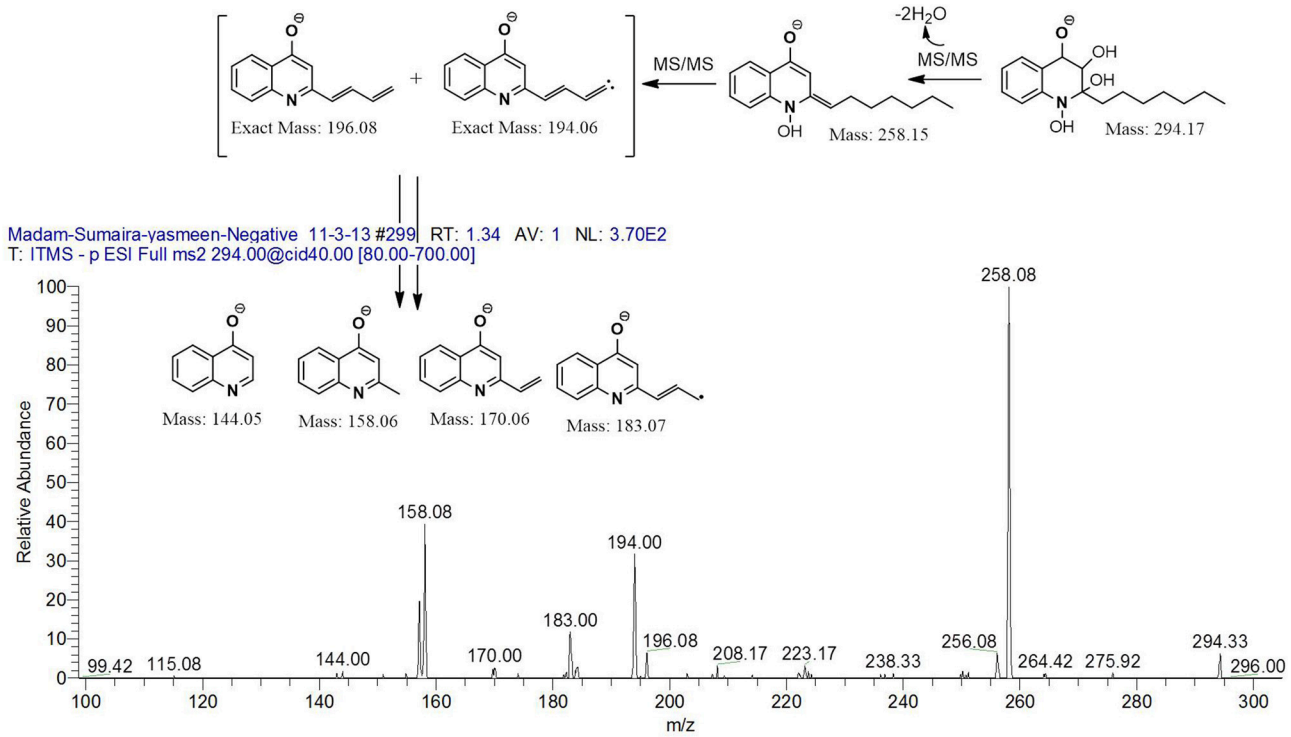


FIGURE 5 | Putative structures of the fragment ion produced by CID of the *m/z* 294 $[M\text{-}H]^-$ of Series 6 compounds.

Sr. No.	Treatment	[a] Experiment 1		[b] Experiment 2	
		%DLA	Plant dry weight (g)	%DLA	Plant dry weight (g)
1	Healthy control	1.9±0.5 D	0.21±0.01 C	5.1±1.2 B	0.15±0.01 C
2	Infected control	37.3±2.5 A	0.21±0.01 C	23.3±3.1 A	0.11±0.01 C
3	Antibiotic	11.6±2.1 B	0.33±0.02 B	7.1±1.6 B	1.23±0.02 A
4	BRp3	3.7±1.4 C	0.44±0.03 A	4.7±0.7 B	0.47±0.04 B



FIGURE 6 | Effects of rice rhizosphere associated *Pseudomonas aeruginosa* BRp3 for suppression of bacterial leaf blight (BLB) in pot experiments under net house conditions. Antagonistic bacteria i.e., BRp3 was applied both as seed treatment and foliar spray 1 day before clip inoculation of Xoo pathogen. Antibiotic i.e., Streptocyclin @ 5 mg/ pot was sprayed 1 day before clip inoculation (Positive control). Means are an average of four biological replicates and there were 15 plants per replicate. Means followed by the same letter differ non-significantly at $p = 0.01$ according to DMRT. [a]Experiment was conducted in small pots and [b]experiment was conducted in large earthen pots. Different letters show statistical significance of treatments while similar letters show non-significant differences.

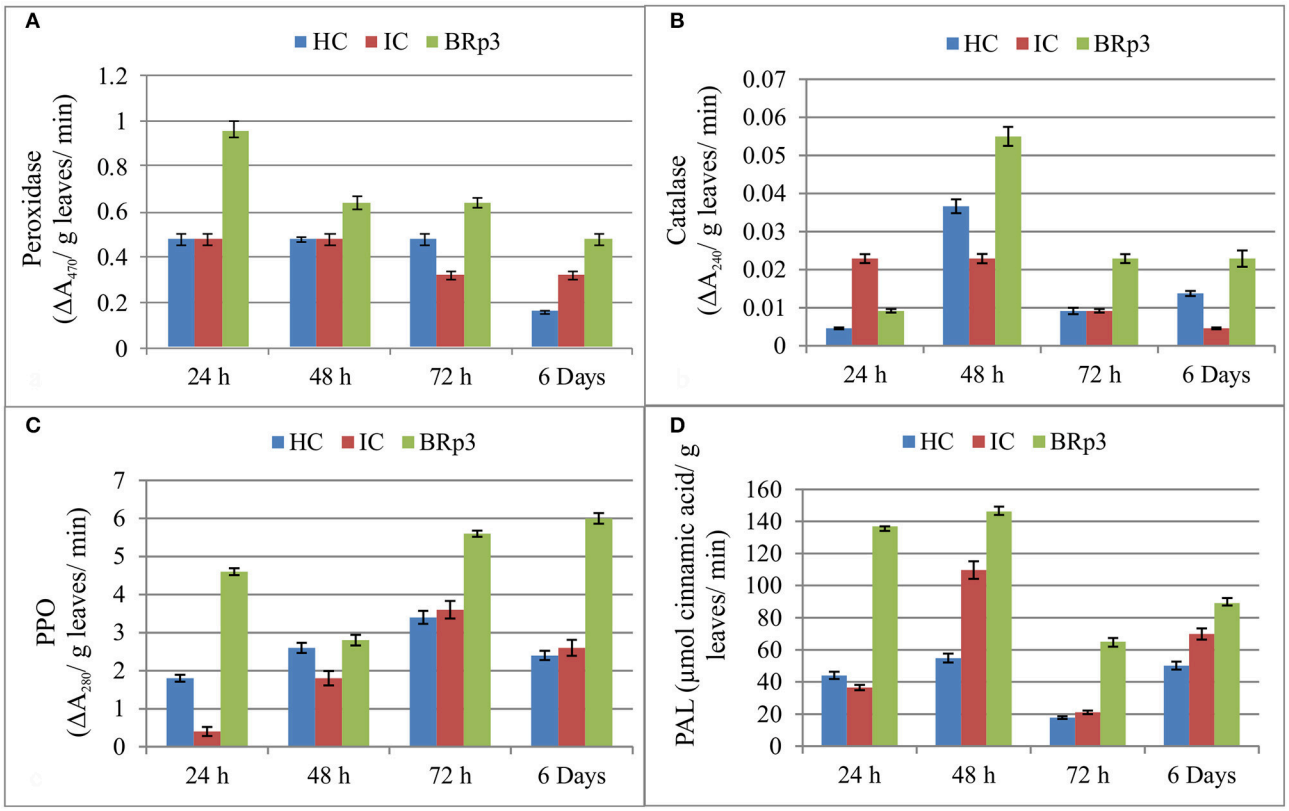


FIGURE 7 | Induction of defense related enzymes **(A)** Peroxidase **(B)** Catalase **(C)** Polyphenol oxidase (PPO) and **(D)** Phenylalanine ammonia lyase (PAL) in rice plants inoculated with *Pseudomonas aeruginosa* BRp3 and challenge inoculated with bacterial leaf blight causing pathogen. Healthy control (HC): Leaves of plants clip inoculated with distilled water, Infected control (IC): Leaves clip inoculated with BLB pathogen i.e., *Xanthomonas oryzae* pv. *oryzae* (Xoo). Forty leaves/ treatment were clip inoculated. Bars show standard deviation of four biological replicates and each replicate has 15 plants per replicate.

DISCUSSION

Plant-associated beneficial bacteria are important growth promoters or biocontrol agents in modern agriculture where eco-friendly and sustainable approaches are growing and have more acceptance than ever before. Rice is grown on large area of Pakistan where a lot of chemical fertilizers as well as pesticides are applied. This study was carried out to evaluate the potential for rhizobacterial inoculum to promote growth and to control disease in rice.

The pathogenicity of *X. oryzae* strains to rice confirmed their identity as causal pathogen of BLB. Xoo1 and Xoo2 were used for further studies due to their aggressive behavior. *In vitro* plate assay for screening the rhizobacterial isolates (512) for inhibition of growth of Xoo led to the selection of *P. aeruginosa* BRp3 for further studies. In a previous study, only LB medium was used for isolation of antagonistic bacteria and the frequency of antagonistic bacteria among the total isolated bacterial population was low (Yasmin et al., 2016). A relatively higher number of antagonistic bacteria was obtained in the present study when different growth media (Nutrient Agar, King's B, and Gould's S1) were used for isolation of bacterial populations. As the biocontrol agents with broad spectrum antagonism are found to be more effective against phytopathogens in rhizosphere, therefore, the antagonistic bacteria isolated in the present study were also screened against different phytopathogens, other than the target pathogen (Table S1). Several rice varieties are infected by various fungal pathogens, including *Fusarium* spp. that cause bakanae disease in rice (Desjardins et al., 2000). The suppression of *in vitro* growth of the *Fusarium* spp. by the antagonistic bacteria showed its potential use as an inoculant for biocontrol of these fungal pathogens.

Phosphate solubilizing and IAA producing microbes are a vital fraction of the microbes that improve the development and growth of their host plant. Vassilev et al. (2006) reported that the solubilization of insoluble phosphates by microbial activity usually induce the secretion of certain metabolites mainly siderophores, lytic enzymes and phytohormones that are involved in the control of phytopathogens. The intrinsic resistance of strain BRp3 against different antibiotics may aid survival in the rhizosphere whenever used as a biological control agent (Dobereiner and Baldani, 1997). In our previous study, siderophore production and P-solubilization were found to be involved in growth promoting activities of antagonistic *P. aeruginosa* Rh323 (Yasmin et al., 2016) whereas the present study suggests that P solubilization accompanied with the production

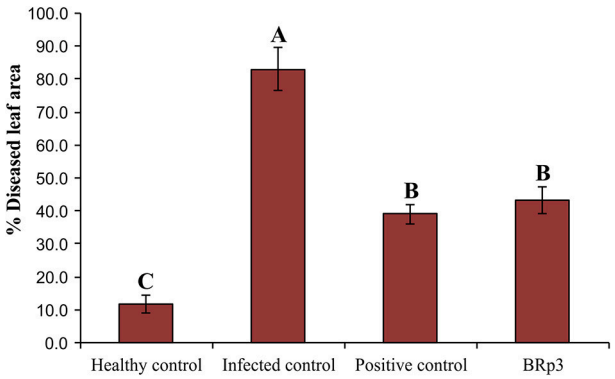


FIGURE 8 | Evaluation of *Pseudomonas aeruginosa* BRp3 for suppression of bacterial leaf blight (BLB) under field conditions. Healthy control: Leaves of plants clip inoculated with distilled water, Infected control: Leaves clip inoculated with prevalent BLB pathogen i.e., *Xanthomonas oryzae* pv. *oryzae* (Xoo) strain isolated in the present study; Positive control: Leaves clip inoculated with BLB pathogen and plants sprayed with streptocyclin and *Pseudomonas* sp. BRp3: Leaves clip inoculated with BLB pathogen and plants sprayed with liquid culture of strain BRp3. Sixty leaves/ treatment were clip inoculated. Four plants per replicate were sown in 1 m row. Suppression of BLB was measured in terms of reduction in the mean bacterial blight lesion length on treated leaves compared to those of non-inoculated control. Bars show standard deviation of three biological replicates. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT.

of IAA, siderophore and ACC may contribute to the growth promoting activities of BRp3, even in the presence of Xoo. ACC deaminase activity of this strain may help to lower the level of ACC caused by ethylene under stress conditions and protect the host plant. Bacterial ACC-deaminase is known to regulate plant growth under biotic and abiotic stress conditions (Singh and Jha, 2016).

It is necessary to understand the mechanisms involved in the suppression of pathogens by the application of biocontrol agents for effective disease management. The bio-antagonistic bacterium used in the present study was characterized for different biocontrol determinants i.e., HCN, chitinases, proteases, starch hydrolysis and siderophores production. Strain BRp3 produced volatile compounds such as HCN which is a known suppresser of phytopathogens (Brimecombe et al., 2001). Recent literature reported that cyanide producing bacteria can be considered as effective biocontrol agents because cyanide production by the bacterial strains induces resistance in the plant (Devi and Kothamasi, 2009; Spence et al., 2014). Gandhi et al. (2009) reported the production of an antifungal protease by rice rhizosphere associated *Chryseobacterium aquaticum* PUPC1 and its effects on the mycelial growth, germination of spores, and sclerotia of phytopathogenic fungi. It has been reported that chitinase can function in defense against many fungal pathogens and also correlated with induced resistance (Perez et al., 2002). Starch hydrolysing ability of the strains indicated their capability to produce amylase and to use a complex carbon source, which aids in the defensive mechanisms of bacterial strains (Marten et al., 2000).

There are many reports that highlight the importance of secondary metabolites in the biocontrol of plant pathogens (Heeb et al., 2011; Khare and Arora, 2011; Jayaseelan et al., 2014). *P. aeruginosa* BRp3 has the capability to produce a variety of metabolites, which include siderophores (1-hydroxy-phenazine, pyocyanin and pyochellin), rhamnolipids and a series of already characterized 4-hydroxy-2-alkylquinolines (HAQs) as well as novel 2,3,4-trihydroxy-2-alkylquinolines and 1,2,3,4-tetrahydroxy-2-alkylquinolines. Mass spectrometric analysis has confirmed their structures (**Figures 1**, **2**, **Table 2**).

Siderophores have also been reported to induce systemic acquired resistance (Trivedi et al., 2008; Sulochana et al., 2014). *P. aeruginosa* BRp3 produces 1-hydroxy-phenazine, pyocyanin (**Figure 1**) and pyochelin (*m/z* 325, **Figure 2**). Phenazine produced by non-pathogenic strain of *P. aeruginosa* has been documented as an antifungal metabolite on the basis of NMR and MS analyses (Kumar et al., 2011). Pyochelin produced by *P. aeruginosa* showed antagonistic activity against *Botrytis cinerea,* the phytopathogen of groundnut (Khare and Arora, 2011). Purified pyocyanin produced by *P. aeruginosa* TO3 was reported for its inhibitory effect against *Macrophomina phaseolina* in tomato (Audenaert et al., 2002).

Rhamnolipids are widely reported to be produced by various microbes including the *Pseudomonas* spp. (Chong and Li, 2017). *P. aeruginosa* BRp3 demonstrated the production of mono- and di-rhamnolipids (ranged from *m/z* 500 to 650, **Figure 2**). LCMS analysis confirmed that the rhamnolipids produced by *P. aeruginosa* DR1 inhibited the growth of different plant pathogens like *F. oxysporum, Sclerotium rolfsii, Phytophthora nicotianae, and M. phaseolina* (Reddy et al., 2016).

P. aeruginosa BRp3 is capable of producing a large repertoire of HAQ and HAQ-related compounds in addition to the siderophores and rhamnolipids. Mass spectrometric analysis of cell free extract revealed the presence of nine analogs of 4-hydroxy-2-alkyl HAQs, having saturated and unsaturated alkyl carbon chains, varied from C_5 to C_{13} chain length, exhibiting *m/z* values of 216–326 $[M+H]^+$ (**Table 2**, Serial No. 1). Their fragmentation data, can confirm their structures, which is well correlated with previously reported literature data (Deziel et al., 2004; Lepine et al., 2004; Vial et al., 2008). Only one analog of the structure representing 3,4-dihydroxy-2-heptyl (HHAQ) was identified (**Figure 3**). HHAQ has demonstrated the highest peak intensity at *m/z* 258 (**Figure 2**), this compound also termed as "*Pseudomonas* Quinolone Signal" (PQS) and is involved in the mechanisms of cellular communication, to detect the pseudomonas cell density (Deziel et al., 2004).

Interestingly, putative polyhydroxy HAQ analogs, i.e., a 2,3,4-trihydroxy-2-alkylquinoline *m/z* 278 $[M-H]^-$ and two analogs of 2,3,4-trihydroxy-2-alkylquinoline *N*-oxideat *m/z* 294 and 306 $[M-H]^-$, respectively, were found to be present in crude extract (Series 5 and 6 compounds, **Table 2**). The fragmentation data correlated with the putative structures of these polyhydroxy HAQs (**Figures 4**, **5**). Budzikiewicz and Kesselmeier (1979) and Neuenhaus et al. (1979) reported the putative 3-alkyl-3-hydroxy-2, 4-dioxo-1, 2, 3, 4-tetrahydroquinoline derivatives in *Pseudomonas*. To support the theme of 3-alkyl substitution, they further synthesized these compounds (Figure S5). Similarly, in a detailed study conducted by Lepine et al. (2004) on

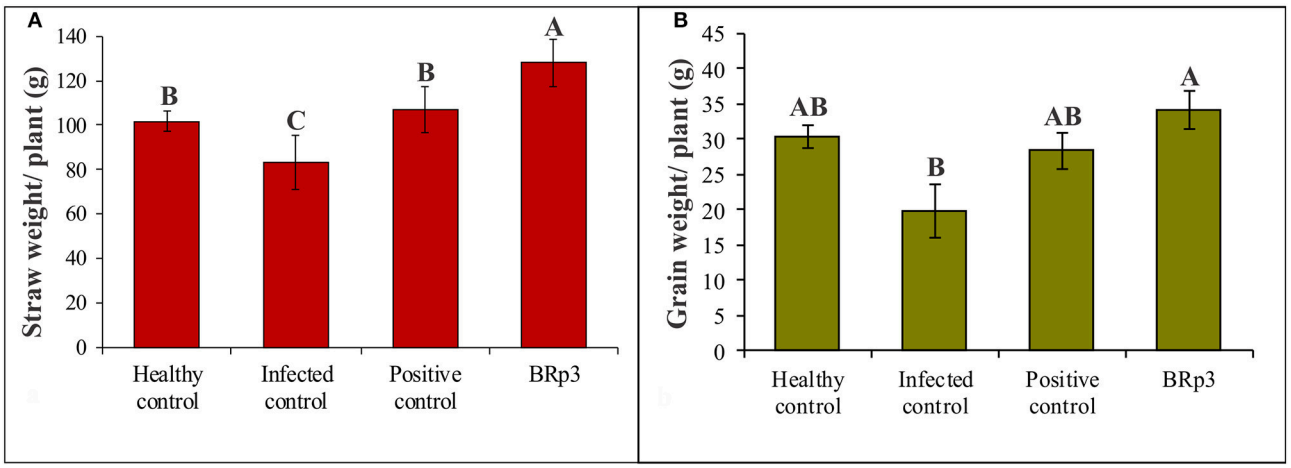


FIGURE 9 | Effects of *Pseudomonas aeruginosa* BRp3 on yield parameters of rice in the presence of Xoo pathogen. **(A)** Straw weight and **(B)** grain weight. Four plants per replicate were sown in 1 m row; Bars show standard deviation of three biological replicates. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT.

metabolic profiling of HAQs from *Pseudomonas*, polyhydroxy HAQs were spotted but the alkyl side chain was reported on 3-position. However, to the best of our knowledge the polyhydroxy HAQs representing alkyl groups at 2-position of the heterocyclic rings, have not been reported in *Pseudomonas* species before this study. To support our theory of alkyl substitution at 2-position, the proposed 2,3,4-trihydroxy-2-alkylquinoline at *m/z* 278 was fragmented through CID that generated predominant daughter ion at *m/z* 242, which on further fragmentation yielded similar finger prints of ions as produced by 4-hydroxy-2-heptylquinolines (**Table 2**, Serial 1; Figure S6). Finally, a 4-hydroxy-2-dodecenylquinoline *N*-oxide analog at *m/z* 340 $[M\text{-}H]^-$ was identified, whose MS/MS data was correlated with the proposed structure (**Table 2**, Serial No. 7). The production of this HAQ by *Pseudomonas*, has also been reported by Lepine et al. (2004). *Pseudomonas aeruginosa* and other related bacteria produced 2-alkyl-4(1H)-quinolones, which exhibited antimicrobial activity (Heeb et al., 2011).

This work demonstrates that *P. aeruginosa* BRp3 is capable of producing a large repertoire of HAQ and HAQ-related compounds in addition to the siderophores and rhamnolipids. Identification of polyhydroxy HAQs may provide insight about the biosynthetic pathway of these interesting compounds. These HAQs along with siderophores in the presence of rhamnolipids (as emulsifying and bacterial cell membrane disrupting agents), may be collectively responsible for the profound antibacterial activity of BRp3 against Xoo. Further studies are needed to establish the individual role of each detected secondary metabolite entity as an active biocontrol agent against BLB pathogen. But the challenge to such study is to get these metabolites in measurable quantity in their purified form. Since these metabolites are being produced in nano grams concentration, the optimization of their production through fermentation would have to be optimized prior to their purification process. Secondly, their purification is challenging, owing to the slight variation in their structures, polarity, hydrophobicity etc. (especially the HAQs), and a routine silica column chromatography was failed to purify them. Although, Naureen et al. (2017) have successfully purified the secondary metabolites (2-pentyl-4-quinolinecarboxylic acid and 1- methylcyclohexene) from *Lysinibacillus sphaericus* ZA9 but the chemistry of these metabolites is significantly different from the HAQs identified in the present study. A high-quality preparatory HPLC system may be required to purify various analogs of HAQs (Wang et al., 2011).

Due to the aforementioned technical challenges, obtaining the sufficient quantities of purified metabolites for plant assays looks difficult. However, crude supernatant from pure cultures of BRp3 were shown to suppress the BLB pathogen (Figure S7). The extracted supernatant of antagonistic bacterial culture has been reported for the control of phytopathogens during *in-planta* evaluation (Simonetti et al., 2012). The extracellular filtrates of biocontrol bacteria having different secondary metabolites such as cyclic lipopeptides were found to be responsible for their antifungal activity (Petatan-Sagahon et al., 2011). Few reports have documented the significant pathogen suppression by supernatants of fermentation without extraction (Garcia et al., 2014). The mechanistic suppression of BLB can be supported by the fact that rice plants inoculated with the strain BRp3 and challenge inoculated with BLB pathogen resulted in an increased activity of defense related enzymes in the host plant. In a previous study, accumulation of defense related enzymes was observed in rice plant up to 48 h after foliar application of antagonistic bacteria Rh323 (Yasmin et al., 2016). Plants inoculated with BRp3 exhibited an increase in the activity of POD, CAT, and PAL after 24 and 48 h post inoculation while, PPO activity was observed even after 72 h and up-to 6 days after challenge inoculation. The production of plant defensive enzymes i.e., POD, PPO, CAT, and PAL in response to inoculated PGPR bacteria is associated with ISR in plants against the pathogen (Saikia et al., 2006; Liang et al., 2011).

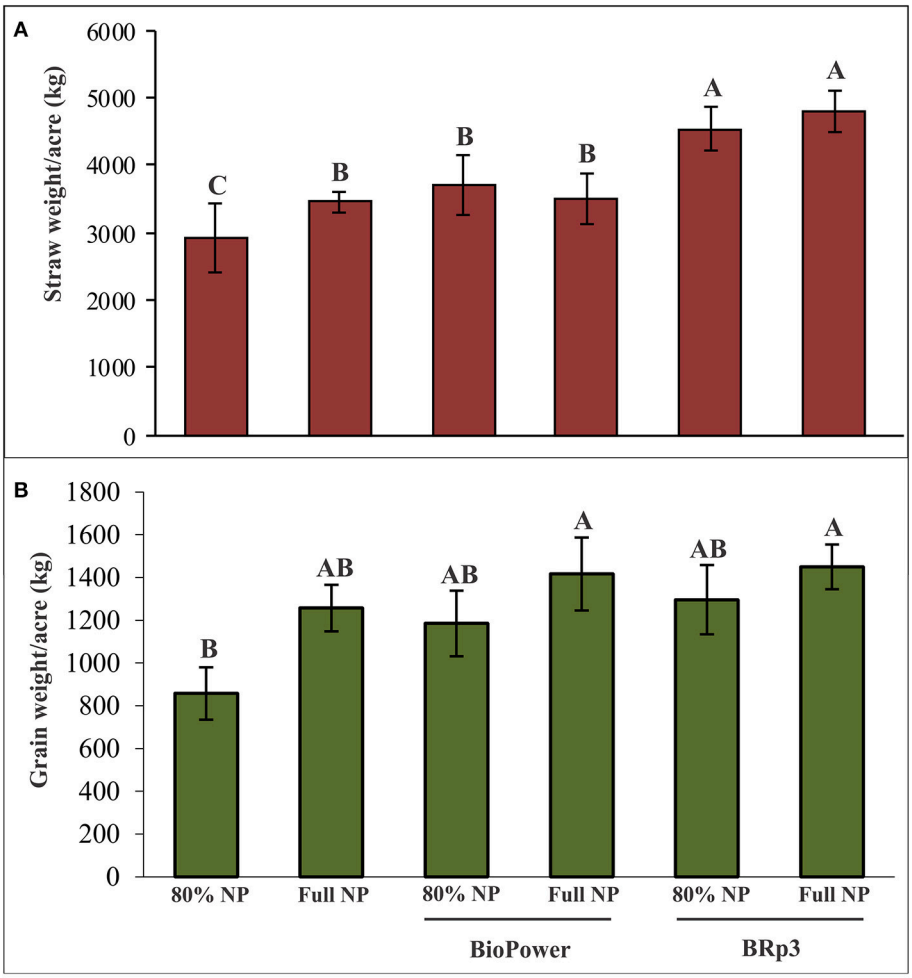


FIGURE 10 | Effects of *Pseudomonas aeruginosa* BRp3 on yield parameters of rice variety *Super Basmati* in a field experiment in the absence of Xoo pathogen. **(A)** Straw weight and **(B)** grain weight. Un-inoculated with 80% of the recommended doses of nitrogen (N) and Phosphorus (P) fertilizers. Un-inoculated with full dose of the recommended N and P fertilizers. "*BioPower*" a consortium of bacterial strains is a commercial biofertilizer product of NIBGE. Bars show standard deviation of four biological replicates. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT. Plot size was 28 m^2.

A better evaluation of plant growth promoting efficiency of the bacterial agents under net house conditions is one of the prerequisites for transferring a strategy from the laboratory into the field. *P. aeruginosa* BRp3 showed effective and consistent pathogen suppression in different pot experiments conducted under net house conditions with different strains of BLB pathogen as indicated by significantly reduced diseased leaf area as compared to the respective non-inoculated control (infected control) as well as compared to positive control of streptocyclin treated plants. Velusamy et al. (2006) reported 58.8 and 64.5% BLB suppression by a DAPG producing *P. fluorescens* PTB9 in net house and field experiments, respectively. Literature showed that the application of *P. fluorescens* Pf1 treatment effectively controlled BLB and the treatment was found to be more effective than the standard streptocyclin in controlling the disease as well as in increasing the yield (Vidhyasekaran et al., 2001).

Field experiments conducted in the presence of Xoo indicated that inoculation with *P. aeruginosa* BRp3 significantly reduced diseased leaf area compared to respective non-inoculated control (infected control) but non-significantly compared to positive control of streptocyclin treated plants. Strain BRp3 showing suppression of BLB equivalent to the positive control under field conditions can be considered as a promising biological control agent to suppress Xoo in rice. Root or seed inoculation of rice plants with this bacterium provided protection to the plant against BLB at early growth stages while its foliar spray at later growth stage or maximum tillering stage protected the rice plant from subsequent disease incidence. Various secondary metabolites produced by this strain along with HCN, may be responsible for the suppression of phytopathogens. The production of increased biomass by inoculated BRp3 may be induced by the production of IAA or siderophores. Different studies reported enhanced growth of root-shoot length

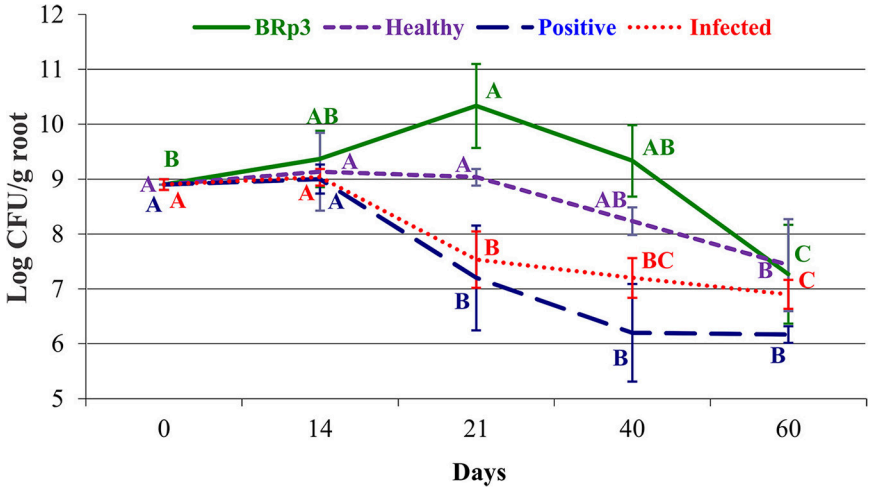


FIGURE 11 | Rice root colonization in response to different treatments in a field experiment. *Pseudomonas aeruginosa* BRp3 was applied both as seed treatment and foliar spray 1 day before clip inoculation of rice bacterial blight pathogen. Foliar spray of antibiotic streptocyclin was used as positive control while non-inoculated plants with and without pathogen, were used as infected and healthy controls, respectively. Bars represent the standard deviation of three biological replicates. Four plants per replicate were sown in 1 m row and three root samples from each replicate were collected. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT. CFU, Colony forming units represent total viable count.

of cucumber, lettuce, potato and tomato due to inoculated *Pseudomonas* strains (Weller, 2007). Khare and Arora (2010) reported the role of IAA produced by *P. aeruginosa* in the suppression of charcoal rot disease of chickpea. Different types of siderophores such as pesudobactin and pyoverdine produced by rhizospheric bacteria chelate to the available form of iron present in the soil and suppress the pathogens by reducing the availability of iron for the phytopathogen (Wensing et al., 2010; Sulochana et al., 2014). Notably, the number of rhizosphere-associated *Pseudomonas* species involved in yield enhancement (Combes et al., 2011) and/or reduction in plant diseases, are increasing (Couillerot et al., 2009; Beneduzi et al., 2012).

The inoculated bacteria BRp3 supplemented with 80% of the recommended doses of N and P significantly enhanced the grain and straw yield with 51 and 55% increase, respectively as compared to the respective control. The single inoculated strain BRp3 showed yield increase comparable to that of commercial biofertilizer (*BioPower*). Application of strain BRp3 either with 80% of the recommended doses or at full/ recommended doses of N and P, significantly improved the growth parameters in rice variety Super Basmati in comparison with control plants without inoculation provided with the same doses of N and P. *BioPower* treatment also significantly increased the grain weight and straw weight even with 80% of the recommended doses of nitrogen and phosphorus (**Figure 10**). The inoculation effect of BRp3 on straw and grain yield with different levels of fertilization indicated that the inoculated bacteria may contribute equal to that of the 80% of the recommended doses of N and P indicating that it may save 20% of urea and DAP fertilizers during crop growth season. These results suggest that the rhizobacteria-inoculants can be applied after further evaluation for nutrient management programs. Adesemoye et al. (2009) reported that supplementing 75% of the recommended fertilizer rate with inoculants produced plant growth, yield, and nutrient (N and P) uptake that were statistically equivalent to the full fertilizer rate without inoculants. The use of fertilizer at rates below the recommended dose, in the absence of bioinoculants, resulted in inconsistent effects on the plant with significant reduction in nutrient uptake and yield. In a previous study by Yasmin et al. (2016), inoculation of *Pseudomonas* spp. and *Serratia* sp. had a significant effect on rice growth under net house conditions and the grain yield was increased in the field too when used in mixed consortia but the effect of field inoculation was non-significant compared to the respective control. In the present study, BRp3 as a single inoculum, not only suppressed the pathogen under field conditions but also improved the rice yield significantly. The factors contributed for higher grain yield due to strain BRp3 as compared to the previously isolated antagonistic strains may be due to its higher potential for IAA and siderophores production. The siderophores produced were also evidenced by LCMS analysis.

Several studies have demonstrated better plant protection when the inoculated bacteria with improved rhizosphere-competence were used (Bonaldi et al., 2015). Colonization studies of *P. aeruginosa* BRp3 under field conditions using viable count showed that the strain had the potential to colonize on rice roots as well as on shoots up to 60 days (**Figures 12**, **13**). Green colored colonies and pigmentation were the main characteristics of *P. aeruginosa* BRp3 that facilitated its detection from the roots and shoots of rice among indigenous soil populations under field conditions using viable count method. Colonization studied by IF and CLSM was in accordance with the results of viable counts of strain BRp3. It appeared that strain BRp3 colonized the rhizoplane and was also found as a shoot endophyte of rice plant. The number of most of the biological control agents decreases with time after their application in the environment,

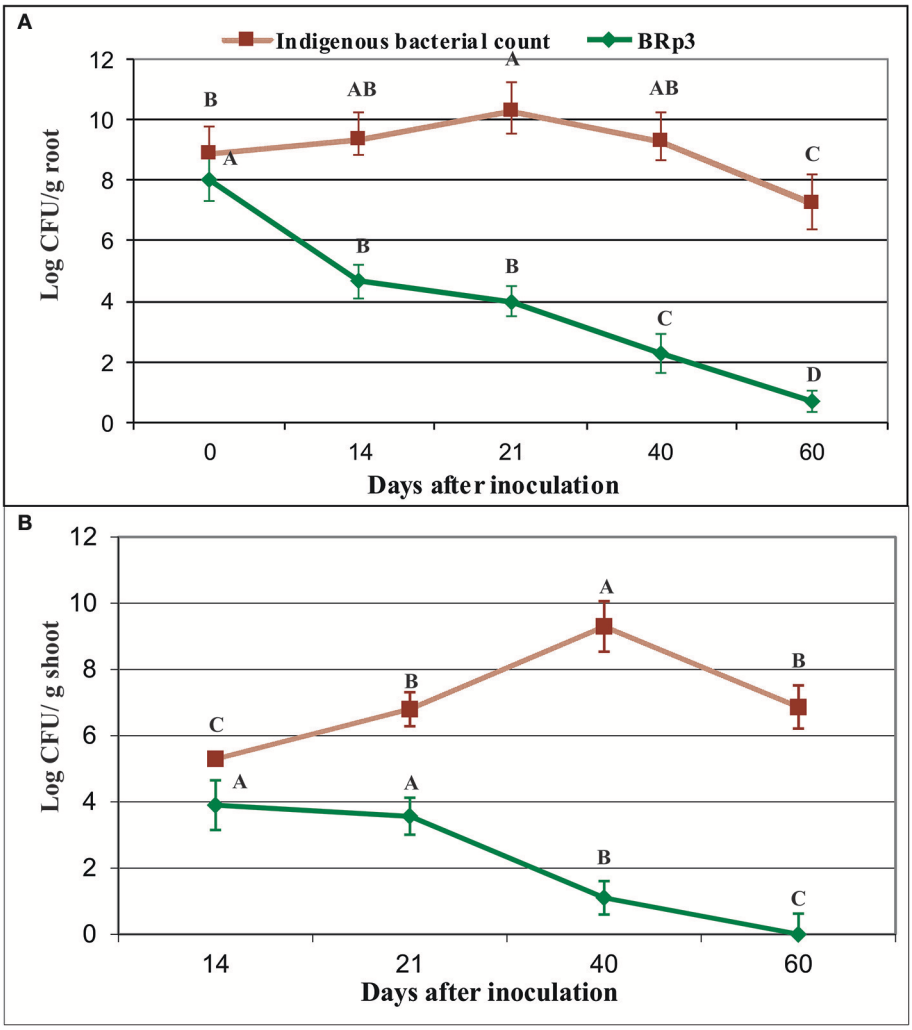


FIGURE 12 | Survival of *Pseudomonas aeruginosa* BRp3 on the roots **(A)** and shoots **(B)** of field-grown rice plants. Enumeration of bacterial population was determined by viable count method. Root and shoot samples were collected from field-grown rice plants. Four plants per replicate were sown in 1 m row and three root/shoot samples from each replicate were collected. Bars represent the standard deviation. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT.CFU, Colony forming units.

which affect the synthesis of inhibitory metabolites produced by these bacteria (Ji et al., 2008). Efficacy of the biocontrol agent depends on the proportion of root-colonized bacterial cells of the antagonistic bacteria. The influence of the introduced strain BRp3 on indigenous microbiota was estimated by comparing the total culturable bacterial population levels with those of different treatments under field conditions. Detection of higher number of culturable bacterial population indicated that BRp3 inoculation did not decrease the native bacterial population, which may be involved to a certain extent for promoting the plant growth (Susana et al., 2007).

To study plant-interacting bacteria, such as phytopathogens, symbionts, endophytes, biocontrol agents, and rhizospheric bacteria requires demonstration of re-infection and establishment of the inoculated strain in or on field-grown plants (Berg et al., 2014). Efficient root colonization by fluorescent *Pseudomonas* spp. has been reported to play an important role in their biocontrol activity against various plant pathogens (Bonaldi et al., 2015).

The significance of this study is that functionally characterized antagonistic *P. aeruginosa* BRp3 may be used for biocontrol of BLB along with enhanced rice growth. Even though, *Pseudomonas* spp. are indigenous and present in various rhizomicrobiomes but some of these can grow above 37°C and may become opportunistic pathogens, hence suitable biosafety regulations are needed to practically implement this technology for field application (Vilchez et al., 2016). *P. aeruginosa* BRp3 will be subjected to acute toxicity tests on mice to study the biosafety of this novel bacterial biocontrol agent. Absence of hemolytic activity on blood agar plates (Unpublished data) indicated that rhizospheric *P. aeruginosa* BRp3 may be unlikely to be a human pathogen (Radhapriya et al., 2015) but whole genome of this

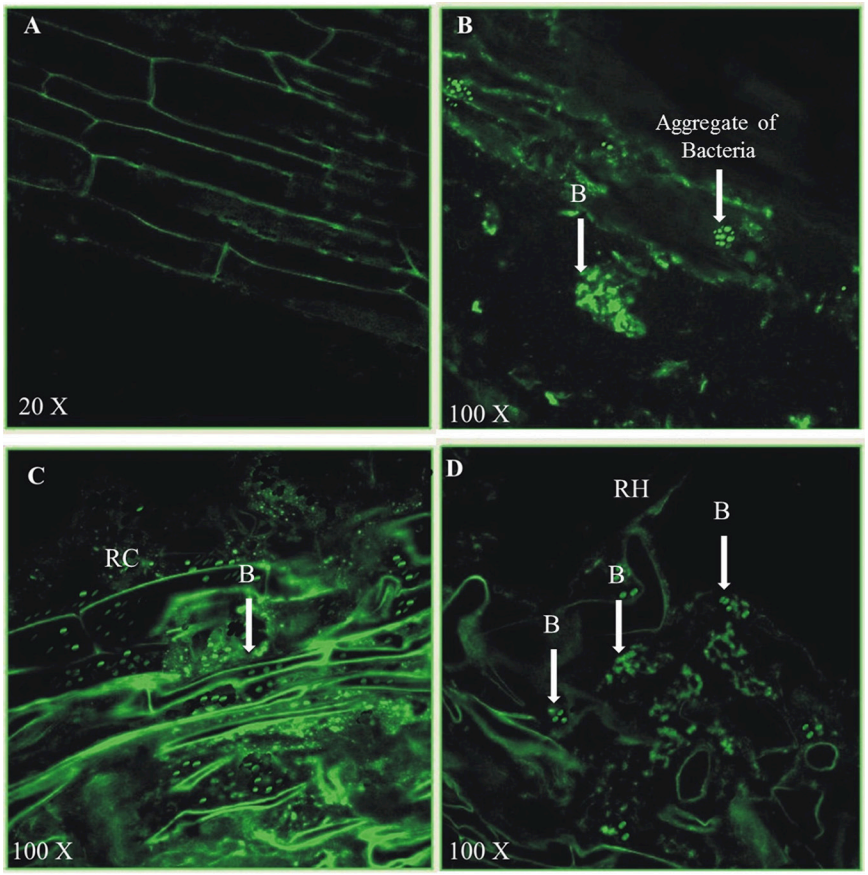


FIGURE 13 | Colonization of 21-days old rice roots by *Pseudomonas aeruginosa* BRp3 studied by fluorescence antibodies staining and CLSM. **(A–D)** FITC-Immunofluorescence images by confocal laser scanning microscopy (CLSM) of whole root after staining by fluorescent antibody (FA) technique. Un-inoculated control **(A)**, Rice roots inoculated with *Pseudomonas aeruginosa* BRp3 **(B,C)** grown in sterile sand under net house conditions and **(D)** under field conditions. B, Bacteria; RC, Root cell; RH, Root hair.

bacterium will be sequenced and compared with non-pathogenic *P. aeruginosa* strains like ATCC 15442 (Wang et al., 2014) to better understand the pathogenicity for its safe application.

CONCLUSIONS

The study presents the detailed physiological characterization and effect of *P. aeruginosa* BRp3 inoculation on rice variety Super Basmati in the presence as well as in the absence of BLB pathogen. This bacterium produced a series of already characterized and novel analogs of HAQs, siderophores and rhamnolipids. The discovery of polyhydroxy HAQs may provide the insight about the biosynthesis pathway of these interesting compounds. The resistance was also induced in rice plants by BRp3 as the activity of defense related enzymes increased after pathogen inoculation. Collectively, the induction of defense related enzymes, HAQs along with siderophores in the presence of rhamnolipids and HCN, were responsible for the profound antibacterial activity of BRp3 against Xoo pathogen. The increased biomass production by strain BRp3 may be attributed to the production of IAA and siderophores. The colonization of the inoculated BRp3 and its re-isolation from rhizosphere indicated its better survival and rhizosphere-competence. On the basis of overall results achieved during this study, bacterial strain BRp3 may be an effective bio-inoculant for Super Basmati rice after ensuring its biosafety aspects. This is perhaps the first systematic effort to use functionally well-characterized beneficial *Pseudomonas* sp. capable of diverse secondary metabolite production for biocontrol of BLB pathogen in the country.

AUTHOR CONTRIBUTIONS

SY was involved in conducting whole research work, data analysis, and write up. FH gave the basic idea to use beneficial bacteria as biocontrol agent and supervised the study. MM helped in 16S rRNA gene sequencing and edited the manuscript. MR helped in lab/field experiments and data analysis. HA provided reference strains of pathogen and involved in conducting experiments at NIAB. MZ helped in LC-MS analysis. MI did LC-MS analysis and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01895/full#supplementary-material

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Rhizosphere Microbiomes Modulated by Pre-crops Assisted Plants in Defense Against Plant-Parasitic Nematodes

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Plant-parasitic nematodes cause considerable damage to crop plants. The rhizosphere microbiome can affect invasion and reproductive success of plant-parasitic nematodes, thus affecting plant damage. In this study, we investigated how the transplanted rhizosphere microbiome from different crops affect plant-parasitic nematodes on soybean or tomato, and whether the plant's own microbiome from the rhizosphere protects it better than the microbiome from fallow soil. Soybean plants growing in sterilized substrate were inoculated with the microbiome extracted from the rhizosphere of soybean, maize, or tomato. Controls were inoculated with extracts from bulk soil, or not inoculated. After the microbiome was established, the root lesion nematode *Pratylenchus penetrans* was added. Root invasion of *P. penetrans* was significantly reduced on soybean plants inoculated with the microbiome from maize or soybean compared to tomato or bulk soil, or the uninoculated control. In the analogous experiment with tomato plants inoculated with either *P. penetrans* or the root knot nematode *Meloidogyne incognita*, the rhizosphere microbiomes of maize and tomato reduced root invasion by *P. penetrans* and *M. incognita* compared to microbiomes from soybean or bulk soil. Reproduction of *M. incognita* on tomato followed the same trend, and it was best suppressed by the tomato rhizosphere microbiome. In split-root experiments with soybean and tomato plants, a systemic effect of the inoculated rhizosphere microbiomes on root invasion of *P. penetrans* was shown. Furthermore, some transplanted microbiomes slightly enhanced plant growth compared to uninoculated plants. The microbiomes from maize rhizosphere and bulk soil increased the fresh weights of roots and shoots of soybean plants, and microbiomes from soybean rhizosphere and bulk soil increased the fresh weights of roots and shoots of tomato plants. Nematode invasion did not affect plant growth in these short-term experiments. In conclusion, this study highlights the importance of the rhizosphere microbiome in protecting crops against plant-parasitic nematodes. An effect of pre-crops on the rhizosphere microbiome might be harnessed to enhance the resistance of crops towards plant-parasitic nematodes. However, nematode-suppressive effects of a particular microbiome may not necessarily coincide with improvement of plant growth in the absence of plant-parasitic nematodes.

Keywords: phytobiome, rhizosphere, microbiome, induced resistance, plant-parasitic nematodes

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INTRODUCTION

Cultivated plants in agroecosystems are part of multi-organismal associations called phytobiomes that may support them in nutrient acquisition, production of growth factors, and defense against pathogens depending on its composition (Sánchez-Cañizares et al., 2017). Plant-parasitic nematodes are microscopic worms that migrate through soil in search of a host plant where they feed from the liquid content of root cells. Root damage in association with the withdrawal of plant nutrients finally leads to plant damage and yield losses. Globally, crop losses associated with plant-parasitic nematodes are estimated to be 12.6% corresponding to $216 billion per year (Nyaku et al., 2017). However, damage by plant-parasitic nematodes often remains unnoticed as aboveground symptoms are rare and a proper diagnosis for nematodes is lacking. Plant-parasitic nematodes vary in their life cycle and type of parasitism. The root lesion nematode *Pratylenchus penetrans* is an endoparasite that invades and migrates through roots as juvenile or adult without becoming sedentary, and escapes to soil under adverse conditions inside roots. In contrast, the root-knot nematode *Meloidogyne incognita* is a sedentary endoparasite, where the infectious second-stage juveniles (J2) enter the root near the tip and establish a feeding site near the vascular system. Once done, the juvenile loses its motility and completes its life cycle within the root.

Management of plant-parasitic nematodes is a challenge because nematicides have been mostly banned, and resistant varieties or non-host crops are often not available or not profitable. Novel methods with high potential and sustainability have become an urgent need. Biocontrol agents are quite expensive and often do not provide consistent control. Interestingly, there is mounting evidence that the indigenous plant microbiome plays a vital role in suppressing soil-borne diseases (Besset-Manzoni et al., 2018). Soil microbiomes that suppressed root invasion and reproduction of plant-parasitic nematodes have been described (Bent et al., 2008; Adam et al., 2014; Elhady et al., 2017). It has been suggested to harness synthetic consortia of microbes (Julien-Laferrière et al., 2016), or beneficial microbiomes (Nyaku et al., 2017) to increase the sustainability and productivity of agriculture. To avoid expensive and inconsistently efficient inoculation of plants with microbes for improvement of plant growth and health, engineering of the soil microbiome was suggested (Finkel et al., 2017). Naturally, the plants itself modulate the microbiome in the rhizosphere (Venturi and Keel, 2016). This may be effective for a prolonged period of time and affect the next plant growing in the same soil (Lapsansky et al., 2016). Agronomically, a preceding crop can affect the yield of a following crop (Jacobs et al., 2018). Understanding plant-microbiome feedbacks was suggested to be one of the keys for exploiting the yield potential of cropping systems (Ahkami et al., 2017). It is yet unclear how different crops affect each other by their specific microbiomes. In monoculture systems soils eventually become disease-suppressive to plant-parasitic nematodes over time (Hamid et al., 2017). However, it was not investigated whether plants enrich a microbiome in their rhizosphere that is more beneficial to them than the microbiome from bulk soil or a different pre-crop.

In this study, we investigated how the rhizosphere microbiomes of different plants affect root invasion of plant-parasitic nematodes on soybean and tomato, and whether the plant's own microbiome protects it better than the microbiome from bulk soil or a different pre-crop. It was analyzed in split-root systems whether observed effects of the microbiome on the nematodes were plant-mediated, and whether induced resistance against the plant-parasitic nematodes has a trade-off in plant growth.

MATERIALS AND METHODS

Plants, Growth Conditions, and Microbiome Inocula

In order to obtain the soil and rhizosphere microbiome inocula, soybean (*Glycine max*) cv. Primus; maize (*Zea mays*) cv. Colisee and tomato (*Solanum lycopersicum*) cv. Moneymaker were grown in 12 cm diameter plastic pots filled with 500 ml field soil as donor crop. The soil was a loamy sand, braunerde, pH 6.5, from a field in Braunschweig, Germany (52°17′57″ N, 10°26′14″ E). Cultivation of soybean in the previous year (tillage, NPK fertilization) resulted in a density of *Pratylenchus* of 599 ± 11 infective stages per 100 ml soil, while other plant-parasitic nematodes had low adundances (*Paratylenchus* 76 ± 29 per 100 ml, *Tylenchorhynchus* 140 ± 21 per 100 ml). The field soil was sampled in May before planting and kept at 4°C until preparation of rhizosphere and bulk soil microbiomes. Five plants per pot were grown to guarantee sufficient root biomass and raise the potential of modulating the tested soil by the donor crops. Pots with only field soil were left fallow to provide the microbiome of bulk soil serving as non-modulated control. All pots were watered as needed every 2–3 days and fertilized weekly with 5 ml per 100 ml substrate with commercial fertilizer (WUXAL Super NPK fertilizer, 8-8-6 with micronutrients, 2.5 g/l, AGLUKON, Düsseldorf, Germany). Pots were kept in the greenhouse at 24°C and 16 h photoperiod. Two weeks after the donor crops were planted, soybean and tomato was planted as recipient hosts for the rhizosphere and bulk soil microbiomes. The soybean and tomato seeds were surface sterilized with 1.5% sodium hypochlorite for 15 min and then rinsed 5 times with sterile deionized water. The seeds were germinated for 5 days on paper tissue under sterile conditions. Seedlings were then planted into two times autoclaved sand as an artificial growth substrate, i.e. in pots containing 100 ml sand for the nematode penetration assay and in pots containing 500 ml sand for the reproduction assay. Recipient plants were grown for 10 days before inoculation of a microbiome suspension from donor crops or bulk soil. For that, the microbiomes of roots with 15 g rhizosphere soil of 6 week-old donor crops, or 15 g bulk soil were extracted in a Stomacher blender (Seward, London, United Kingdom) three times with 15 ml sterile 0.85% NaCl at high speed for 60 s. Soil particles were sedimented and the microbial suspensions of the supernatant were passed through a 5 μm sieve to remove remaining particles, nematodes, and root debris. The microbes were pelleted for 10 min at 4000 g and re-suspended in 45 ml sterile tap water. Each pot with the recipient plant received 15 ml of this suspension per 100 ml autoclaved

sand. The transplanted microbiomes were established for 2 weeks in the rhizosphere of the recipient plants before plant-parasitic nematodes were inoculated.

Growth and Surface Sterilization of Plant-Parasitic Nematodes

Adults and juveniles of *P. penetrans* were multiplied for 2–4 months on carrot disks and extracted by Baermann funnel (EPPO, 2013). The root knot nematode *M. incognita* was multiplied on tomato cv. Moneymaker for 2 months in the greenhouse at 16 h photoperiod and 26°C. Second-stage juveniles (J2) were collected by picking egg masses from tomato roots and transferring them into sterile tap water at room temperature to facilitate hatch of J2. For surface disinfection, nematodes were placed first on 5 μm sieves (Cell-Trics1 filters, Sysmex, Norderstedt, Germany) and washed with 10 ml sterilized tap water. Nematodes were then treated with 0.02% $HgCl_2$ for 3 min and with 4000 ppm streptomycin sulfate for another 3 min. Next, nematodes were incubated for 4 h in 5 ml 1x CellCultureGuard (AppliChem, Darmstadt, Germany) on a rotary shaker at 150 rpm. Finally, the nematodes were washed on a 5 μm sieve and incubated overnight in sterilized tap water. Prior to use in the experiments, nematodes were checked for their sterility by plating them on R2A (Merck, Darmstadt, Germany) for bacterial growth and on potato extract glucose agar (Carl Roth, Karlsruhe, Germany) for fungal growth. Inoculation of plants with nematodes was done by digging eight ca. 5 cm deep half an inch wide holes in 5 cm distance around the shoot, and equally distributing the nematode suspension.

Nematode Invasion and Reproduction Assays

Ten days after nematode inoculation, roots were sampled and washed to remove soil. Nematode invasion was quantified by staining the roots with 1% acid fuchsin (Bybd et al., 1983). Roots were stored in the staining solution at 4°C until counting of nematodes at 20× magnification under a stereomicroscope. To determine the number of living and dead nematodes in the growth substrate, 250 g soil was washed over a sieve combination of 100 μm and 5 μm. The soil particles on the 100 μm sieve were discarded and the nematodes on the 5 μm sieve counted under a stereomicroscope. Alive and dead nematodes were distinguished based on their active mobility and body style.

To determine the reproduction of *M. incognita*, roots were sampled 2 months after nematode inoculation. Roots were gently washed to clean them from adhering soil. The number of galls on the root was determined. To estimate the number of eggs, roots were cut to 2 cm pieces and macerated in 1.5% sodium hypochlorite twice for 15 s each with a commercial blender (Waring, Torrington, CT, United States). The macerate was passed through a 100 μm sieve nested over a 5 μm sieve. Plant debris collected on the 100 μm sieve was discarded and eggs collected on the 5 μm sieve were washed with tap water into 50 ml falcon tubes. A 1 ml aliquot was transferred into a nematode-counting slide and nematodes were counted at 40× magnification under a stereomicroscope.

Invasion of *P. penetrans* Into Soybean Roots Affected by Transplanted Microbiomes

In order to investigate how the transplanted rhizosphere microbiome from different crops affect invasion of *P. penetrans* into roots, soybean plants were grown in two times steam-sterilized sand. Two weeks old soybean plants were inoculated with microbiomes from soybean, tomato, or maize rhizosphere, or from bulk soil in randomized complete block design with 12 replicates. Plants were kept for 2 weeks for microbiome establishment and colonization before each pot was inoculated with 1000 mixed stages of *P. penetrans*. The number of *P. penetrans* in roots, and the living and dead *P. penetrans* in soil were determined 10 days after inoculation.

Susceptibility of Tomato to *P. penetrans* and *M. incognita* Affected by Transplanted Microbiomes

An analogous experiment with tomato was used to confirm whether the plant's own microbiome protects it better than the microbiome from bulk soil or a different pre-crop. Each pot was inoculated with 500 mixed stages of *P. penetrans* or J2 of *M. incognita*. The invasion of both nematodes was determined after 10 days while the reproduction of *M. incognita* and their galls was determined 2 months after incubation. Each treatment had 10 replicates.

Split-Root Experiment

The potential of the rhizosphere microbiome to induce systemic resistance was studied in a split-root system as described by Dababat and Sikora (2007). Three square pots of 7 cm × 7 cm × 8 cm were arranged as follows: Two pots were attached to each other (inducer pot and responder pot, respectively) and one pot was placed in the center above those two pots. Two-week old seedlings of tomato or soybean were transplanted in the center of the upper pot, which was half filled with sterile sand. The number of replicates was 10 for tomato and 12 for soybean. The inducer pot was inoculated with the microbiomes of the donor crops. After establishment of the microbiome, the responder pot was inoculated with 500 mixed stages of *P. penetrans*. The plants were watered and fertilized. The roots were weighted and the number of invaded *P. penetrans* was determined. Roots of the inducer side were frozen in liquid nitrogen for later determination of phenolic compounds. The total phenolic compounds in 0.5 g root were quantified using a Folin Ciocalteu assay (Ainsworth and Gillespie, 2007), with gallic acid (Sigma-Aldrich, Darmstadt, Germany) as reference for quantification.

Statistical Analysis

Analysis of variance was done using the procedure GENMOD of the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, United States) to fit generalized linear models. For count data (numbers of galls, eggs, nematodes) the procedure was used to perform a Poisson regression analysis with a log link function and specification of a scale parameter (Pearson) to fit overdispersed

distributions. Class variables were treatment (microbiome or uninoculated control) and block (accounting for the randomized block design of experiments). For multiple comparisons, the *p*-value was adjusted by the method of Tukey. Graphs were generated using Prism 7 (GraphPad Software, La Jolla, CA, United States).

RESULTS

Rhizosphere Microbiomes of Different Crops Affected the Invasion of *P. penetrans* Into Soybean Roots

Microbes extracted from the rhizosphere of soybean, maize, tomato and from bulk soil were inoculated to the roots of soybean to investigate their effect on root invasion by *P. penetrans*. The type of inoculated microbiome significantly affected the number of nematodes that penetrated the root (**Figure 1**). Compared to the sterile control the microbiomes from soybean, maize and bulk soil all significantly reduced the invasion of *P. penetrans* (**Figure 1**). The tomato microbiome showed a similar trend, although not significantly. The inoculated microbiomes from soybean and maize rhizospheres affected the nematodes stronger than the microbiome from bulk soil, while the effect of the tomato microbiome did not significantly differ from that of bulk soil. The strongest effect on *P. penetrans* was exerted by the microbiome of the maize rhizosphere that reduced invasion significantly more than the microbiome from the soybean rhizosphere. Regarding dead and alive specimen of *P. penetrans* outside the root, dead nematodes were relatively higher in the substrates treated with the microbiomes of bulk soil, maize rhizosphere and soybean rhizosphere than in the uninoculated control, suggesting a role of direct antagonism of the microbiome to the nematode (**Figure 2**). For the microbiome of the tomato rhizosphere such an effect was not observed. The numbers of live *P. penetrans* in the substrate were significantly higher in the pots with microbiomes from maize and soybean compared to all other treatments. Thus, the lower invasion rates of these treatments could not be explained by microbe-induced death of *P. penetrans* outside the root but rather by preferential partitioning of the active nematodes.

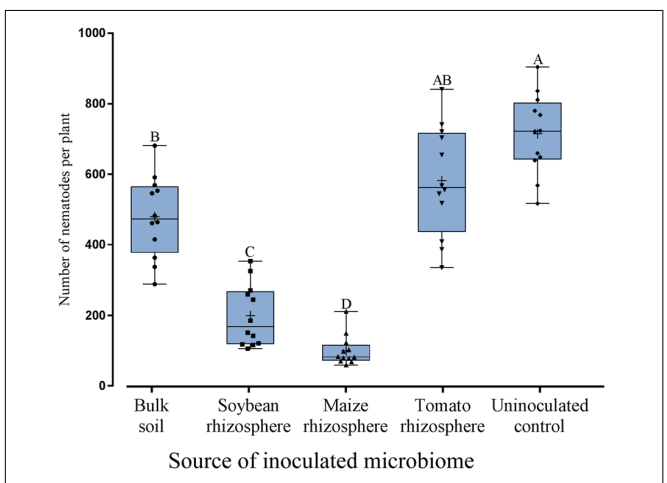


FIGURE 1 | Effect of different microbiomes transplanted from the rhizosphere of different donor crops or bulk soil to the rhizosphere of soybean on the invasion of *Pratylenchus penetrans* into roots. Mean numbers of *P. penetrans* in the root 10 days after inoculation of the nematodes into soil are shown as (+) for each treatment, the medians are shown as (—), whiskers indicate quartiles. Different letters above whiskers indicate significant differences among treatments in Tukey's test (n = 12).

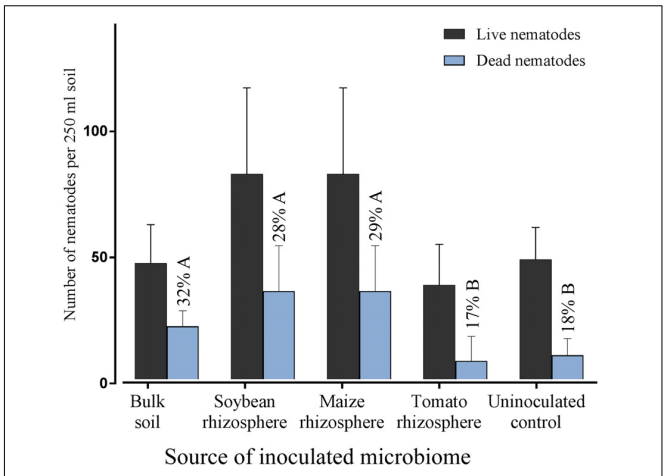


FIGURE 2 | Densities of living and dead *Pratylenchus penetrans* in the soil fraction of pots with soybean plants that were inoculated with microbiomes from different sources, or not inoculated with microbes, 10 days after inoculation of the nematodes. Percentages of dead *P. penetrans* in each treatment are shown above the blue bars, different letters indicate significant differences in Tukey's test (n = 12). Error bars represent standard deviations.

Effect of Different Rhizosphere Microbiomes on Invasion of *P. penetrans* and *M. incognita* Into Tomato Roots

To investigate whether the defense supportive effect of a plant's own microbiome in the rhizosphere is specific to soybean, tomato plants were used as a microbiome recipient instead of soybean. The effect of transplanted microbiomes on tomato root invasion was analyzed for two species of plant-parasitic nematodes that differ in their parasitism and life cycles, i.e., the migratory endoparasite *P. penetrans* and the sedentary endoparasite *M. incognita*. The invasion of tomato roots by both nematodes significantly depended on the inoculated microbiome (**Figure 3**). The trend of the interactions with the different microbiomes was similar for both nematodes. All inoculated microbiomes except from soybean significantly reduced the invasion of *P. penetrans* and *M. incognita* into tomato roots compared to the uninoculated control (**Figure 3**). The soybean rhizosphere microbiome failed to hamper both *P. penetrans* and *M. incognita* to invade into tomato roots. The microbiomes from maize and tomato rhizospheres significantly reduced the invasion of *P. penetrans* into the tomato roots compared to the bulk soil microbiome. For *M. incognita* only the treatment with maize microbiome and not tomato microbiome differed from the effect of the bulk soil microbiome on root invasion of the

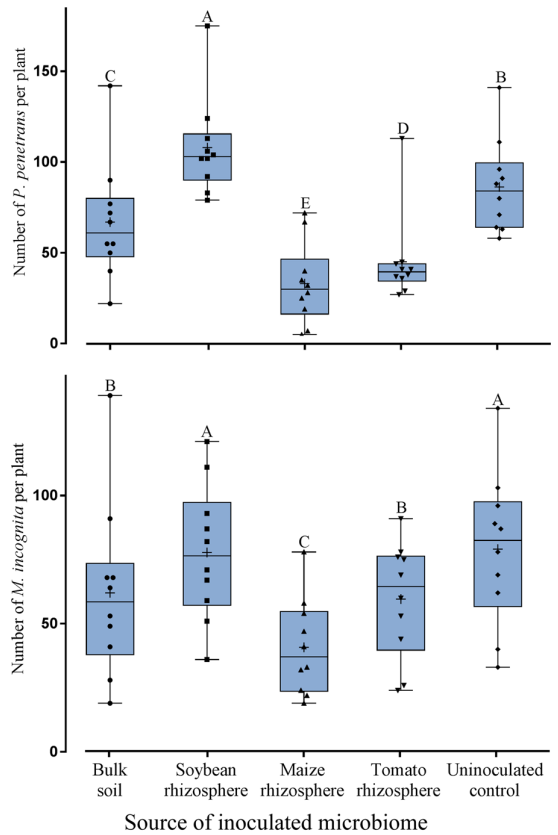


FIGURE 3 | Effect of transplanted microbiomes from the rhizosphere of different donor crops or bulk soil on the number of *Pratylenchus penetrans* (migratory endoparasitic nematodes) or *Meloidogyne incognita* (sedentary endoparasitic nematodes) in tomato roots 10 days after inoculation of the nematodes into soil. Mean numbers are shown as (+) for each treatment, medians are shown as (—), whiskers represent quartiles. Different letters indicate significant differences among treatments in Tukey's test (n = 10).

nematode. The microbiome extracted from maize rhizosphere had the highest suppressive effect among the tested microbiomes against both *P. penetrans* and *M. incognita*. Comparison with the previous experiment showed that soybean plants are better protected from invasion of the nematodes by the soybean microbiome rather than the tomato microbiome, and that tomato plants are better protected by the tomato microbiome rather than the soybean microbiome.

To investigate the effect of different rhizosphere microbiomes of tomato plants on the reproduction of *M. incognita*, the number of galls and eggs were determined 2 months after nematode inoculation of tomato plants previously inoculated with the microbiome from soybean rhizosphere, maize rhizosphere, tomato rhizosphere, bulk soil, or uninoculated. The number of galls on tomato roots was significantly reduced in the treatments with transplanted microbiomes compared to the control, except for the microbiome from soybean rhizosphere (**Figure 4**). The number of eggs was only on those tomato plants significantly reduced which received the rhizosphere microbiome from tomato plants (**Figure 4**). On tomato plants that received the microbiome from soybean plants the largest offspring was produced by *M. incognita*.

Plant-Mediated Effect of Microbiomes on *P. penetrans* Invasion Into Soybean and Tomato Roots Analyzed in Split-Root Systems

To investigate whether the observed effect of some microbiomes on nematode invasion of roots is plant-mediated or rather based on a direct antagonism of microbes towards the nematodes, microbes and *P. penetrans* were inoculated spatially separated in split root systems of tomato and soybean plants. Again, the microbiomes extracted from the rhizospheres of plants of the receiving crop or maize significantly reduced the number of invaded *P. penetrans* in the roots compared to the treatment with the microbiome from bulk soil (**Figure 5**). This suggested an involvement of systemic resistance of the plant specifically induced by these microbiomes as a basis for the observed effects. The invasion rates of nematodes into the roots did not differ between the treatments with host and maize microbiomes. Soybean and tomato plants showed the same trend to stronger suppress root invasion by nematodes when inoculated with the rhizosphere microbiome from the same plant species compared to inoculation with the bulk soil microbiome.

Effect of Rhizosphere Microbiomes on Plant Growth

Although treatment effects on plant growth could not be expected due to the short experimental period, a trend for higher plant weight was observed for tomato and soybean plants inoculated with microbiomes compared to the uninoculated control (**Figure 6**). A slightly better growth than in the uninoculated control was determined for tomato with microbiomes from

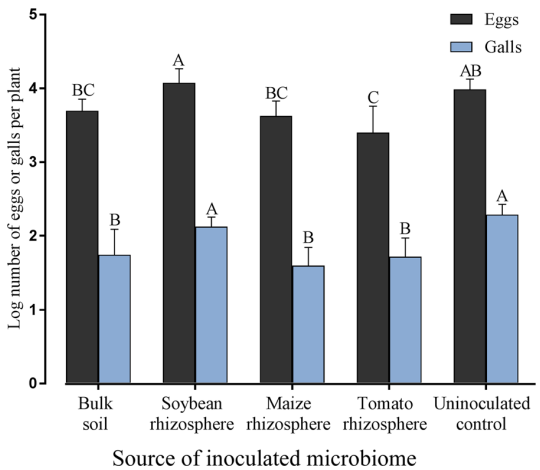


FIGURE 4 | Effect of transplanted microbiomes from the rhizosphere of different donor crops or bulk soil on galls and eggs produced by *Meloidogyne incognita* on tomato plants two months after inoculation of the nematodes. Different letters indicate significant differences among treatments in Tukey's test (n = 10). Error bars represent standard deviations.

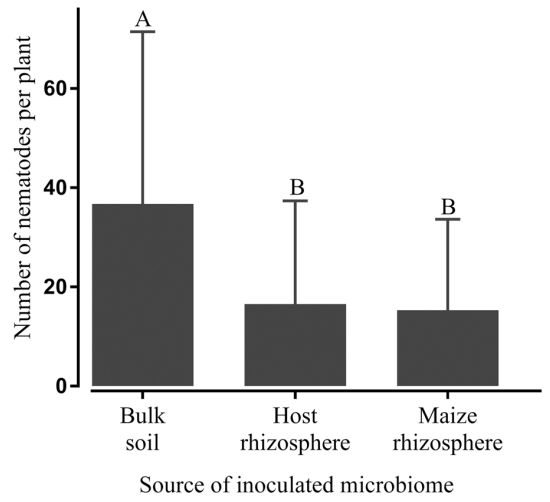


FIGURE 5 | Systemic effect of microbiomes transplanted from the rhizosphere of the host plant (tomato or soybean) or maize, or bulk soil on the number of *Pratylenchus penetrans* in the root of the host plant 10 days after inoculation of the nematodes. Microbiomes were inoculated on one side of a split-root system and nematodes on the opposite side. Different letters indicate significant differences among treatments in Tukey's test (n = 10). Error bars represent standard deviations.

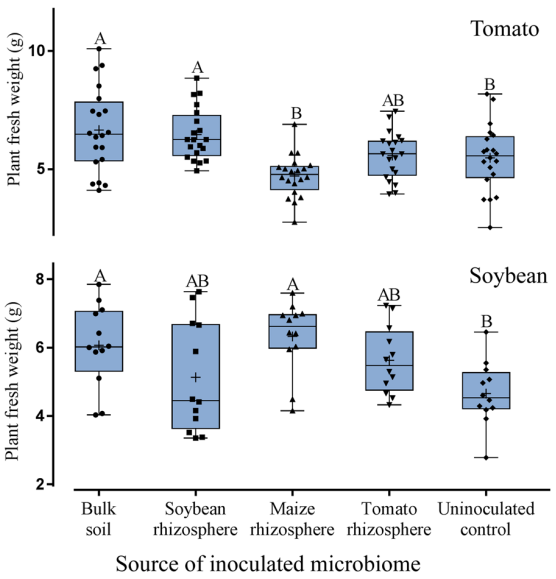


FIGURE 6 | Effect of microbiomes transplanted from the rhizospheres of different donor crops or bulk soil on the weight of tomato plants (upper panel, n = 10) or soybean plants (lower panel, n = 12). Mean numbers are shown as (+) for each treatment, medians are shown as (—), whiskers represent quartiles. Different letters indicate significant differences among treatments in Tukey's test.

bulk soil or from soybean rhizosphere, and for soybean with microbiomes from bulk soil or maize rhizosphere (**Figure 6**). Overall, by applying a generalized linear model, the type of microbiome had a statistically significant effect on plant weight but this effect was rather weak when looking at the root and shoot weights of the single experiments (Supplementary Table S1). Numbers of leaves per plant showed no significant differences among all the treatments. Notably, the microbiome from maize rhizosphere supported the least growth of tomato plants while it well protected tomato plants from nematodes, suggesting a trade-off between growth and defense. The plant weight was not negatively influenced by the number of nematodes in the root but showed rather a slight positive correlation.

In the split-root experiment, the root weight of tomato was significantly increased on the side of the inoculated microbiomes compared to the side without inoculated microbiome (**Table 1**). The tomato microbiome supported root growth of tomato significantly better than microbiomes from maize rhizosphere or bulk soil on the inoculated side of the root system, while no significant difference was observed on the other side, which was inoculated with nematodes instead of microbiome. For soybean root weight determined on the nematode side, no significant difference was found between treatments. To investigate whether the microbiomes induced stress responses of the root that might affect growth of the plant, the concentration of total phenolic compounds in the roots on the microbiome side was determined. The type of the inoculated microbiome and the recipient plant had a statistically significant effect on the concentration of total phenolics (**Table 1**). However, the means differed by maximally 7%. In tomato roots, the total phenolics were increased by its own microbiome compared to bulk soil and maize microbiomes. In soybean, the maize microbiome increased the accumulated phenolics more than other microbiomes. Overall, the rhizosphere microbiomes showed a trend for higher induction of phenolic compounds production than the bulk soil microbiome (**Table 1**).

DISCUSSION

In this study, rhizosphere microbiomes of different plants affected the invasion of the plant-parasitic nematodes *P. penetrans* and *M. incognita* into roots of soybean and tomato plants, and reduced reproduction of *M. incognita* in tomato roots. This suppressive effect depended on the plant species from which the microbiome was transplanted. Most efficient in suppression of the nematodes on both plants was the microbiome enriched in the rhizosphere of maize. We tested the effect of the different microbiomes in a standardized pot system containing sterile substrate to avoid confounding factors of physico-chemical soil properties. Indigenous plant-parasitic nematodes were removed by sieving because inter-species competition might affect root invasion and reproduction assays (Umesh et al., 1994). Some evidences of an effect of soil microbiota on root-knot nematodes were reported in earlier studies (Pyrowolakis et al., 2002; McSorley et al., 2006). Adam et al. (2014) found significantly lower reproduction of *Meloidogyne hapla* on tomato plants growing in native soils compared to disinfected soils which showed the importance of soil microbiota in the process. The suppressive effect of the soil microbiota differed between soils from several fields with different crop rotations, and a soil with maize as pre-crop was

TABLE 1 | Total phenolics and root fresh weight of soybean and tomato inoculated with different microbiomes from the rhizosphere of different donor crops or bulk soil, and uninoculated control (different letters in a column indicate significant differences in Tukey's test).

Source of inoculated microbiome	Tomato plants (n = 10)			Soybean plants (n = 12)	
	Total phenolics[1]	Root fresh weight (g)		Total phenolics[1]	Root fresh weight (g) in nematodes side
		Microbiome side	Nematodes side		
Bulk soil	1.63 ± 0.09b	0.70 ± 0.23b	0.49 ± 0.25a	1.80 ± 0.12b	0.54 ± 0.23a
Host rhizosphere	1.75 ± 0.10a	1.59 ± 0.62a	0.84 ± 0.49a	1.85 ± 0.08b	0.66 ± 0.15a
Maize rhizosphere	1.64 ± 0.05b	0.97 ± 0.24b	0.64 ± 0.27a	1.94 ± 0.07a	0.69 ± 0.15a

[1] *In roots on the microbiome side (mg gallic acid equivalent per gram root).*

most suppressive. However, it remained unclear in how far the effect was due to an influence of the pre-crop on the microbiome. In addition, a bias by differing abiotic properties of the soils or native plant-parasitic nematodes could not be ruled out. In our study, the suppressive effect of rhizosphere microbiota was more pronounced than that of bulk soil microbiota. This might be explained by the composition of the microbiome in the plant rhizosphere but also by the higher density of microbes with a higher metabolic activity compared to the microbiome of the bulk soil (Philippot et al., 2013).

For tomato and soybean plants we showed that the plant's own microbiome protected it better from plant-parasitic nematodes than the microbiomes from bulk soil or from the respective other plant. Each plant species recruits a specific set of root associated microbes when planted in the same soil (Turner et al., 2013; Haichar et al., 2014; Ofek et al., 2014). The plant's own specific rhizosphere microbiome might have coevolved with the plant to assist in growth and defense (Sánchez-Cañizares et al., 2017). Enrichment of the plant's own microbiome in mono-cropping soils might explain the often observed development of suppressiveness to specific pathogens of that crop (Zhu et al., 2013; Hamid et al., 2017). However, in our study not the plant's own microbiome but the microbiome from the maize rhizosphere had the most suppressive effect against *P. penetrans* and *M. incognita* on soybean and tomato plants. This leads to the conclusion that maize might be a good pre-crop in rotations with soybean and tomato with respect to managing the soil microbiome. The rhizosphere microbiome of maize was shown to harbor a higher functional diversity than bulk soil (Li et al., 2014a,b) and is enriched with bacterial taxa of the orders Burkholderiales, Oceanospirillales, Sphingobacteriales, Actinobacteria, and Bacteroidetes containing several beneficial species (Chauhan et al., 2011; Peiffer et al., 2013). Furthermore, it was shown that root exudates of maize can stimulate rhizosphere colonization by *Bacillus amyloliquefaciens* SQR9 resulting in enhanced plant growth and reduced infestation by soil pathogens (Zhang et al., 2015). In addition, secondary metabolites of maize like benzoxazinoid induce plant defense mechanisms against soil pathogens and contribute to the recruitment of plant beneficial bacteria in the maize rhizosphere (Neal et al., 2012).

Plant-parasitic nematodes migrate through the soil in search of roots, directed by communication signaling, and thereby interfere with indigenous microbes. This could be a direct antagonism, or microbes in the rhizosphere can stimulate plant defenses and thus interfere with plant-parasitic nematodes indirectly. In this study, we observed that the suppressive effect of the microbiome on *P. penetrans* was at least partially mediated by the plant as shown in the split-root experiment. This was also evidenced by the observation that *P. penetrans* partitioned more into the compartment outside the root in the pots with suppressive microbiomes, and less into the root, compared to less suppressive treatments. At the same time the death rate of *P. penetrans* in soil did not contribute to suppressiveness. The plant-mediated effect of the rhizosphere microbiomes could be caused by stimulation of the biosynthesis of phytohormones, defense proteins and secondary metabolites that are involved in plant defense responses (Pieterse et al., 2014; Rashid and Chung, 2017). A major role in the regulation of plant defenses play phenolic compounds (Ma et al., 2016). Our results showed that the type of the inoculated microbiome significantly affected accumulation of total phenolics in the roots, i.e., accumulation of phenolic compounds was highest in soybean roots treated with the rhizosphere microbiome of maize and in tomato roots treated with the microbiome of tomato. In a recent study, the microbiome of a suppressive soil increased resistance of tomato plants against *Fusarium oxysporum* f. sp. *lycopersici* compared to steam disinfected soil by inducing a state of alert which included increased levels of phenolic compounds in the roots (Chialva et al., 2018).

In our study, the plant-mediated effect on the parasitic nematodes depended on the plant species from which the microbiome was transplanted. This effect might be harnessed to engineer soil microbiomes by selected crops towards increased plant resistance against plant-parasitic nematodes (Nyaku et al., 2017). However, a trade-off between induced resistance and plant growth was often reported (Huot et al., 2014; Vyska et al., 2016). With the exception of tomato plants treated with the rhizosphere microbiome of maize, plants inoculated with microbiomes showed a higher plant fresh weight than non-inoculated plants. On the contrary, the plant growth of tomato plants was negatively affected by the maize microbiome, which coincided with a higher suppression of the nematodes. That raises the importance of balancing plant immunity and plant growth when managing rhizosphere microbiomes (Albrecht and Argueso, 2017; Ning et al., 2017). Prolonged effects of crop rotations on soil microbial communities are well documented (Smith et al., 2016; Ashworth et al., 2017; Benitez et al., 2017; Wubs and Bezemer, 2018). If the derived changes in microbial

communities are associated with improved crop yield and yield stability, both in the presence and absence of biotic stress, then crop rotations and cover crops might be harnessed to manage soil microbiomes. This tool for sustainable agricultural intensification could be even more promising if the responsiveness of modern crops to beneficial microbiota was enhanced as a target of breeding programs. In addition, crop varieties could be selected that better support beneficial soil microbiota. However, any successful implementation in the near future requires a deeper understanding of the main taxa responsible for pathogen suppression and how different plant genotypes stimulate those taxa.

AUTHOR CONTRIBUTIONS

HH and JH designed the research. AE and SA performed the research. HH and AE performed the analyses. HH, JH, and AE wrote the paper.

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SUPPLEMENTARY MATERIAL

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Klebsiella pneumoniae SnebYK Mediates Resistance Against *Heterodera glycines* and Promotes Soybean Growth

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Soybean is an important economic crop that is often adversely affected by infection in the field with the soybean cyst nematode *Heterodera glycines*. Biological control is an eco-friendly method used to protect the crop against disease. The bacterium *Klebsiella pneumoniae* has been reported to protect rice from sheath blight and seedling blight, but its role in the control of nematode is unclear. In this study, the effect of *K. pneumoniae* SnebYK on the control of *H. glycines* was assessed. Potting experiment results showed that coating soybean seeds with *K. pneumoniae* SnebYK not only reduced the infection rate of *H. glycines* but also decreased the proportion of adult female nematodes. Field experiment results showed that *K. pneumoniae* SnebYK reduced both the number of *H. glycines* in soybean roots and the number of adult females. However, *K. pneumoniae* SnebYK caused low juvenile mortality in an *in vitro* assay. To further analyze the role of *K. pneumoniae* SnebYK in the inhibition of *H. glycines* infection, split root experiments were conducted. The results indicated that *K. pneumoniae* SnebYK controls *H. glycines* via induced systemic resistance, which reduces *H. glycines* penetration. *Klebsiella pneumoniae* SnebYK treatment also significantly increased the proportion of second-stage juveniles and decreased the proportions of third- and fourth-stage juveniles in the *H. glycines* population. Moreover, 48 h after inoculation with *H. glycines*, the expression levels of *PR1*, *PR2*, *PR5*, and *PDF1.2* were significantly higher in soybeans pretreated with *K. pneumoniae* SnebYK than in control soybeans. Interestingly, besides providing protection against nematodes, *K. pneumoniae* SnebYK fixed nitrogen, produced ammonia, solubilized phosphate, and produced siderophores, leading to well-developed root system and an increase in soybean seedling fresh weight. These results demonstrate for the first time that *K. pneumoniae* SnebYK not only promotes soybean growth but also inhibits the invasion and development of *H. glycines* by inducing systemic resistance.

Keywords: induced systemic resistance, plant growth-promoting bacteria, *Klebsiella pneumoniae*, *Heterodera glycines*, soybean

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INTRODUCTION

Soybean (*Glycine max*) is an important crop worldwide due to its nutritive and commercial value. The soybean cyst nematode (*Heterodera glycines* Ichinohe) is a soil-borne plant-parasitic nematode that disturbs the root growth of soybean and causes the early yellowing of plants (Subbotin et al., 2010). As a result of the widespread distribution of *H. glycines* and its ability to reduce seed yield, *H. glycines* is considered the most harmful pest that endangers soybean yield globally (Koenning and Wrather, 2010; Rincker et al., 2017). Depending on the severity of the infection, *H. glycines* generally decreases soybean production by 5–100% in northeast China, a major soybean production region (Duan, 2011). Meanwhile, in the United States, the loss caused by *H. glycines* has been estimated to exceed one billion USD annually (Koenning and Wrather, 2010). Thus, finding a simple, eco-friendly, and effective approach to control *H. glycines* is imperative for modern agriculture.

The use of plant growth-promoting bacteria (PGPB) is an ideal approach for the prevention and control of nematodes. PGPB promote plant growth and also induce systemic resistance (Wei et al., 1996; Kloepper et al., 2004). Induced systemic resistance (ISR) is a phenomenon whereby resistance to an infectious disease is systemically induced by localized infection, by treatment with microbial components or products or with a diverse group of structurally unrelated organic and inorganic compounds (Kuć, 2001). Jasmonic acid (JA)/ethylene (ET), and/or salicylic acid (SA) signaling pathways are important in the regulation of beneficial microbes-mediated ISR (Pieterse et al., 2014; Vryzas, 2016). In recent years, ISR mediated by PGPB (e.g., *Bacillus* sp., *Bacterium* sp., *Methylomonas methanica*, *Rhizobium etli*) has been intensively studied against a wide range of important plant-parasitic nematodes, such as *Meloidogyne incognita* (Anter et al., 2014; Alfianny et al., 2017), *H. glycines* (Xiang et al., 2013), and *Globodera pallida* (Reitz et al., 2000). Tian et al. (2014) reported that *Sinorhizobium fredii* decreased the infection rate of *H. glycines* by triggering ISR and also prolonged the developmental stage of *H. glycines* in the root to 30 days, compared with 27 days in the control. Xiang et al. (2013) also reported that treating soybean seeds with *B. simplex* reduced the *H. glycines* population by triggering ISR. Therefore, seed coating with PGPB is a simple and cost-effective strategy that elicits ISR in host plants against pathogens (Van Loon et al., 1998; Ramamoorthy et al., 2001; Pathak et al., 2016). After seed coating, these plants acquire broad-spectrum resistance without any changes to their genome sequences, and respond more rapidly and strongly to various stresses (Pathak et al., 2016).

Klebsiella pneumoniae is a strain of the PGPB that promotes plant growth by fixing nitrogen (Iniguez et al., 2004); producing 1-aminocyclopropane-1-carboxylate deaminase (Singh et al., 2015), indole-3-acetic acid (Sachdev et al., 2009), gibberellic acid (Singh et al., 2015), and siderophores; and solubilizing phosphate (Ji et al., 2014). *Klebsiella* sp. KW7-S06 elicited ISR to the pathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani* in rice, alleviating disease symptoms (Ji et al., 2014). However, reports of *K. pneumoniae*-elicited ISR to nematodes are few. Furthermore, finding a candidate biocontrol agent against *H. glycines* would have great significance for the soybean industry. In the present study, we aimed to (1) assess the plant growth-promoting properties of the *K. pneumoniae* strain SnebYK and its effect on soybean growth, (2) evaluate the effect of the *K. pneumoniae* strain SnebYK on the control of *H. glycines* via seed coating, and (3) investigate the ability of the *K. pneumoniae* strain SnebYK to induce systemic resistance to *H. glycines* in soybean.

MATERIALS AND METHODS

Strain, Fermentation Broth, and Nematode

Klebsiella pneumoniae strain SnebYK, subsequently referred to as SnebYK, is a Gram-negative facultatively anaerobic bacterium that was isolated from mud at the outlet of a pesticide manufacturing factory in Liaoning Province, China. SnebYK was identified by Luo (2010) and stored at −80°C at the Nematology Institute of Northern China at Shenyang Agricultural University. Before use, the strain was streaked onto a nutrient agar medium and incubated at 28°C to confirm purity. A single colony was selected, transferred to nutrient agar medium, and incubated for 48 h at 28°C. The SnebYK fermentation broth (1×10^9 CFU ml^{-1}) was prepared according to the method previously described by Chen et al. (2014).

The population of *H. glycines* (race 3, HG type 0) was maintained on soybean [*Glycine max* (L.) Merr. 'Liaodou15'] grown in the experimental field of the Nematology Institute of Northern China. *Heterodera glycines* specimens were morphologically identified following the method previously described by Subbotin et al. (2010) prior to use in the experiment. Cysts were extracted from soybean rhizosphere soil in the experimental field using a floating sieve, and then surface-sterilized with 0.5% sodium hypochlorite. Second-stage juveniles (J2) were incubated as described by Tian et al. (2014). The hatched J2 were assessed for survival and collected in a beaker for inoculation.

Biochemical Assays of Putative Plant Growth-Promoting Properties

The siderophore production of SnebYK was determined as described by Schwyn and Neilands (1987). SnebYK was inoculated in the center of a chrome azurol S agar plate and incubated at 28°C for 7 days; plates were observed to determine whether a yellow–orange halo appeared around the colony. Assays of inorganic and organic phosphate solubilization by SnebYK were performed using the National Botanical Research Institute's phosphate growth medium and Mongina organic culture medium with lecithin, respectively (Hao et al., 2012; Ji et al., 2014). SnebYK was spot-inoculated onto the plate and incubated at 28°C for at least 7 days. A clear zone around the colony indicated that the strain tested had phosphate solubilization activity. The phosphate-solubilizing ability of SnebYK was evaluated using the phosphomolybdate blue colorimetric method (Murphy and Riley, 1962). Ammonia

production was detected with Nessler's reagent (Singh et al., 2015).

The nitrogen-fixing ability of SnebYK was tested using ACCC55 agar plates (Gao et al., 2015). The nitrogenase activity of SnebYK was evaluated through an acetylene reduction assay, following the protocol of Boddey and Knowles (1987). To analyze the *nifH* gene of SnebYK, genomic DNA was extracted from the SnebYK strain using a bacterial genomic DNA extraction kit (Tiangen Biotech, China). The primers nifH1 (5′-ADNGCCATCATYTCNCC-3′) and nifH2 (5′-TGYGAYCCNAARGCNGA-3′) were used for polymerase chain reaction (PCR) amplification of the *nifH* gene (Gaby and Buckley, 2012). The PCR amplification conditions were 5 min at 94°C for initial denaturation; 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, followed by a 72°C extension for 10 min (Liu et al., 2011). PCR products were analyzed by electrophoresis on 1.2% agarose gels and then sequenced by Sangon Biotech Co. Ltd., Shanghai, China. The obtained sequences were compared with those in the GenBank database (**RRID**: *SCR_002760*) using the NCBI BLAST algorithm (Pinto-Tomás et al., 2009; Liu et al., 2011; Lin et al., 2012). The phylogenetic tree was established using the neighbor-joining method in Mega 7.0.26. To detect the expression of *nifH*, reverse transcription (RT)-PCR analysis was conducted. SnebYK colonies grown on ACCC55 agar plates were collected, and colonies grown on nutrient agar plates were also collected as controls. Total RNA was extracted from the samples using a bacterial total RNA extraction kit (Tiangen Biotech, China), and the PrimeScript RT Reagent kit (TaKaRa, China) was used for cDNA synthesis following the manufacturer's instructions. The primers used to amplify *nifH* were PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′) (Poly et al., 2001). The 16S rRNA gene was used as an internal control. For the PCR, 100 ng cDNA was used, with the following program: 94°C for 4 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by an extension at 72°C for 5 min. PCR products were visualized using the GoldView DNA staining reagent (Aidlab Biotech, China) following agarose gel electrophoresis and analysis.

Growth Promotion Assay in Soybeans Treated With SnebYK

Soybean seeds were surface-sterilized with 0.5% sodium hypochlorite, washed with sterilized water at least three times, and then dried at room temperature. Treated soybean seeds were coated with the SnebYK fermentation broth at a 70:1 mass ratio. Control soybean seeds were coated with sterilized water. All seeds were sown in 10 cm × 10 cm plastic pots containing a sterilized mixture of soil and sand (1:1, *v/v*) and grown in a greenhouse at 26/21°C with a 16/8 h photoperiod. The soybean seedlings were thinned to one plant per pot once two euphylla were observed. Each treatment included 10 replicates and was arranged in a completely randomized design. Thirty days after sowing, soybean seedlings were harvested from all pots, the soil adhered to the roots was carefully removed with tap water, and the shoot length, taproot length, and fresh weight of the plants in each treatment were measured. Root morphology images were obtained using a scanner (Expression 10000 XL, Epson, Japan) and then analyzed using the WinRHIZO image analysis system (V4.1c, Regent Instruments, Canada).

Effect of SnebYK on *H. glycines* Control

Juvenile Mortality *in Vitro* Assay

To obtain the culture filtrate, SnebYK fermentation broth (1×10^9 CFU ml^{-1}) was centrifuged for 30 min at 12,000 rpm. SnebYK filtrate (1 ml) was added to each well of a 12-well tissue culture plate containing 1 ml suspension of freshly hatched *H. glycines* J2 (35–50 juveniles ml^{-1}, surface-sterilized). Filtrate of *B. megaterium* fermentation broth (1×10^9 CFU ml^{-1}), which showed high contact toxicity to *H. glycines* J2 in our previous work (Zhou et al., 2017), was used as a positive control; sterile water was used as a negative control. The plate was incubated in the dark at 25 ± 2°C. Immotile J2 were considered dead when they were needled without reaction (Cayrol et al., 1989). The number of dead J2 was counted under a microscope (SMZ800, Nikon, Japan) after 12 and 24 h of incubation. Each treatment included three replicates, and the experiment was repeated three times.

Potting and Field Experiments

Soybean seeds were surface-sterilized and coated with SnebYK as described above, and the control seeds were coated with sterilized water. When soybean seedlings grew to the two-euphylla stage, their roots were inoculated with 2,000 J2 of *H. glycines*. Thirty days after nematode inoculation, the soybean roots were harvested, and the adult female nematodes on the entire root surface were counted. The roots were then washed with tap water, stained with NaClO-acid fuchsin, and the number of *H. glycines* in each root was counted (Bybd et al., 1983). Adult females and nematodes in the soil were screened by sieving and counted under a stereomicroscope (Liu, 1995). To ascertain the effect of SnebYK on soybean seedlings growth under *H. glycines* stress, the shoot length, taproot length, and fresh weight of seedlings were measured. The root morphology was analyzed using the WinRHIZO image analysis system as previously described. In each case, there were seven replicates, and the entire experiment was repeated twice.

Field trials were carried out in a field infested with *H. glycines* at the experimental test site of the Modern Agricultural Industry Technology System of China in Kangping, located at 42°43′N, 123°24′E, during the growing seasons of 2016 and 2017. Soybean seeds were coated with SnebYK or sterile water in a 70:1 mass ratio. Seeds for each treatment were then sown in six rows of 70 seeds with 10 cm intervals between seeds, in a 3.5 m × 7 m plot. A randomized complete block design was adopted in this experiment, and each treatment included five plots. Thirty days after sowing, 12 seedlings were randomly selected from the inner four rows of each plot and nematode infections were evaluated. Adult females of *H. glycines* on the roots and in the rhizosphere soil (100 ml), and the nematodes in the roots, were counted as described above. The shoot length, taproot length, and fresh weight of the soybean seedlings were also measured. The field

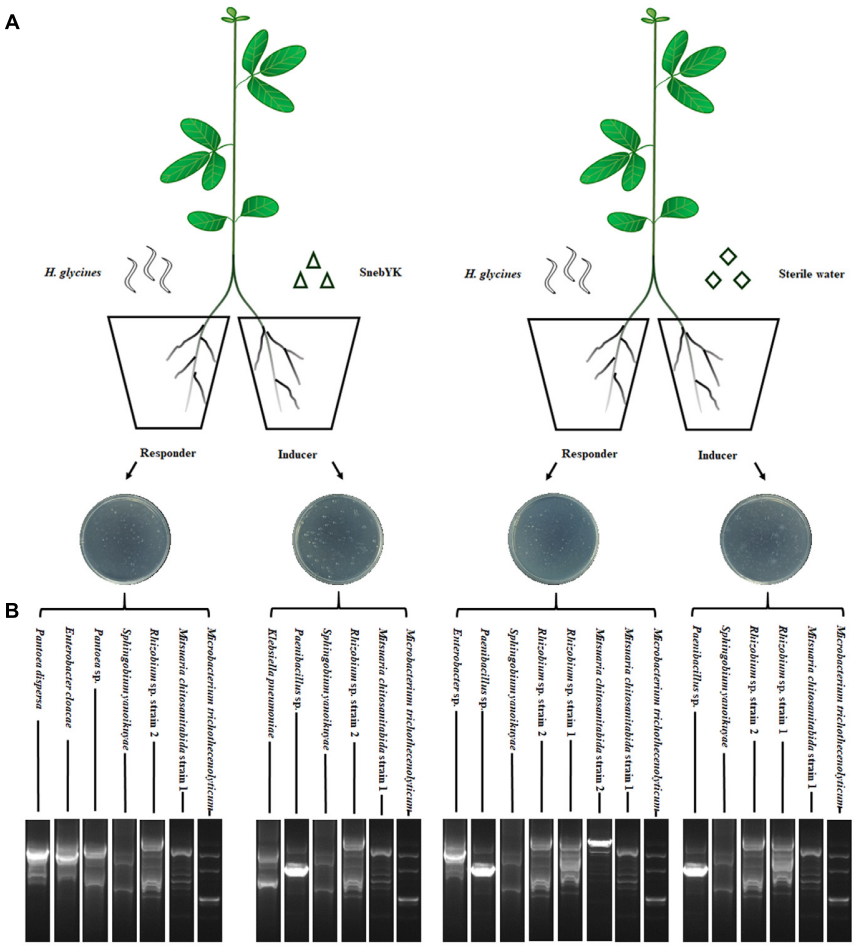


FIGURE 1 | Split root system used to study induced systemic resistance against *Heterodera glycines* in soybean. **(A)** SnebYK and *H. glycines* were inoculated into opposite pots containing the inducer and responder root systems, respectively. Controls were inoculated with sterile water. **(B)** BOX-PCR fingerprints and 16S rRNA identification of rhizosphere/endophytic bacteria in the split root system.

experiment was conducted in a fenced test field kept separate from animals and other crops.

Split Root Experiment

Soybean seeds were surface-sterilized as described and sown in pots containing the sterilized soil mixture, with one seed per pot. When soybean seedlings had two euphylla, the roots were removed from the mixture and washed thoroughly with running tap water. Roots of approximately the same size were selected and divided into two equal parts with a scalpel: one part was designated the inducer root system, and the other part was designated the responder root system (Adam et al., 2014). The inducer and responder roots were transplanted into two plastic pots, with 200 ml of sterilized mixture in each. When the two parts of the root systems had been successfully fixed, the inducer root system was inoculated either with SnebYK fermentation broth (10 ml, 1×10^9 CFU ml^{-1}) or with sterile water as a control. After 5 days of treatment, all responder roots were inoculated with 2,000 J2 of *H. glycines* as shown in **Figure 1A**. Fifteen days after nematode inoculation, the responder roots were stained with NaClO-acid fuchsin (Bybd et al., 1983). The number of *H. glycines* in the roots was counted, and the developmental stages of *H. glycines* were recorded simultaneously. Each treatment had four repetitions, and the entire experiment was repeated twice.

TABLE 1 | Plant growth-promoting properties of SnebYK.

Properties	Activity[a]	Concentration[b]
Inorganic phosphate solubilization	+	0.14 ± 0.00
Organic phosphate solubilization	–	–
Ammonia production	+	258.39 ± 35.13
Siderophore production	+	49.83 ± 1.45
Nitrogen fixation	+	2.60 ± 0.13

[a] *Positive result (+); negative result (–).* [b] *The values are means ± SD (n = 3); –, no data available due to a lack of activity; nitrogen fixation ability is expressed as μmol C_2H_4/h/10^9 cells; other values are expressed as μg ml^{-1}.*

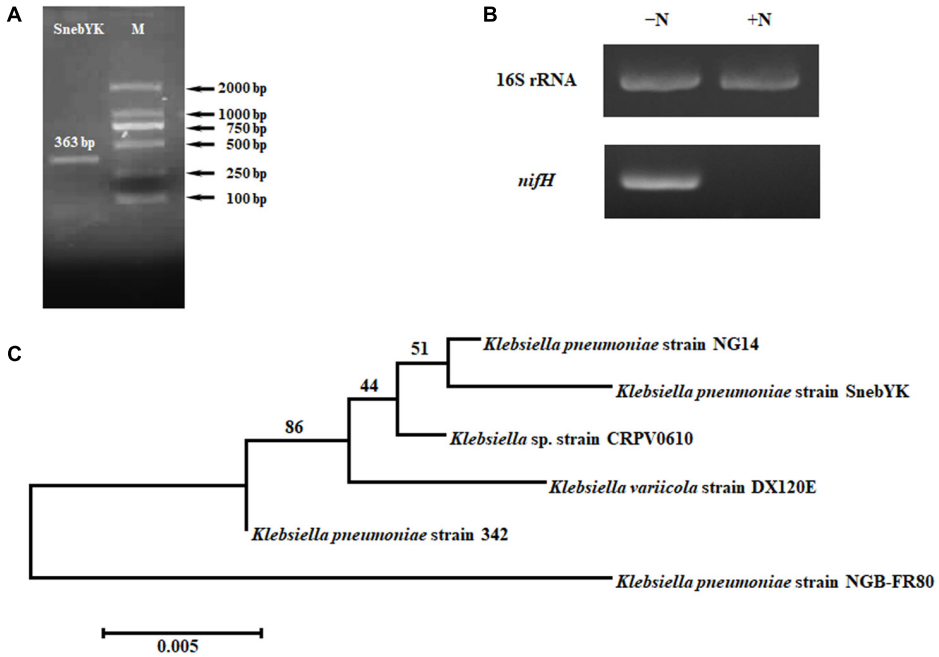


FIGURE 2 | Amplification and analysis of the *nifH* gene of SnebYK. **(A)** Amplicon of SnebYK *nifH* (363 bp) in the left lane and DNA ladder in the right lane. **(B)** Transcriptional analysis of the SnebYK *nifH* gene in nitrogen-free medium determined with RT-PCR. The lane labeled "−N" shows the expression of the 16S rRNA and *nifH* gene of SnebYK grown in ACCC55 nitrogen-free medium; the lane labeled "+N" shows the expression of the 16S rRNA and *nifH* gene of SnebYK grown in nutrient agar medium. The transcript level of the 16S rRNA was used as a loading control; the transcript level of *nifH* of SnebYK grown in nutrient agar medium was used as a negative control. **(C)** Dendrogram based on the *nifH* sequences of SnebYK and other strains in the genus *Klebsiella* with similar *nifH* sequences. This analysis was performed using the neighbor-joining method in Mega 7.0.26 with a bootstrap value of $n = 1000$.

To verify the validity of the split root system (SRS), BOX-PCR analysis and 16S rRNA gene sequencing were performed to test the colonization of the root system by SnebYK. Twenty days after the inducer roots were inoculated with SnebYK or sterile water, the responder and inducer root systems were removed. Immediately, 0.5 g of each root was collected and ground with a tissue grinder to release the rhizosphere and endophytic bacteria. A bacterial suspension was obtained after adding 1 ml of sterile water to the tissue grinder. After further dilution with sterile water (1,000×), 100 μl of the suspension was spread onto an ACCC55 agar plate and incubated at 28°C. Each treatment included three replications, and all steps were conducted under sterile conditions. After 72 h, morphologically distinct colonies of bacteria were selected and cultured separately. The genomic DNA of each strain was extracted with a bacterial genomic DNA extraction kit (Tiangen Biotech, China). The electrophoretograms of the BOX-PCR products were used to screen out different bacterial strains and could also be used to investigate the colonization of SnebYK by comparing them with the SnebYK electrophoretogram. The primer used for the BOX-PCR was BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic et al., 1994). The amplification was conducted following the method reported by Ateba and Mbewe (2013) in a 25 μl reaction mixture with the DNA samples obtained from the bacterial strains found in the SRS. Strains with different fingerprinting profiles were identified by 16S rRNA gene sequencing as described by Sarma and Saikia (2014).

RNA Isolation and Real-Time PCR Analysis of Gene Expression

Soybeans were treated with SnebYK and grown as previously described for the pot experiment. Sterilized water was used as control. When soybean seedlings reached the two-euphylla stage, their roots were inoculated with 2,000 J2 of *H. glycines*. To measure the transcript levels of defense-related genes in real time, soybean roots were collected at 0, 24, and 48 h after nematode inoculation, froze immediately in liquid nitrogen and stored at −80°C until use. Total RNA was extracted from the soybean roots using the MiniBEST Universal RNA Extraction kit (TaKaRa, China). Total RNA from each sample (1 μg) was reverse-transcribed using the PrimeScript RT Reagent kit (TaKaRa, China). Quantitative real-time PCR (qRT-PCR) was performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad, United States) using a SYBR green I quantitative PCR master mix (TaKaRa, China). The reaction conditions were 95°C for 30 s followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s (fluorescence measurement step). After 40 cycles, a melting-curve analysis was performed (15 s at 95°C, 30 s at 60°C, and 15 s at 95°C, fluorescence measurement step). Each sample included three replicates. Expression of the soybean gene *Actin11* was used as an internal standard for normalization, and the data were

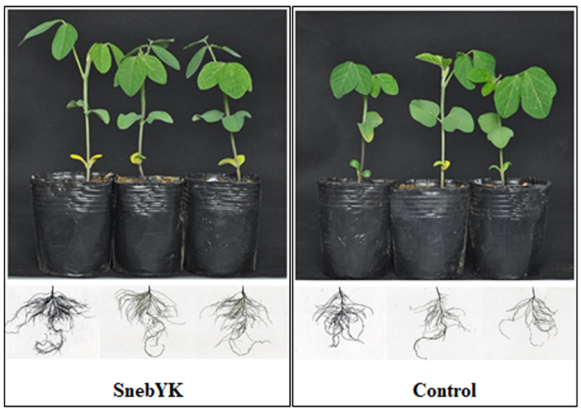


FIGURE 3 | Effect of SnebYK on the aboveground and belowground growth of soybean.

quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All data were analyzed using SPSS Statistics 19.0 (**RRID**: *SCR_002865*) and expressed as the mean ± standard deviation. Significant differences among treatments in the *in vitro* juvenile mortality assay were determined according to Duncan's multiple range test ($P < 0.05$). Significant differences between treatments in other experiments were evaluated using *t*-tests ($P < 0.05$).

RESULTS

Plant Growth-Promoting Properties of SnebYK

Klebsiella pneumoniae has been reported to possess many plant growth-promoting properties. Therefore, several such properties, including nitrogen fixation, ammonia production, phosphate solubilization, and siderophore production, were analyzed in SnebYK (**Table 1**). An orange halo zone was observed on the chrome azurol S agar plate, indicating that SnebYK produced siderophores. The ability of SnebYK to solubilize inorganic phosphate was confirmed by the clear zone around SnebYK on the National Botanical Research Institute's phosphate agar plate. However, nothing was observed on the Mongina agar plate, which indicates that SnebYK could not solubilize organic phosphate. SnebYK grown on the nitrogen-free medium showed high nitrogenase activity. The yellow color observed following the addition of Nessler's reagent to culture filtrate indicated that SnebYK could produce ammonia. A 363 bp amplicon specific to *nifH* (GenBank accession number: MF580385), a functioning gene encoding nitrogenase iron protein, was obtained from the genomic DNA of SnebYK (**Figure 2A**). The RT-PCR results showed that the *nifH* gene was expressed (1.24-fold the level of the internal control) when SnebYK was cultured under nitrogen-free conditions but was not expressed when SnebYK was cultured in a medium with sufficient nitrogen (**Figure 2B**). Phylogenetic analysis revealed that the *nifH* sequence of SnebYK was closely related to that of the *K. pneumoniae* strain NG14 and other strains in the genus *Klebsiella* (**Figure 2C**).

TABLE 2 | Effect of SnebYK on the growth of soybean seedlings.

Parameters	Treatments	
	SnebYK	Control
Shoot length (cm)	11.30 ± 0.31a	10.35 ± 0.27b
Taproot length (cm)	33.65 ± 2.69a	25.70 ± 3.25a
Total root length (cm)	1031.35 ± 43.52a	678.09 ± 53.43b
Total root surface area (cm^2)	194.91 ± 7.50a	131.94 ± 9.25b
Total root volume (cm^3)	2.94 ± 0.12a	2.06 ± 0.14b
Fresh shoot weight (g)	1.76 ± 0.07a	1.27 ± 0.05b
Fresh root weight (g)	3.36 ± 0.11a	2.40 ± 0.14b
Fresh seedling weight (g)	5.12 ± 0.15a	3.67 ± 0.17b
Root–shoot ratio	1.93 ± 0.08a	1.88 ± 0.08a

The values are means ± SD (n = 10). Different letters (a, b) following the values indicate significant differences between the treatments as determined by a t-test (P < 0.05).

Effect of SnebYK on the Growth of Soybean Seedlings

Since SnebYK exhibits a variety of growth-promoting properties, the effect of SnebYK on soybean growth was evaluated. SnebYK-treated soybean plants had well-developed second compound leaves and roots (**Figure 3**). The second compound leaves of untreated soybean were not fully expanded, and their roots were sparse. In the presence of SnebYK, soybean seedlings exhibited increased biomass (**Table 2**). SnebYK caused significant increases in the shoot length and fresh weight, which were 9.18% and 39.51% higher, respectively, than those in the control. However, SnebYK did not affect taproot length or the root–shoot ratio. SnebYK also had a considerable impact on root growth. SnebYK increased the total root length, total root surface area, and total root volume by 52.10%, 47.73%, and 42.72%, respectively, compared to those of the untreated control.

Effect of SnebYK on the Control of *H. glycines* in the Pot and the Field

SnebYK had low contact toxicity to *H. glycines*, as the mortality rate after 24 h was only 18.23% (**Figure 4A**). To evaluate whether coating seeds with SnebYK provided protection against *H. glycines*, seeds were sown in pots after coating and then the soybeans were inoculated with *H. glycines*. The number of females significantly influences the nematode population. Thus, we evaluated the effect of SnebYK on the control of both the adult female and the total population of *H. glycines*. Thirty days after *H. glycines* inoculation, juveniles and adults, especially adult females, in the soil and the soybean roots were counted. The nematode population of SnebYK-treated soybean was significantly smaller than that of the control, with inhibition rates reaching 47.32% (**Figure 4B**). Analysis of the population revealed that SnebYK dramatically decreased the

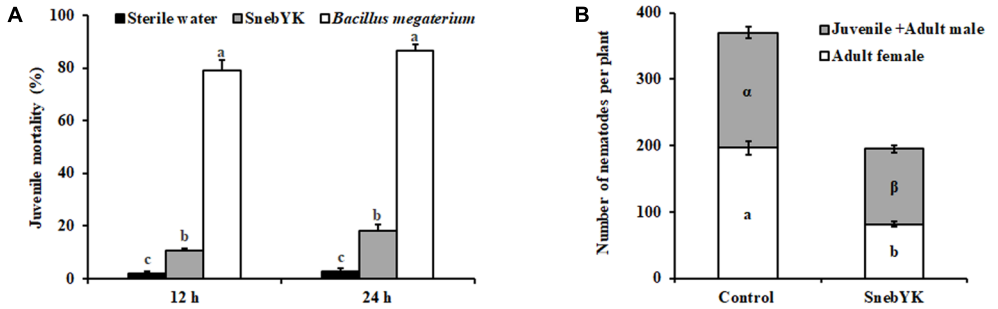


FIGURE 4 | Effects of SnebYK on the control of *Heterodera glycines in vitro* and in the potting experiment. **(A)** *In vitro* juvenile mortality of *H. glycines* at 12 and 24 h after treatment with culture filtrates of SnebYK. Sterile water was used as a negative control; *Bacillus megaterium* was used as a positive control. The error bars represent standard deviations, and different letters (a, b) above the bars indicate significant differences at $P < 0.05$ according to Duncan's multiple range test ($n = 3$). **(B)** Effect of SnebYK on the control of *H. glycines* in pots following seed coating. The numbers of *H. glycines* in the roots and soil were counted 30 days after inoculation. Different letters (a, b or α, β) indicate statistically significant differences (*t*-test with $P < 0.05$); a or b indicates a significant difference in the number of adult females, and α or β indicates a significant difference in the number of juveniles + adult males between treatments.

numbers of both juveniles and adult males, as well as adult females. Interestingly, the proportion of adult females in the *H. glycines* population was 40.88% (±4.77%) after treatment with SnebYK, while that in the control was 52.42% (±2.19%). This indicates that SnebYK significantly decreased the proportion of adult females, demonstrating that SnebYK effectively restrained the population of *H. glycines* and prevented their development into females. In the presence of *H. glycines*, SnebYK-treated soybean maintained a higher fresh root weight than the untreated control, concomitantly with a significantly increased root–shoot ratio (**Table 3**). After analysis of the root system, we found that the total root volume of SnebYK-treated soybean was significantly higher, by 28.74%, than that in the control (**Table 3**).

To determine the effect of SnebYK on the control of *H. glycines* in the field, experiments were conducted at the experimental test site in Kangping. We observed that SnebYK effectively reduced the number of *H. glycines* in per root by 53.23% and 33.75% in 2016 and 2017, respectively (**Figure 5A**). The 2016 data showed that SnebYK did not reduce the number of adult females on the soybean roots but did reduce their abundance in the soil by 40.11%. The 2017 data showed that SnebYK greatly reduced the number of adult females both on the roots and in the soil (**Figures 5B,C**). **Figures 5D–F** summarize the effect of SnebYK on soybean growth under *H. glycines* infection in the field trials. The results indicated that, although SnebYK-treated soybeans had a higher fresh weight than that of the untreated soybeans, the difference was not significant.

TABLE 3 | Effects of SnebYK on the growth of soybean seedlings in the presence of *Heterodera glycines*.

Parameters	Treatments	
	SnebYK	Control
Shoot length (cm)	30.71 ± 1.16a	32.29 ± 1.83a
Taproot length (cm)	25.14 ± 1.74a	24.36 ± 2.29a
Total root length (cm)	581.31 ± 40.22a	480.42 ± 51.22a
Total root surface area (cm^2)	90.46 ± 6.85a	72.09 ± 6.01a
Total root volume (cm^3)	1.12 ± 0.10a	0.87 ± 0.05b
Fresh shoot weight (g)	2.10 ± 0.14a	2.21 ± 0.13a
Fresh root weight (g)	1.58 ± 0.12a	1.27 ± 0.06b
Fresh seedling weight (g)	3.68 ± 0.22a	3.48 ± 0.18a
Root–shoot ratio	0.76 ± 0.05a	0.58 ± 0.03b

The values are means ± SD (n = 7). Different letters (a, b) following the values indicate significant differences between the treatments as determined by a t-test (P < 0.05).

Effect of SnebYK on Inducing Systemic Resistance to *H. glycines* in Soybean

While SnebYK resulted in low *H. glycines* mortality in the *in vitro* assay, it exhibited the capacity to control *H. glycines* in both the potting and field experiments when used to coat soybean seeds. Therefore, we conducted experiments to verify whether SnebYK could elicit systemic resistance in soybean against *H. glycines*. The SRS is considered an effective tool for demonstrating induced resistance, and in this study, we confirmed the validity of the SRS before using it. Rhizosphere and endophytic bacteria from the SRS were cultured on ACCC55 agar plates. After 1 day, several bacterial colonies (with morphologies similar to that of SnebYK) were observed on only the agar plate that was inoculated with the bacteria from the inducer root treated with SnebYK. More bacterial colonies appeared after 2 days. When comparing the BOX-PCR fingerprints and 16S rRNA identifications of rhizosphere and endophytic bacteria from the SRS, we found *K. pneumoniae* in only the inducer roots treated with SnebYK (**Figure 1B**). Since the responder roots were not colonized by SnebYK, the SRS used in this experiment was valid. Next, we conducted a split root experiment to determine whether SnebYK elicited ISR to *H. glycines* in soybean. Penetration of the responder roots of the SRS, assessed 15 days after *H. glycines* inoculation, was significantly reduced when the inducer roots of the soybean were treated with SnebYK. This treatment caused a 61.78% reduction in the total nematode penetration when compared with that in the untreated control. Specifically, the numbers of J2, third-stage juveniles (J3), and fourth-stage juveniles (J4)

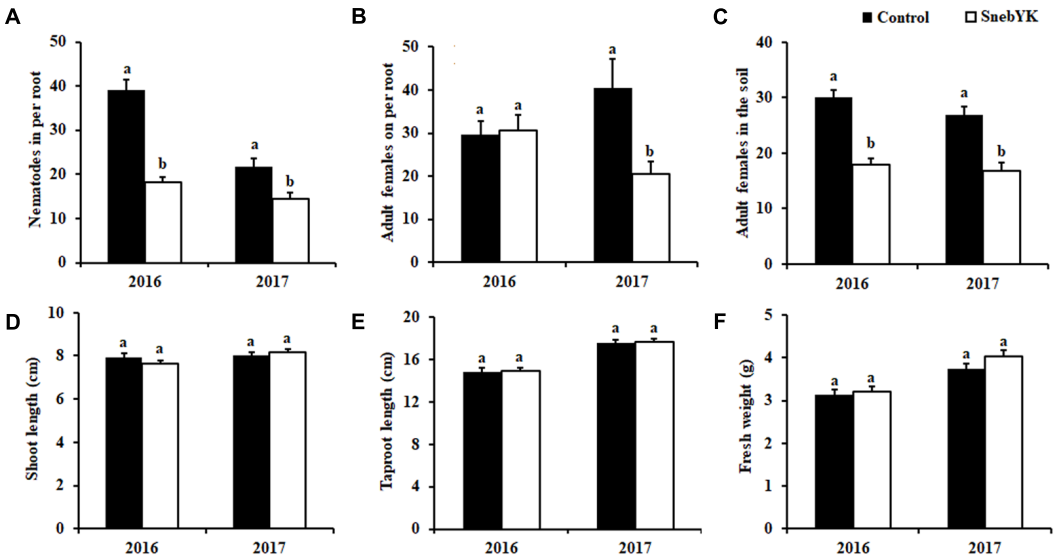


FIGURE 5 | Effects of SnebYK on the control of *Heterodera glycines* and on soybean growth in the field following soybean seed coating. **(A)** Effect of SnebYK on the control of *H. glycines* in soybean roots. **(B)** Effect of SnebYK on the control of adult females of *H. glycines* on soybean roots. **(C)** Effect of SnebYK on the control of adult females of *H. glycines* in 100 ml rhizosphere soil. **(D)** Effect of SnebYK on the shoot length of soybean infested with *H. glycines* in the field. **(E)** Effect of SnebYK on the taproot length of soybean infested with *H. glycines* in the field. **(F)** Effect of SnebYK on the fresh weight of soybean infested with *H. glycines* in the field. Error bars represent standard deviations, and different letters (a, b) above the bars indicate significant differences at $P < 0.05$ according to a *t*-test ($n = 60$).

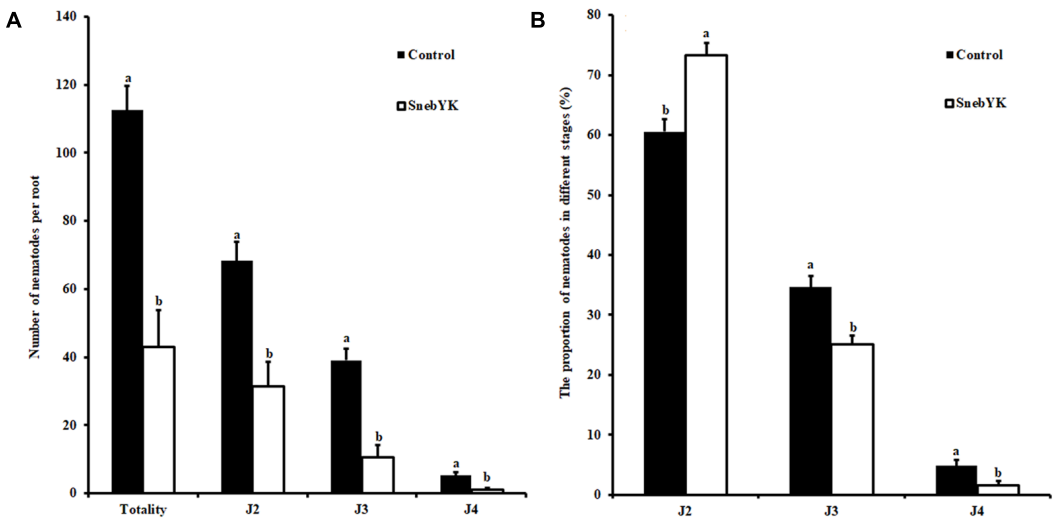


FIGURE 6 | Effect of SnebYK on inducing systemic resistance to *Heterodera glycines* using a split root system in soybean. **(A)** Effect of SnebYK on the penetration and development of *H. glycines* in the responder root system. **(B)** Effect of SnebYK on the proportion of each developmental stage of *H. glycines* in the responder root system. Numbers of *H. glycines* in responder roots were counted 15 days after inoculation. J2, J3, and J4 indicate second-, third-, and fourth- stage juveniles of *H. glycines*, respectively. Error bars represent standard deviations, and different letters (a, b) above the bars indicate significant differences at $P < 0.05$ according to a *t*-test ($n = 4$).

were significantly lower in the responder roots than in the control group (**Figure 6A**). The proportion of *H. glycines* juveniles of each stage in the responder roots is summarized in **Figure 6B**. The proportions of J2, J3, and J4 in the control group were 60.59%, 34.62%, and 4.8%, respectively. Meanwhile, the proportions in the SnebYK-treated group were 73.27%, 25.1%, and 1.63%, respectively. Therefore, inoculation with SnebYK significantly increased the proportion of J2 by 12.68% and decreased the proportions of J3 and J4 by 9.52% and 3.17%, respectively.

To investigate whether SnebYK-mediated ISR was accompanied by primed defense responses to *H. glycines* in the roots, the expression levels of *PR1*, *PR2*, *PR3*, *PR5*, *PR9*, *PR10*, *PDF1.2*, *NPR1-1*, and *NPR1-2* in soybeans treated or not treated

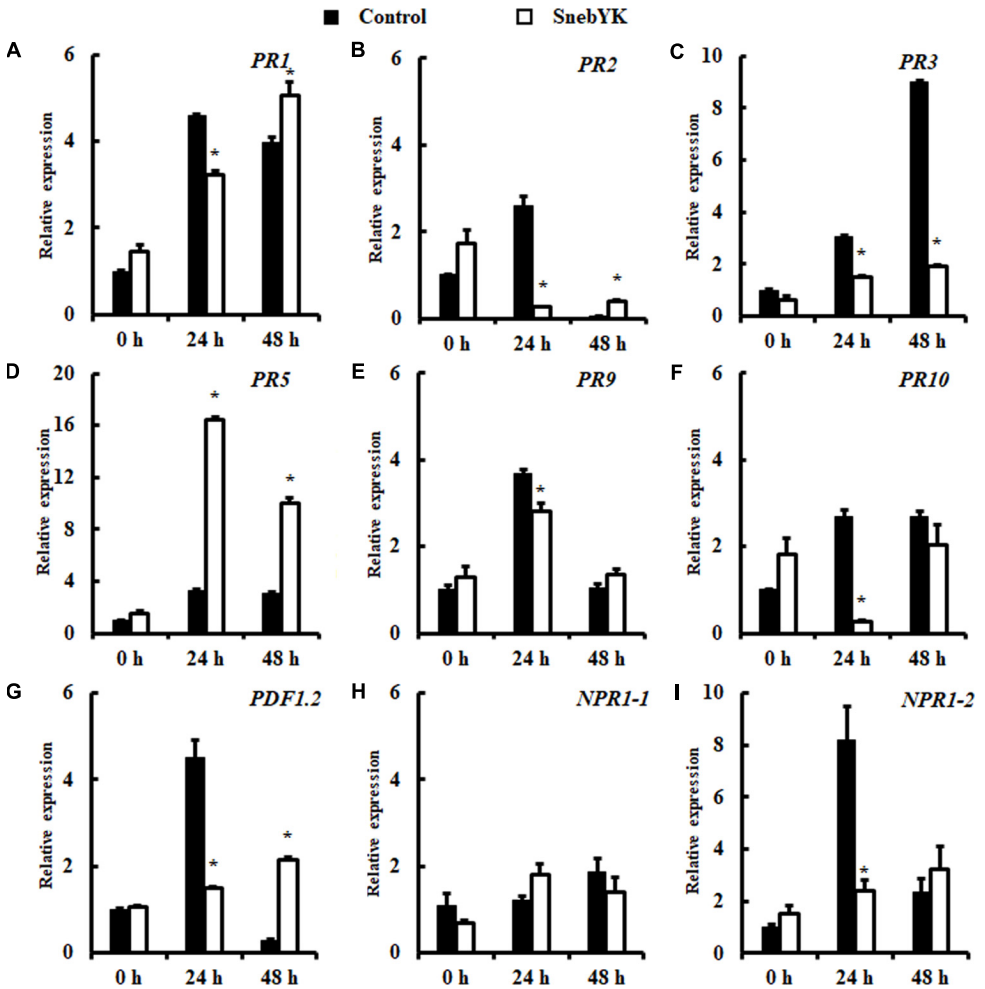


FIGURE 7 | Induction of defense genes in soybean roots after *Heterodera glycines* inoculation. Soybean root samples were taken at 0, 24, and 48 h after *H. glycines* inoculation, and the expression levels of nine defense genes were analyzed by qRT-PCR: **(A)** *PR1*, **(B)** *PR2*, **(C)** *PR3*, **(D)** *PR5*, **(E)** *PR9*, **(F)** *PR10*, **(G)** *PDF1.2*, **(H)** *NPR1-1*, and **(I)** *NPR1-2*. Error bars represent standard deviations; a *t*-test was used to determine significant differences between the SnebYK-treated sample and the control (*$P < 0.05$).

with SnebYK were assessed using qRT-PCR. Transcript levels of the defense genes analyzed did not differ in the non-challenged soybeans regardless of the presence of SnebYK. After *H. glycines* inoculation, elevated *PR5* expression was detected in control roots, indicating that soybean responds rapidly to *H. glycines* infection. Moreover, transcription of *PR5* in soybeans pretreated with SnebYK was 5.04-fold and 3.23-fold higher at 24 and 48 h after inoculation, respectively, than those in control soybeans. Differences were also found in the expression levels of *PR1*, *PR2*, *PR3*, *PR9*, *PR10*, *PDF1.2,* and *NPR1-2* between untreated soybeans and soybeans pretreated with SnebYK upon challenge with *H. glycines*. Twenty-four hours after *H. glycines* inoculation, these genes were induced in control soybeans, whereas the increase upon *H. glycines* challenge was lower in soybeans previously treated with SnebYK. However, 48 h after inoculation of *H. glycines*, the expression levels of *PR1*, *PR2* and *PDF1.2* were significantly higher in soybeans pretreated with SnebYK than in control soybeans; the transcript levels of *PR9*, *PR10* and *NPR1-2* did not differ significantly between the treatments; and the expression of *PR3* was much lower in SnebYK-treated soybean roots than in untreated soybean roots (**Figure 7**). The transcript levels of *NPR1-1* did not differ between soybeans pretreated with SnebYK and soybeans not treated with SnebYK following the inoculation of *H. glycines*.

DISCUSSION

Soybean is an important legume, and its production is greatly threatened by *H. glycines*. PGPB provide one of the most efficient control strategies in modern agriculture. In this study, the effect of SnebYK on the control of nematodes was evaluated. Data collected in potting experiment demonstrated that the population of *H. glycines* in SnebYK-treated soybean roots decreased by 47.32%; concomitantly, the number of adult females was reduced by 58.56% compared to that in the control. The number of

female nematodes has an important effect on population size (Triantaphyllou, 1973) and was therefore a focus of this study. The results of the field trials also confirmed the ability of SnebYK to control *H. glycines*. By coating soybean seeds, SnebYK not only reduced the number of *H. glycines* in the soybean roots but also suppressed the number of adult females. Our data showed that SnebYK could effectively delay the development of juvenile nematodes into adult females, hindering mating and reproduction and thereby decreasing the abundance of *H. glycines*. These results are consistent with previous reports of PGPB and other beneficial microbes (Martinuz et al., 2013; Séry et al., 2016; De Medeiros et al., 2017; Kumari, 2017; Zhou et al., 2017). However, we found that SnebYK exhibited only a weak ability to kill *H. glycines in vitro*. Therefore, we speculated that SnebYK prevented *H. glycines* by inducing resistance.

Although some studies have demonstrated that *K. pneumoniae* can elicit ISR to pathogenic fungi and phytotoxicity in plants (Luo, 2010; Ji et al., 2014), these studies employed seed treatment. By establishing a split root experiment, we gained better insight into *K. pneumoniae*-elicited induced resistance. In the present study, we designed experiments to confirm that no colonization of SnebYK was present in the responder root system, to guarantee that bioprotection occurred when SnebYK and *H. glycines* were spatially separated. Indeed, SnebYK in the inducer roots reduced the *H. glycines* population in the responder roots, which clearly demonstrates that SnebYK-mediated bioprotection was at least partially systemically induced. The results of the split root experiment showed that SnebYK effectively triggered ISR in soybean against *H. glycines* and resulted in significantly reduced penetration. Similar results have been observed in *R. etli* (Martinuz et al., 2012), *B. subtilis* (Adam et al., 2014), and *S. fredii* (Tian et al., 2014) in defense against nematodes. In the split root experiment, the proportion of J2 *H. glycines* in SnebYK-treated soybean was significantly higher than that in the untreated control; correspondingly, the proportions of J3 and J4 decreased substantially. This result suggests that SnebYK plays a prominent role in delaying the development of *H. glycines* via ISR. Martinuz et al. (2013) reported similar results following *R. etli* G12 treatment, which reduced *M. incognita* penetration, delayed J2 development to J3, and reduced the numbers of females and eggs. Plants accumulate pathogenesis-related (PR) proteins in response to various pathogens, such as fungi, bacteria, viruses, insects, and nematodes (Kitajima and Sato, 1999; Van Loon et al., 2006). During *H. schachtii* infection of *Arabidopsis*, the expression of *PR1*, *PR2*, and *PR5* was induced, and that of *PR3* and *PR4* was not altered in roots (Hamamouch et al., 2011). Non-expressor of pathogenesis related genes-1 (NPR1) is a transcriptional co-activator of *PR* gene expression (Dong, 2004), and transgenic tobacco plants constitutively expressing *AtNPR1* exhibited resistance to *M. incognita* (Priya et al., 2011). In our study, SnebYK-mediated resistance to *H. glycines* was also accompanied by boosts in the expression of defense genes. Forty-eight hours after inoculation with *H. glycines*, the transcript levels of *PR1*, *PR2*, *PR5,* and *PDF1.2* increased in SnebYK-pretreated soybeans compared with those in non-pretreated soybeans; however, the expression of *PR3* decreased. Plant resistance to pathogens involves the plant hormones SA, JA, and ET. *PR1*, *PR2,* and *PR5* are considered to be markers of SA-dependent signaling pathways (Delaney et al., 1994; Penninckx et al., 1996; Thomma et al., 1998), while *PR3*, *PR4,* and *PDF1.2* are marker genes for JA/ET-dependent signaling pathways (Penninckx et al., 1998; Thomma et al., 1998). The *PR* genes with significant expression differences in the present study are all marker genes in the SA or JA/ET signaling pathways, including *PR1*, *PR2*, *PR3*, *PR5,* and *PDF1.2*. ISR often relies on pathways regulated by JA/ET (Van Oosten et al., 2008; Pieterse et al., 2009). However, dependence on both SA and JA/ET signaling pathways has also been reported (Niu et al., 2011; Salas-Marina et al., 2011). Our results implied that SnebYK might also activate SA and JA/ET signaling pathways to induce plant defense against *H. glycines*.

Treatment of soybean seeds with SnebYK significantly improved soybean seedling growth, especially that of the root. Several mechanisms may contribute to the increase in biomass. SnebYK carries the nitrogen-fixing gene *nifH* and expresses it under nitrogen-deficient conditions. SnebYK fixes atmospheric nitrogen, as shown by its ability to reduce acetylene. Non-symbiotic N_2-fixing bacteria interact with plants to provide available nitrogen, increasing their vegetative growth and yield (Elkoca et al., 2010; Hayat et al., 2010). *Klebsiella pneumoniae* 342 is well known for its nitrogen-fixing capabilities. When *K. pneumoniae* 342 was applied to wheat, the dry weight of the plant increased; when the *nifH* gene of this strain was knocked out, the growth-promoting effect disappeared (Iniguez et al., 2004). Several experiments have examined the effects of *K. pneumoniae*, as a non-symbiotic N_2-fixing bacterium, on the growth of monocotyledonous plants, such as rice (Ji et al., 2014; Banik et al., 2016), maize (Kuan et al., 2016), and wheat (Remus et al., 2000). However, few data are available on the effects of *K. pneumoniae* on the dicotyledonous plants growth. Our results demonstrated that SnebYK exhibited growth-promoting effects on the dicotyledonous soybean. Soybean roots treated with SnebYK were larger than those of the controls. PGPB affect root morphology, particularly by increasing root surface area (Larraburu et al., 2007; Wang et al., 2015) and weight (Islam et al., 2016; Singh et al., 2017), thereby improving nutrient uptake. This mechanism is even more important than nitrogen fixation in some PGPB (Vessey, 2003). SnebYK produces large amounts of ammonia, which may play an important role in the suppression of nematode populations (Rodriguez-Kabana, 1986; Khan et al., 2012). Phosphorus is also a key nutrient element for plants. SnebYK can convert tricalcium phosphate to a form that is accessible to the host. SnebYK also produces siderophores, which block the proliferation of pathogenic microorganisms by chelating Fe^{3+} and act as one of the major determinants of ISR (Hiifte et al., 1994; Ramamoorthy et al., 2001). The main signaling steps during the development of ISR require gene expression and consume resources (Buell, 1999; Heil, 2001), and SnebYK treatment activated the expression of *PR* genes under *H. glycines* infection. Allocation of limited plant resources to defense, means they cannot be used for plant growth (Herms and Mattson, 1992; Heil, 2001). Our data indicated that SnebYK fixes nitrogen and

solubilizes inorganic phosphate, implying that SnebYK might provide nutrition to plants. This additional nutrition might be a reason that SnebYK-treated soybean exhibited a higher biomass and resistance to *H. glycines* at the same time. Further experiments are required to explore the regulatory mechanism in detail.

CONCLUSION

Our results support a role for the PGPB strain *K. pneumoniae* SnebYK in the induction of ISR against *H. glycines* in soybean. This study is the first report of *K. pneumoniae* eliciting ISR against *H. glycines*. SnebYK showed an ability to inhibit both the invasion and the development of nematodes. It induced the expression of defense genes in soybean following *H. glycines* infection and suppressed *H. glycines* in the field after coating soybean seeds, suggesting this may be an efficient and economical method to control *H. glycines*. SnebYK also presented remarkable plant growth-promoting properties, which promoted the growth of soybean. The overall results of the present study support *K. pneumoniae* SnebYK as a potential biocontrol agent for *H. glycines*.

AUTHOR CONTRIBUTIONS

DL, LC, and YD conceived and designed the research. DL, LC, XZ, YW, and YX conducted all experiments and data analyses. The manuscript was written by DL and YD. XL and LJC provided the instruments and critically revised the manuscript. YD provided the final approval of the version to be published. All authors read and approved the final manuscript.

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Chitosan Increases Tomato Root Colonization by *Pochonia chlamydosporia* and Their Combination Reduces Root-Knot Nematode Damage

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The use of biological control agents could be a non-chemical alternative for management of *Meloidogyne* spp. [root-knot nematodes (RKN)], the most damaging plant-parasitic nematodes for horticultural crops worldwide. *Pochonia chlamydosporia* is a fungal parasite of RKN eggs that can colonize endophytically roots of several cultivated plant species, but in field applications the fungus shows a low persistence and efficiency in RKN management. The combined use of *P. chlamydosporia* with an enhancer could help its ability to develop in soil and colonize roots, thereby increasing its efficiency against nematodes. Previous work has shown that chitosan enhances *P. chlamydosporia* sporulation and production of extracellular enzymes, as well as nematode egg parasitism in laboratory bioassays. This work shows that chitosan at low concentrations (up to 0.1 mg ml^{-1}) do not affect the viability and germination of *P. chlamydosporia* chlamydospores and improves mycelial growth respect to treatments without chitosan. Tomato plants irrigated with chitosan (same dose limit) increased root weight and length after 30 days. Chitosan irrigation increased dry shoot and fresh root weight of tomato plants inoculated with *Meloidogyne javanica*, root length when they were inoculated with *P. chlamydosporia*, and dry shoot weight of plants inoculated with both *P. chlamydosporia* and *M. javanica*. Chitosan irrigation significantly enhanced root colonization by *P. chlamydosporia*, but neither nematode infection per plant nor fungal egg parasitism was affected. Tomato plants cultivated in a mid-suppressive (29.3 ± 4.7% RKN egg infection) non-sterilized clay loam soil and irrigated with chitosan had enhanced shoot growth, reduced RKN multiplication, and disease severity. Chitosan irrigation in a highly suppressive (73.7 ± 2.6% RKN egg infection) sterilized-sandy loam soil reduced RKN multiplication in tomato. However, chitosan did not affect disease severity or plant growth irrespective of soil sterilization. Chitosan, at an adequate dose, can be a potential tool for sustainable management of RKN.

Keywords: endophytic colonization, nematophagous fungi, root-knot nematodes, suppressive soil, *Solanum lycopersicum*

INTRODUCTION

Plant-parasitic nematodes are a major problem for agriculture, causing crop losses of ca. $157 billion yearly (Abad et al., 2008). Among these, root-knot nematodes (RKN, *Meloidogyne* spp.) are the most damaging for vegetable crops. *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* are the most widely distributed species affecting horticultural crops worldwide (Sikora and Fernández, 2005). Management of RKN has been mainly based on the use of chemical nematicides (Talavera et al., 2012), but these have been banned or restricted due to their harmful effects on the environment, as well as on wildlife and human health. Consequently, there is an urgent need for environmentally friendly and effective alternatives for RKN management, such as the use of biocontrol agents. To this respect, nematophagous fungi represent the most diverse group of nematode antagonists (Stirling, 2014).

Pochonia chlamydosporia Goddard (Zare and Gams; syn. *Metacordyceps chlamydosporia*) is a fungal parasite of nematodes eggs (Kerry and Hirsch, 2011). This fungus is a key component of soils suppressive to cyst (Kerry, 1982; Kerry et al., 1984) and RKN (Bent et al., 2008; Giné et al., 2016). *P. chlamydosporia* has been found to be able to colonize endophytically roots of *Arabidopsis* (Zavala-Gonzalez et al., 2016), barley (Monfort et al., 2005; Maciá-Vicente et al., 2009), potato (Manzanilla-Lopez et al., 2011), and tomato (Bordallo et al., 2002). Some isolates can also induce plant growth and reduce flowering time in tomato (Zavala-Gonzalez et al., 2015). The fungus can elicit plant defense mechanisms (Larriba et al., 2015). Jasmonate modulates root colonization (Zavala-Gonzalez et al., 2016) and influences the biocontrol capacity of *P. chlamydosporia* (Vieira Dos Santos et al., 2014). However, the presence of the fungus in roots decreases with time after inoculation (Maciá-Vicente et al., 2009; Escudero and Lopez-Llorca, 2012). Consequently, the ability of the fungus to control RKN populations could also be compromised because RKN are embedded in roots or near the root surface.

Previous studies show that a single application of *P. chlamydosporia* at a rate of 5000 chlamydospores per gram of soil can reduce damage by *M. incognita* on tomato growing in pot experiments (Bourne and Kerry, 1999; Atkins et al., 2003; Yang et al., 2012). In contrast, multiple applications of the fungus up to 1.2 10^7 chlamydospores per plant were necessary to reduce RKN damage in the field (Sorribas et al., 2003). Attempts to increase the efficacy of RKN management in field conditions combining the application of *P. chlamydosporia* with the nematicides oxamyl (Tzortzakakis, 2000; Verdejo-Lucas et al., 2003) or fosthiazate (Tobin et al., 2008) were not successful. In this work, we test *P. chlamydosporia* combined with a 70 kDa chitosan for RKN management. Chitosan is a deacetylated and soluble form of chitin that is not toxic to plants, animals, and mammalian cells (Lopez-Moya et al., 2015). Chitosan is also biodegradable and environmentally safe (Kumar, 2000). Chitin and chitosan elicit plant defenses inhibitory to pathogenic fungi and bacteria (Chittenden and Singh, 2009; López-Mondéjar et al., 2012). Chitosan reduces RKN infection and disease severity on tomato (Khalil and Badawy, 2012). Chitosan increases *P. chlamydosporia* sporulation (Palma-Guerrero et al., 2008) and expression of VCP1, the main serine protease (Palma-Guerrero et al., 2010a) used by the fungus to parasitize nematode eggs (Escudero et al., 2016). Consequently, chitosan increases *M. javanica* egg parasitism by *P. chlamydosporia* in laboratory bioassays (Escudero et al., 2016). Thus, chitosan at an adequate dose can be a promising candidate to safely enhance effectiveness of *P. chlamydosporia* against RKN. Thus, the aim of this work was to determine the effect of chitosan on: (i) viability and germination of *P. chlamydosporia* chlamydospores; (ii) tomato plant growth; (iii) the tritrophic interaction tomato–*M. javanica*–*P. chlamydosporia* in micropots; and (iv) RKN multiplication in pot experiments using soils in which *P. chlamydosporia* occurs naturally. Our final goal was to define a concentration for chitosan irrigation compatible with tomato and *P. chlamydosporia* development for sustainable management of RKN.

MATERIALS AND METHODS

Fungal Isolate, Root-Knot Nematode Populations, Plant Materials, and Chitosan

The strain of *P. chlamydosporia* Pc123 (ATCC MYA-4875; CECT 20929) used in this study was isolated from infected *Heterodera avenae* eggs collected in SW Spain (Olivares-Bernabeu and Lopez-Llorca, 2002). The fungus was maintained in corn meal agar (CMA; Becton Dickinson and Company, United States) at 25°C in the dark for mycelial growth. For chlamydospore production, mycelial plugs from the edge of 21-day-old fungal colonies were transferred to Vogel's solid medium (1× Vogel's salts, 2% sucrose, and 1.5% technical agar) and incubated at 25°C. After 4 weeks, chlamydospores were then extracted following the Kerry and Bourne (2002) procedure.

Root-knot nematode populations belonging to *M. javanica* or *M. incognita* were used in micropot and pot experiments, respectively. The *M. javanica* population was isolated from infected carnation cultivated in Chipiona (S Spain). The *M. incognita* population was isolated from tomato cultivated in Viladecans (NE Spain). Both RKN populations were maintained on susceptible tomato cultivars to produce enough inoculum for experiments. Nematode eggs were extracted by macerating infected tomato roots in 0.5% (v/v) NaClO as in Hussey and Barker (1973). Eggs were placed in Baermann trays (Whitehead and Hemming, 1965) to obtain nematode juveniles (J2).

Susceptible tomato cv. Marglobe was used in micropot experiments. Seeds were surface-sterilized with 10% (v/v) NaClO, plated on Petri dishes with germination medium (Bordallo et al., 2002), and incubated for 1 day at 4°C to favor seed stratification, followed by 5 days at 25°C in the dark and 4 additional days at 16 h:8 h (light:dark) photoperiod. Afterward, tomato plantlets were transplanted individually in 150 ml sterile cylindrical micropot containing 70 cm^3 of sterilized sand. Susceptible

tomato cv. Durinta with three true developed leaves provided by Planters Rovira (Barcelona, Spain) was used in pot experiments.

Chitosan with a deacetylation degree of 80.5% and 70 kDa molecular weight was obtained from Marine BioProducts GmbH (Bremerhaven, Germany) and prepared as in Palma-Guerrero et al. (2008). Chitosan was dialyzed for salt removal against distilled water for laboratory and micropot experiments.

Effect of Chitosan on *P. chlamydosporia* Chlamydospores Viability and Mycelia Growth

Suspensions of 10^5 chlamydospore ml^{-1} including 1, 2, 3, or 4 mg ml^{-1} chitosan (final concentration) were used to assess the effect of chitosan on chlamydospore viability. The suspensions were incubated at room temperature for 4 h and then stained with 5 mg ml^{-1} propidium iodide (PI) which penetrates plasma membrane from dead cells labeling them red (nucleic acid staining), while living cells remain unstained. Fluorescence was recorded with a Leica TCS-SP2 laser-scanning confocal microscope, using 488 and 560 nm excitation and detection wavelengths, respectively (Oparka and Read, 1994; Hickey et al., 2005). Chlamydospore suspensions mixed with sterilized distilled water or hydrogen peroxide were used as negative and positive controls, respectively. Thirty chlamydospores were assessed per treatment.

Chlamydospore germination assays were carried out on 10-well microscope slides (Waldemar Knittel). Each well was filled with 2.5×10^4 chlamydospores and aliquots of chitosan solutions to reach a final concentration of either 0, 0.01, 0.05, 0.1, 0.5, 1, and 2 mg ml^{-1} in a final volume of 25 µl. Higher chitosan concentrations were discarded because chlamydospore viability was compromised. Slides were incubated in moist chambers at room temperature in the dark for 24 h. Percentage of germination in random samples of 200 chlamydospores per well was then scored in an Olympus BH-2 microscope. A chlamydospore was considered germinated when the germ tube length was 1.5 times the chlamydospore diameter (Plascencia-Jatomea et al., 2003). Three slides per treatment were scored and the experiment was carried out twice.

The effect of chitosan on mycelium developing from chlamydospores was carried out as described by Lopez-Moya et al. (2015). Two-hundred microliter aliquots of either 0, 0.01, 0.05, 0.075, 0.1, 1, or 2 mg ml^{-1} chitosan solutions (final concentration) mixed with 2.5 10^3 chlamydospores were dispensed in 96-well microtiter plates (Sterilin Ltd., Newport, United Kingdom). Growth was estimated daily for 8 days by measuring the optical density at 490 nm (OD_{490}) in a GENios (Tecan, Männedorf, Switzerland) spectrofluorometer. Each treatment was evaluated in four wells and the experiment was carried out three times.

Effect of *P. chlamydosporia* and Chitosan on Tomato Growth

Tomato cv. Marglobe plantlets in micropots containing sterilized sand were singly inoculated with four 5-mm-diameter plugs taken from the edge of a 20-day-old *P. chlamydosporia* colony grown on CMA. Corn meal agar plugs without fungus were used in non-inoculated plants. The fungal inoculum was placed 1 cm deep and mixed with the substrate as in Macia-Vicente et al. (2008). Plantlets were irrigated daily with a 0.1× Gamborg's B5 nutrient solution amended with final chitosan concentrations of either 0, 0.01, 0.05, 0.075, 0.1, and 0.3 mg ml^{-1}. Plantlets were randomly distributed in the growth chamber (Fitoclima 10000EHVP) and maintained at 25°C, 65% relative humidity, and 16 h:8 h (light:dark) photoperiod. Dry shoot weight (DSW), maximum shoot length (MSL), fresh root weight (FRW), and maximum root length (MRL) were scored per plant at 10, 20, and 30 days after fungal inoculation. Each fungus-chitosan concentration and sampling time combination was replicated 10 times, and the experiment was performed twice.

Effect of Chitosan on the Tritrophic Interaction Tomato–*M. javanica*–*P. chlamydosporia*

Tomato seeds were germinated, inoculated with *P. chlamydosporia* (Pc) and irrigated with 0.1 mg ml^{-1} chitosan (0.1chi) as described. Twenty-five-day-old plants were inoculated with two *M. javanica* J2 (RKN) per cm^3 of substrate. Treatments containing Pc were re-inoculated with 5000 chlamydospores g^{-1} substrate 30 dai (Kerry and Bourne, 2002). Micropots were placed randomly in a growth chamber (Fitoclima 10000EHVP) at 25°C, 65% relative humidity, and 16 h:8 h (light:dark) photoperiod. After 56 days, DSW, MSL, FRW, and MRL per plant were assessed. Egg masses of *M. javanica* per plant were counted after staining with 1% eosin yellowish hydroalcoholic solution (Panreac) (Roberts et al., 1990). The experiment consisted of four treatments: Tomato (To)+RKN, To+RKN+0.1chi, To+RKN+Pc, and To+RKN+ Pc +0.1chi, with 10 replicates each. The experiment was performed twice (80 plants in total).

Fungal egg parasitism was assessed as in Giné et al. (2013). Briefly, at the end of the experiment, 30 egg masses per treatment were handpicked from tomato roots and divided into six subsamples in 1000 µl sterile distilled water each. Eggs were dispersed from egg masses using a pestle, and 333 µl aliquots of the egg suspensions were spread onto Petri dishes containing a growth-restricting medium for *P. chlamydosporia* slightly modified from Lopez-Llorca and Duncan (1986) (50 µg ml^{-1} streptomycin, 50 µg ml^{-1} chloramphenicol, 50 µg ml^{-1} chlortetracycline, 50 µg ml^{-1} rose bengal, 0.5 % triton X-100, and 1.5% agar). Petri dishes were incubated at 25°C in the dark. The number of parasitized eggs was recorded 96 h after plating using a dissecting microscope. Eggs were considered parasitized when hyphae grew from inside. The percentage of parasitism was calculated as the proportion of the number of parasitized eggs respect to the total number of eggs plated.

Tomato root colonization by *P. chlamydosporia* was estimated using real time quantitative PCR (qPCR) as in Escudero and Lopez-Llorca (2012). Briefly, DNA was extracted independently from three tomato roots per treatment.

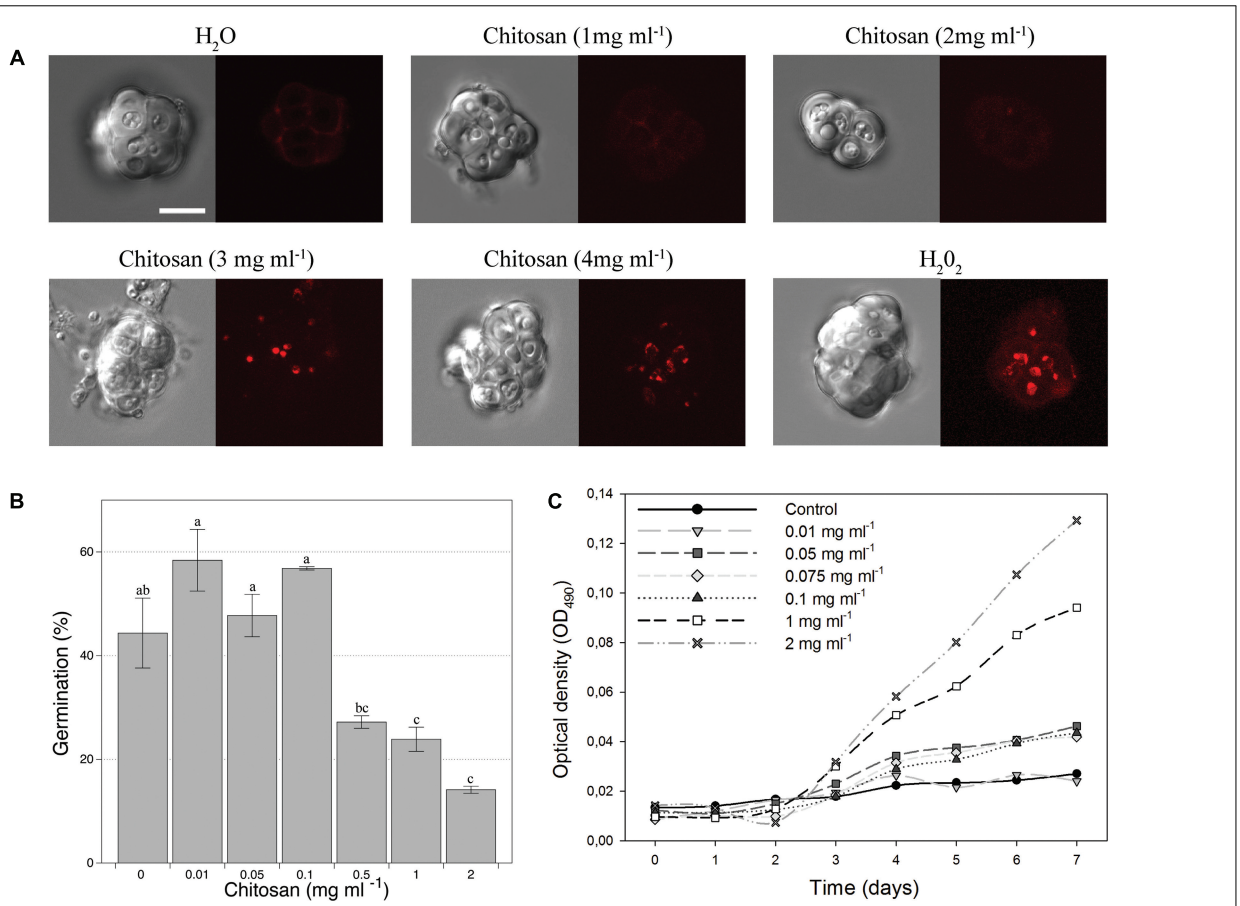


FIGURE 1 | Effect of chitosan on *Pochonia chlamydosporia* chlamydospores. **(A)** Viability. DIC images on the right and fluorescence images of the same chlamydospores on the left. Dead chlamydospores red stained with 5 μg ml^{-1} propidium iodide indicate full plasma membrane permeabilization (cell death). Controls: H_2O (negative), H_2O_2 (positive). Bar = 10 μm. **(B)** Germination. **(C)** Growth kinetics from chlamydospores as initial inoculum in chitosan as sole nutrient. Chitosan concentrations are shown in each graph and asterisks indicate significant differences (p-value < 0.05) vs. control (no chitosan).

Primers for *P. chlamydosporia* detection were VCP1q_F (5′–3′GCCATCGTTGAGCAGCAG) and VCP1q_R (5′–3′ACCGTGACCGTCGTTGTTCT). qPCR reactions were performed using the FastStart Universal SYBR Green Master (Roche) mix in a final volume of 10 µl, containing 100 ng of total DNA and 0.25 µM of each primer. Reactions were performed in triplicate in a Thermal Cycling StepOne Plus (Applied Biosystems) using the following thermal cycles: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Pc123 genomic DNA dilutions were used to define a calibration curve from 30 ng to 3 pg. After each run, a dissociation curve was acquired to check amplification specificity. Fungal DNA was referred to total DNA.

Effect of Chitosan on RKN Multiplication on Tomato in Soils in Which *P. chlamydosporia* Occurs Naturally

Chitosan at 0.1 mg ml^{-1} was applied weekly in a pot experiment using two agricultural soils from NE Spain, henceforth referred to as M10.41 and M10.56. *P. chlamydosporia* and other fungal egg parasites of RKN occur naturally in both soils (Giné et al., 2013). Soil from site M10.41 is clay loam (33% sand, 29% clay, and 38 silt); pH 8.2; electric conductivity 516 µS/cm; 4.4 organic matter (w/w) content, and 33% RKN eggs produced in zucchini–squash in July 2015 were parasitized by *P. chlamydosporia*. Soil from site M10.56 is sandy loam (53% sand, 18% clay, and 29 silt); pH 8.3; electric conductivity 415 µS/cm; 4.3 organic matter (w/w) content, and 70% RKN eggs produced on tomato in July 2015 were parasitized by *P. chlamydosporia*. Soil samples were taken in February 2016 and processed as in Giné et al. (2016). Each soil was sieved through a 4-mm mesh and split into two subsamples. A subsample from each soil was autoclave-sterilized for 1 h at 121°C. The sterilization process was repeated after 24 h. The remaining soil subsamples were stored at 4°C. Sterilized and non-sterilized soils were mixed with sterile sand (1:1; v:v) before being used as plant substrate in pots, to favor aeration and root development. In both soils, *Meloidogyne* sp. population densities were determined from two 500 cm^3 subsamples using Baermann trays after a week of incubation at 25°C. Tomato cv. Durinta

TABLE 1 | Effect of the concentration of chitosan alone applied weekly by irrigation or combined with *Pochonia chlamydosporia* (Pc) on dry shoot weight (DSW), maximum shoot length (MSL), fresh root weight (FRW), and maximum root length (MRL) of tomato plants after 30 days.

Chitosan concentration	DSW (g)		MSL (cm)		FRW (g)		MRL (cm)	
[mg ml^{-1}]	− Pc	+ Pc	− Pc	+ Pc	− Pc	+ Pc	− Pc	+ Pc
0	0.16 ± 0.01	0.19 ± 0.01	9.17 ± 0.44	10.45 ± 0.32*	0.92 ± 0.07	1.06 ± 0.09	10.47 ± 0.35	12.31 ± 0.87
0.01	0.16 ± 0.01	0.19 ± 0.01	7.89 ± 0.50	**7.88 ± 0.38**	1.12 ± 0.12	1.30 ± 0.04	12.57 ± 0.68	11.72 ± 0.82
0.05	**0.21 ± 0.01**	0.20 ± 0.01	8.04 ± 0.10	**8.17 ± 0.22**	**2.34 ± 0.09**	**1.37 ± 0.08**	12.13 ± 0.46	12.45 ± 0.42
0.075	0.18 ± 0.01	0.20 ± 0.01	8.25 ± 0.39	**8.44 ± 0.23**	1.07 ± 0.05	1.28 ± 0.08	**14.11 ± 0.59**	11.95 ± 0.43
0.1	0.17 ± 0.01	0.17 ± 0.01	8.02 ± 0.25	**7.75 ± 0.15**	**1.27 ± 0.09**	**1.42 ± 0.06**	**13.6 ± 0.96**	12.87 ± 0.82
0.3	**0.13 ± 0.01**	**0.08 ± 0.01**	7.41 ± 0.30	**6.85 ± 0.44**	0.84 ± 0.04	**0.50 ± 0.05**	10.56 ± 0.61	**8.24 ± 0.47**

Values are means ± SE (n = 10). Data in bold in the same column differ from 0 mg ml^{-1} (Dunnett's test; p-value < 0.05), asterisk indicates differences due to P. chlamydosporia inoculation for no-chitosan irrigation treatments (Student's t-test; p-value < 0.05).

plantlets with three true developed leaves were individually transplanted to 3 l pots and inoculated with *M. incognita* J2. *Meloidogyne* spp. J2 extracted from each non-sterilized soil were taken into account to inoculate a final amount of 3000 J2 per pot. The experiment included four treatments per agricultural soil: (i) sterile soil mixture, (ii) sterile soil mixture + 0.1 mg ml^{-1} chitosan irrigation, (iii) non-sterile soil mixture with no-chitosan irrigation, and (iv) non-sterile soil mixture + 0.1 mg ml^{-1} chitosan irrigation. Each treatment was replicated 10 times.

Plants were maintained in greenhouse conditions and irrigated as required. Those from the chitosan treatment were irrigated weekly with approximately 150 ml of 0.1 mg ml^{-1} chitosan per plant. The experiment was carried out in 2016, from April 19 to July 4. At the end of the experiment, plants were removed from pots and DSW and RFW were determined as described. Galling index was estimated by the 0–10 Zeck (1971) scale, where 0 is a non-galled root and 10 is a completely galled root system. RKN eggs were extracted from roots as in Hussey and Barker (1973). J2 were extracted from soil using Baermann trays and their number per plant scored. Percentage of fungal egg parasitism was assessed 24 and 48 h after plating.

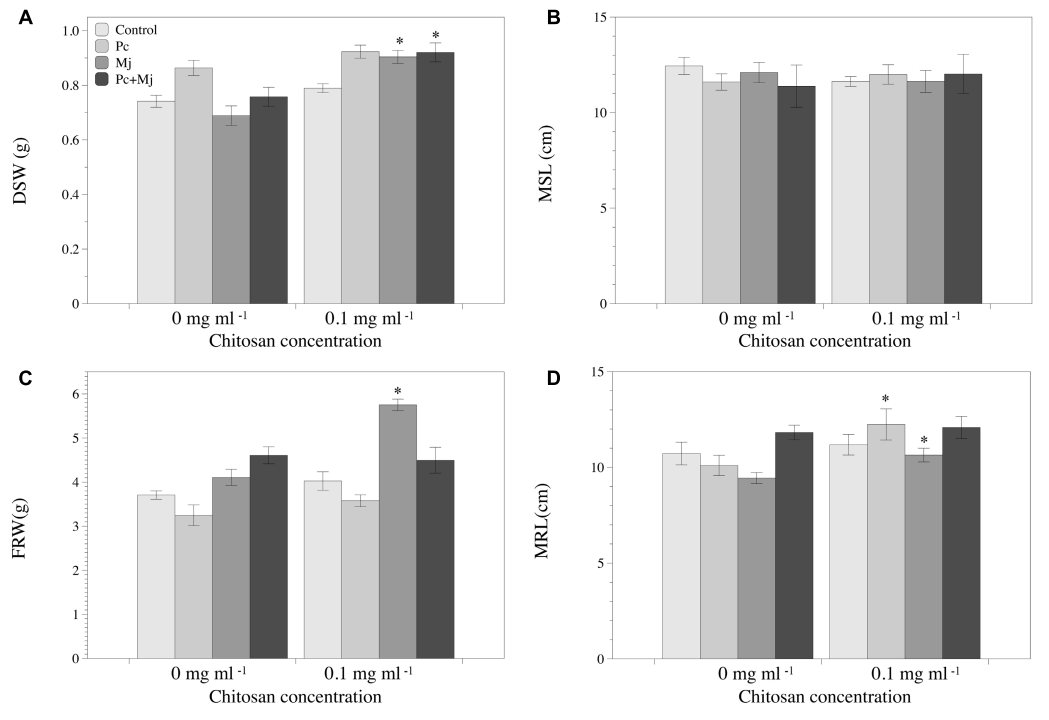


FIGURE 2 | Effect of *P. chlamydosporia*, *Meloidogyne javanica*, and chitosan on growth of tomato plantlets. **(A)** Dry shoot weight (DSW), **(B)** maximum shoot length (MSL), **(C)** fresh root weight (FRW), and **(D)** maximum root length (MRL). Asterisks indicate differences in the chitosan treatments respect to those without chitosan (p-value < 0.05). C, control (non-inoculated); Pc, plants inoculated with *P. chlamydosporia* only; Mj, plants inoculated with *M. javanica* only; and Pc+Mj, plants inoculated with both *P. chlamydosporia* and *M. javanica*.

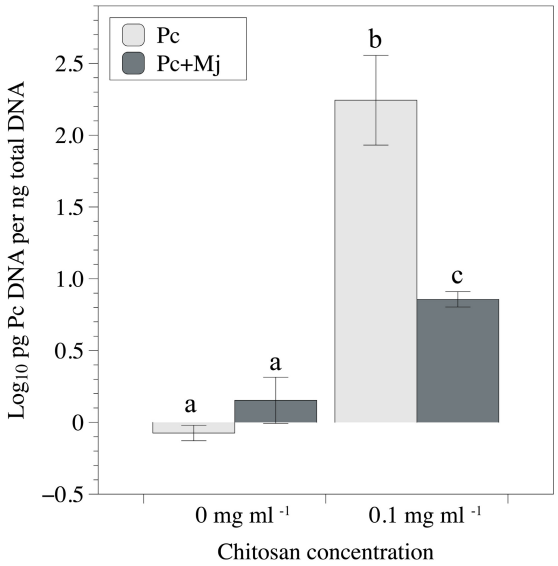


FIGURE 3 | Effect of chitosan irrigation and *M. javanica* inoculation on root colonization of tomato plants by *P. chlamydosporia* estimated by qPCR. Pc, plants inoculated with *P. chlamydosporia* only and Pc+Mj, plants inoculated with both *P. chlamydosporia* and *M. javanica*. Different letters indicate statistical differences (p-value < 0.05).

Statistical Analyses

Statistical analyses were performed in R (v. 3.1.2) (R Core Team, 2014). Variables were $\log_{10}(x + 1)$ or square root$(x + 0.5)$ transformed when required. Homoscedasticity was checked using Levene's test and normality using Shapiro–Wilk's test. Differences between treatments were tested by Dunnett's or Student's t-tests ($p < 0.05$). All data are reported as mean ± standard error (SE).

RESULTS

Effect of Chitosan on *P. chlamydosporia* Chlamydospores Viability and Mycelia Growth

Chitosan solutions at concentrations up to 2 mg ml^{-1} did not affect viability of chlamydospores (**Figure 1A**). Higher chitosan concentrations caused chlamydospore death (PI staining), just as H_2O_2 treatments. Chitosan at concentrations up to 0.1 mg ml^{-1} did not affect chlamydospore germination (**Figure 1B**). Higher concentrations significantly reduced germination. Two milligrams per milliliter chitosan reduced ca. 70% germination respect to untreated controls, but favored mycelial growth after 3 days of incubation. Maximum mycelia growth (measured as OD_{490}) was obtained with 2 mg ml^{-1} chitosan. These results suggest that *P. chlamydosporia* uses chitosan as a nutrient source. Chitosan concentrations between 0.05 and 0.1 mg ml^{-1} did not affect viability/germination of chlamydospores and improved mycelial growth compared to the control (**Figure 1C**).

Effect of *P. chlamydosporia* and Chitosan on Tomato Growth

The effect of *P. chlamydosporia* inoculation on tomato plants, not irrigated with chitosan was first evaluated. *P. chlamydosporia* increased both DSW and FRW 10 days after inoculation (Supplementary Table 1), but this effect was not sustained over time. However, fungal inoculation increased shoot length of tomato plants at 30 days (**Table 1**).

Chitosan irrigation (0.01–0.075 mg ml^{-1}) of tomato plants uninoculated with *P. chlamydosporia* promoted shoot growth (DSW) at 10 days, but the effect was lost with time (**Table 1** and Supplementary Tables 1, 2). The same doses promoted root growth (FRW) at 20 days and to a less extent (0.05 and 0.1 mg ml^{-1} only) at 30 days (Supplementary Table 2 and **Table 1**). However, chitosan applied at 0.3 mg ml^{-1} reduced tomato growth (**Table 1**).

Pochonia chlamydosporia combined with low chitosan concentrations (0.01–0.1 mg ml^{-1}) promoted root growth (FRW) after 20 days, and to a less extent (0.05 and 0.1 mg ml^{-1}) after 30 days. On the contrary, *P. chlamydosporia* combined with the largest chitosan concentration (0.3 mg ml^{-1}) reduced shoot and root weight, MRL, and MSL 30 days after fungal inoculation and chitosan application (**Table 1**). Shoot length was very sensitive to *P. chlamydosporia* combined with chitosan, because all treatments abolished the MSL promotion obtained with the fungus inoculated alone (no chitosan) after 30 days (**Table 1**). The results obtained from this section selected 0.1 mg ml^{-1} of chitosan irrigation for further experiments.

Effect of Chitosan on the Tritrophic Interaction Tomato–*M. javanica*–*P. chlamydosporia*

Chitosan irrigation increased DSW (**Figure 2A**) and FRW and root length (**Figures 2C,D**) in tomato plants inoculated with *M. javanica*. Chitosan also promotes root length in plants inoculated with *P. chlamydosporia* (**Figure 2D**), and DSW in plants inoculated with both *P. chlamydosporia* and *M. javanica* (**Figure 2A**). Differences in maximum shoot length were not found (**Figure 2C**). The number of egg masses per plant was not influenced by chitosan irrigation irrespective of fungal inoculation (data not shown). Fungal egg parasitism was low (2.3–7.2%) and did not differ with chitosan irrigation. Chitosan irrigation significantly ($p < 0.05$) enhanced tomato root colonization by *P. chlamydosporia* (**Figure 3**) by 20-fold. In RKN treatments, chitosan irrigation also significantly increases the colonization of roots by the fungus but to a lesser extent than roots with no RKN.

Effect of Chitosan on RKN Multiplication on Tomato in Soils in Which *P. chlamydosporia* Occurs Naturally

Chitosan irrigation of tomato plants cultivated in the non-sterilized clay loam soil M10.41 enhanced ($p < 0.05$) shoot growth, reduced galling index, and RKN multiplication

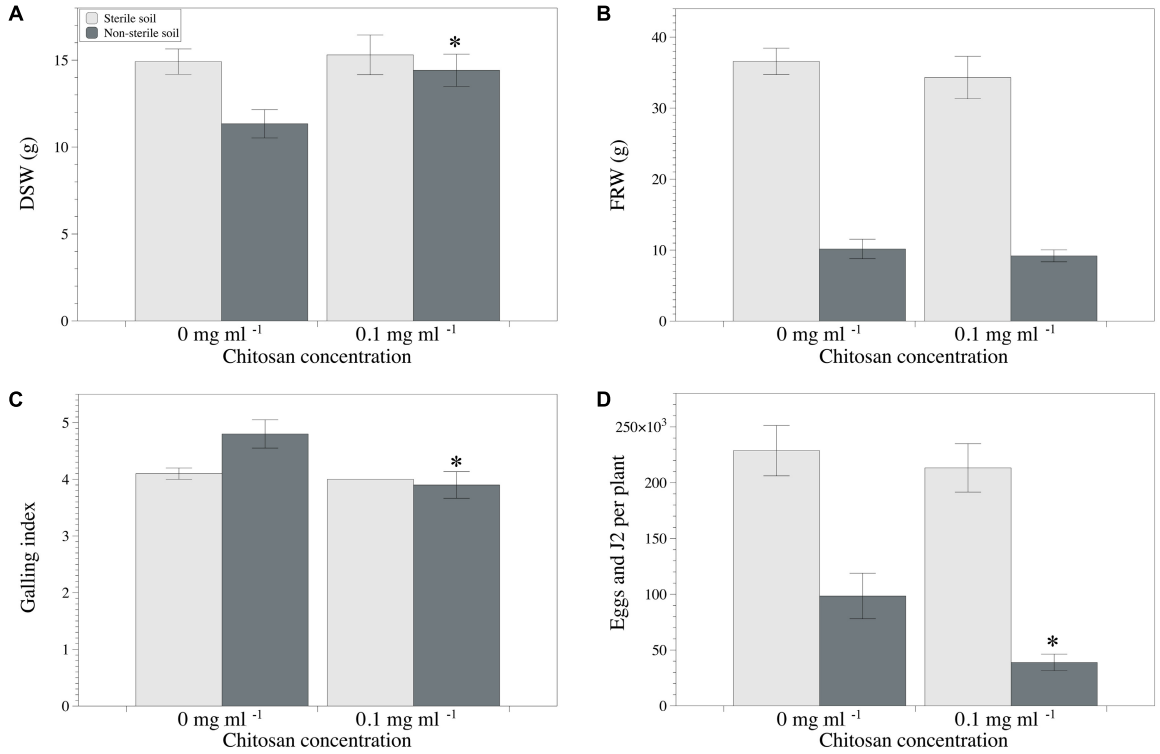


FIGURE 4 | Effect of chitosan irrigation on RKN (*Meloidogyne* spp.) in tomato plants grown in M10.41 clay soil with natural moderate suppression to RKN. **(A)** DSW, **(B)** FRW, **(C)** Galling index, and **(D)** total nematodes per plant. Asterisks show statistical differences between control and chitosan treatments (p-value < 0.05).

(**Figures 4A,C,D**), but no effect was observed in the sterilized soil. No differences were found for fresh root weight (**Figure 4B**). Fungal egg parasitism was 36.6 ± 3.3% and 29.3 ± 4.7% in chitosan irrigated and non-irrigated non-sterilized soil, respectively. *P. chlamydosporia* was the only fungus identified parasitizing nematode eggs.

Regarding the sandy loam soil M10.56, chitosan irrigation reduced RKN multiplication (sterilized soil only), but had not effect ($p < 0.05$) on disease severity or plant growth irrespective of soil sterilization (**Figures 5A–C**). No differences were found for fresh root weight (**Figure 5B**). Fungal egg parasitism was 45.7 ± 6.0% and 73.7 ± 2.6% in the non-sterile soil irrigated and non-irrigated with chitosan, respectively. *P. chlamydosporia* was again the only fungus parasitizing the nematode eggs. No fungal egg parasitism was detected on eggs produced on tomato plants grown in the sterile soil mixtures from both sites.

DISCUSSION

Chitosan enhances *P. chlamydosporia* sporulation, protease induction (Palma-Guerrero et al., 2010a,b), appressorium differentiation, and nematode egg parasitism in laboratory bioassays (Escudero et al., 2016). However, little is known about the effect of chitosan on the tritrophic interaction Plant–RKN–*P. chlamydosporia*, or on RKN suppressiveness when applied to agricultural soils containing natural nematode antagonists.

The first aim of this work was to determine the effect of a 70 kDa chitosan on the viability of *P. chlamydosporia* chlamydospores. These resistant spores are used as inoculum for nematode management and consistently found in suppressive soils (Kerry and Bourne, 2002). Viability of *P. chlamydosporia* chlamydospores was kept at concentrations of chitosan up to 2 mg ml^{-1} which are toxic for several fungal species causing soil-borne diseases, such as *Fusarium oxysporum* f. sp. *radicis-lycopersici, Verticillium dahliae*, *Rhizoctonia solani* (Allan and Hadwiger, 1979; Palma-Guerrero et al., 2008; Xing et al., 2015). Thus, soil irrigation with chitosan could inhibit soilborne fungal pathogens without affecting *P. chlamydosporia* chlamydospores.

Our results show that mycelium of *P. chlamydosporia* derived from chlamydospores can grow with chitosan as the only carbon source. The large expansion of GH75 chitosanase family in the genome of *P. chlamydosporia* (Aranda-Martinez et al., 2016) could explain this feature.

This is, to the best of our knowledge, the first time *P. chlamydosporia* has been applied combined with chitosan against RKN in micropot experiments. Chitosan at 0.1 mg ml^{-1} increased the growth of *M. javanica* infested plants respect to those inoculated the nematode only, as previously reported by Vasyukova et al. (2001). Chitosan combined with *P. chlamydosporia* promoted root colonization by the fungus respect to plants inoculated with *P. chlamydosporia*

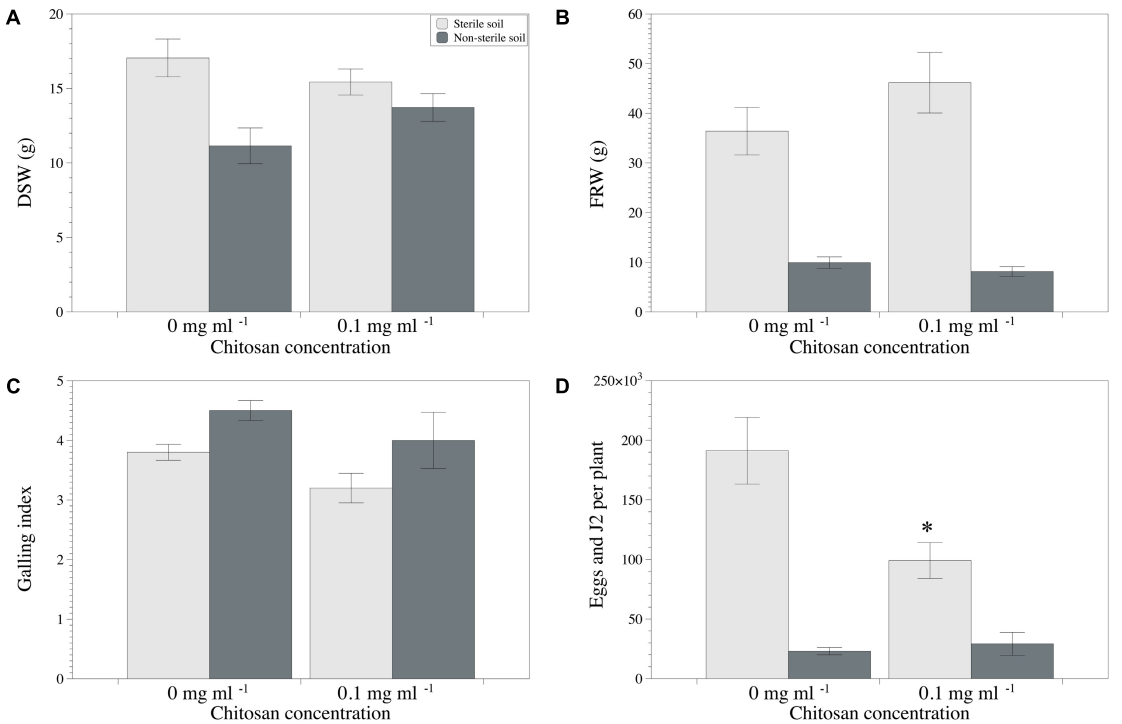


FIGURE 5 | Effect of chitosan irrigation on RKN (*Meloidogyne* spp.) in tomato plants grown in M10.56 sandy soil with natural high suppression to RKN. **(A)** DSW, **(B)** FRW, **(C)** Galling index, and **(D)** total nematodes per plant. Asterisks show statistical differences between control and chitosan treatments (p-value < 0.05).

alone. Chitosan, perhaps acting as an alternative carbon source, is involved in the increase of tomato root colonization by *P. chlamydosporia* as it found in this work. This could help improving rhizosphere competence of the fungus which decreases with time (Maciá-Vicente et al., 2009; Escudero and Lopez-Llorca, 2012). The mechanism involved is unknown but should be further investigated. To this respect, chitosan is a poor substrate for chitinases and a weak inducer of plant immune response (Sanchez-Vallet et al., 2015).

Pochonia chlamydosporia, chitosan, or its combination did not affect nematode infection and development in roots. Therefore RKN juveniles, under our experimental conditions, could infect and develop into mature females laying eggs. In addition, neither *P. chlamydosporia*, chitosan, nor its combination affected fungal egg parasitism. The ability of chitosan to increase root colonization by *P. chlamydosporia* found in this study should be used in future work to increase plant tolerance/resistance to RKN.

Chitosan irrigation of agricultural soils containing natural nematode antagonists had different effects depending on soil properties such as the resident microbiota and soil texture. In the non-sterilized clay loam M10.41 soil, chitosan promoted plant growth and reduced the number of nematodes per plant. These effects were associated with the nematode antagonistic microbiota but mechanisms other than egg-parasitism (only slightly enhanced) were probably involved. The differential effect of chitosan on soil microorganisms could induce rearrangements of microbial communities, which could affect the level of nematode suppression in soils (Tian et al., 2015). Accordingly, chitosan had no effect in M10.41 sterilized soil.

Chitosan irrigation reduced nematodes per plant in sterilized M10.56 sandy loam soil. However, no such effect was found in non-sterilized soil, where high parasitism of RKN egg by *P. chlamydosporia* only was recorder. These results are in concordance with those reported by Khalil and Badawy (2012) and Radwan et al. (2012) who found a reduction of RKN densities in tomato irrigated with chitosan in soils with at least 50% sand.

The present study demonstrates that chitosan applied at low rates does not affect chlamydospores and enhances mycelial growth of *P. chlamydosporia*. However, soil texture and nematode antagonistic microbiota in agricultural soils affect the performance of chitosan against RKN. Microbiota in agricultural soils putative antagonistic to RKN is diverse (Giné et al., 2016). Chitosan properties (e.g., deacetylation degrees and molecular weight) have different effects on microorganisms (Younes et al., 2014). Therefore, the interaction of these features should be analyzed for improving chitosan as a bioactivator of RKN antagonists, such as *P. chlamydosporia*.

AUTHOR CONTRIBUTIONS

This work was a part of the Ph.D. thesis of NE supervised by LL-L. NE design and performance of the research, data collection and data analysis, and writing of the manuscript. FL-M performance of the research, data collection, and writing of the manuscript.

ZG, EZ-G, AA-C, CR-I, and AL performance of the research and technical support. FS design and performance of the research, data collection, data interpretation, and writing of the manuscript. LL-L design of the research, data interpretation, and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01415/full#supplementary-material

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Combined Field Inoculations of *Pseudomonas* Bacteria, Arbuscular Mycorrhizal Fungi, and Entomopathogenic Nematodes and their Effects on Wheat Performance

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In agricultural ecosystems, pest insects, pathogens, and reduced soil fertility pose major challenges to crop productivity and are responsible for significant yield losses worldwide. Management of belowground pests and diseases remains particularly challenging due to the complex nature of the soil and the limited reach of conventional agrochemicals. Boosting the presence of beneficial rhizosphere organisms is a potentially sustainable alternative and may help to optimize crop health and productivity. Field application of single beneficial soil organisms has shown satisfactory results under optimal conditions. This might be further enhanced by combining multiple beneficial soil organisms, but this remains poorly investigated. Here, we inoculated wheat plots with combinations of three beneficial soil organisms that have different rhizosphere functions and studied their effects on crop performance. Plant beneficial *Pseudomonas* bacteria, arbuscular mycorrhizal fungi (AMF), and entomopathogenic nematodes (EPN), were inoculated individually or in combinations at seeding, and their effects on plant performance were evaluated throughout the season. We used traditional and molecular identification tools to monitor their persistence over the cropping season in augmented and control treatments, and to estimate the possible displacement of native populations. In three separate trials, beneficial soil organisms were successfully introduced into the native populations and readily survived the field conditions. Various *Pseudomonas*, mycorrhiza, and nematode treatments improved plant health and productivity, while their combinations provided no significant additive or synergistic benefits compared to when applied alone. EPN application temporarily displaced some of the native EPN, but had no significant

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long-term effect on the associated food web. The strongest positive effect on wheat survival was observed for *Pseudomonas* and AMF during a season with heavy natural infestation by the frit fly, *Oscinella frit*, a major pest of cereals. Hence, beneficial impacts differed between the beneficial soil organisms and were most evident for plants under biotic stress. Overall, our findings indicate that in wheat production under the test conditions the three beneficial soil organisms can establish nicely and are compatible, but their combined application provides no additional benefits. Further studies are required, also in other cropping systems, to fine-tune the functional interactions among beneficial soil organisms, crops, and the environment.

Keywords: plant-growth promoting rhizobacteria, biofertilizer, *Steinernema, Heterorhabditis,* wheat, biological control, insect pest, plant growth promotion

INTRODUCTION

In addition to poor soil fertility, soil pests and pathogens pose major threats to the health and productivity of crops in agricultural ecosystems resulting in important yield losses every year (Oerke, 2006; Kupferschmied et al., 2013). The use of fertilizers, fungicides, nematicides, and insecticides to counter these problems can have important negative consequences, such as the persistence of these agrochemicals in the soil, water, and food with potential negative impacts on the environment and consumers (Bale et al., 2008; Lichtfouse et al., 2009; Kupferschmied et al., 2013; Johnson et al., 2016). Hence, new and more sustainable pest and disease control strategies need to be explored for a next-generation agriculture and the application of beneficial soil organisms (BeSO) presents a promising alternative for maintaining crop health and productivity (Bommarco et al., 2013; Bender et al., 2016).

Various BeSO are known to enhance plant performance, e.g., by directly promoting plant growth, by stimulating plant defenses, by facilitating nutrient acquisition by the plant, or by protecting the plant from pathogens and pests (Philippot et al., 2013; Rasmann and Turlings, 2016; Venturi and Keel, 2016). The three groups of BeSO investigated in the present study fulfill one or several of these beneficial functions, i.e., plant-growth promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF), and entomopathogenic nematodes (EPN). Root-colonizing bacteria belonging to the *Pseudomonas fluorescens* group are well-characterized PGPR that have the ability to induce systemic plant defenses and ward off soil-borne pathogens, in particular pathogenic fungi and oomycetes, including *Gaeumannomyces*, *Thielaviopsis, Rhizoctonia, Fusarium oxysporum*, and *Pythium* (Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Hol et al., 2013; Vacheron et al., 2015). To date, several biocontrol products that are based on PGPR pseudomonads are on the market (Berg, 2009; Kupferschmied et al., 2013). Moreover, certain subgroups, in particular the two species *Pseudomonas protegens* and *Pseudomonas chlororaphis* exhibit potent oral insecticidal activity notably against Lepidopteran pests (Kupferschmied et al., 2013; Ruffner et al., 2013; Flury et al., 2016).

AMF are well-known beneficial symbionts that colonize the roots of the majority of land plants (Schueßler et al., 2001; van der Heijden et al., 2015). AMF form extensive hyphal networks that provide water and nutrients to their host plant. AMF are key actors in processes such as the mineralization of phosphorus and nitrogen and enhancing the nutrient up-take by plant roots (Jakobsen et al., 1992; Mäder et al., 2000; Smith et al., 2004; van der Heijden et al., 2006). AMF primarily improve plant nutrition, but they can also contribute to enhance the tolerance of their host plant against biotic and abiotic stresses (van der Heijden et al., 2015). Numerous AMF species, e.g., *Rhizoglomus irregulare*, are commercialized as inoculum to improve soil fertility (Lekberg and Koide, 2005; Pellegrino et al., 2015) and plant productivity (Hijri, 2016; Köhl et al., 2016). Today, the agronomic use of AMF includes the direct augmentation or inoculation of seedlings in nurseries before transplanting to the field (Jeffries et al., 2003) and seed coating (e.g., Ijdo et al., 2011).

Finally, EPN of the genera *Steinernema* and *Heterorabditis* are well-known biocontrol agents that selectively search their insect hosts and kill them within 2–3 days with the aid of mutualistic bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively (Georgis et al., 2006; Kaya et al., 2006; Dillman et al., 2012; Campos-Herrera, 2015; Lacey et al., 2015). Their wide distribution in soils throughout the world (Adams et al., 2006) and the availability of commercial products (Lacey et al., 2015) make them excellent products in integrated pest management (IPM) programs or in organic production, both for augmentation or restoration of naturally occurring EPN (Campos-Herrera, 2015). However, their performance and activity is affected by biotic and abiotic factors, and hence, their efficacy depends on soil characteristics, agricultural management practices, and competition within the food web (Stuart et al., 2015).

The three groups of organisms—*Pseudomonas,* AMF, and EPN—occur naturally in most arable soils and commercial formulations are available for agronomic use (Stockwell and Stack, 2007; Berg, 2009; Kupferschmied et al., 2013; Lacey et al., 2015). Previous greenhouse and field studies have reported varying effects on plant health and growth when combining inoculants of these BeSO groups. For example, combinations of certain *Pseudomonas* strains provided better control of the wheat disease take-all than did the individual strains alone (Pierson and Weller, 1994). Positive effects have been also recorded when combining bacteria, such as *Pseudomonas* or *Azospirillum*

TABLE 1 | Beneficial soil organisms applied individually or in combinations in the field experiments.

Beneficial group/species	Strain	Treatment code	Application type	GenBank accession no.	Reference or source
***PSEUDOMONAS* BACTERIA**					
Pseudomonas chlororaphis	PCL1391[a]	B2	Aqueous	NZ_LFUT01000004	Chin-A-Woeng et al., 1998
Pseudomonas protegens	CHA0[a]	B1	Aqueous	NC_021237	Stutz et al., 1986
ARBUSCULAR MYCORRHIZAL FUNGI					
Claroideoglomus claroideum	SAF12[c]	F4	Substrate	n.a.[d]	Swiss collection of arbuscular miychorrizal fungi (SAF)
Funneliformis mosseae	SAF11[c]	F3	Substrate	n.a.[d]	SAF
Rhizoglomus irregulare[b]	INOQ Top	F1	Substrate	n.a.[d]	Inog GmbH, Schnega
Rhizoglomus irregulare[b]	SAF22[c]	F2	Substrate	DQ377990	Germany, SAF
ENTOMOPATHOGENIC NEMATODES					
Heterorhabditis bacteriophora	Andermatt	N2	Aqueous	KJ938576	Andermatt Biocontrol AG, Grossdietwil, Switzerland
Heterorhabditis megidis	Andermatt	N1	Aqueous	KJ938577	Andermatt Biocontrol AG, Grossdietwil, Switzerland
Steinernema carpocapsae	D-83	N3	Aqueous	KJ818295	Jaffuel et al., 2016
Steinernema feltiae	RS-5	N4	Aqueous	KJ938569	Jaffuel et al., 2017

[a] *Rifampicin-resistant variants of strains CHA0 and PCL1391 were used as inoculants in the field trials (see Materials and Methods).*
[b] *Rhizoglomus irregulare was previously referred to as Rhizophagus irregularis and earlier as Glomus intraradices (Sieverding et al., 2015).*
[c] *Strain ID referring to the Agroscope AMF strain collection, http://www.agroscope.ch/saf.*
[d] *n.a., not available.*

strains, with fungi, including the AMF *Glomus* (Frey-Klett et al., 2007; Couillerot et al., 2012; Walker et al., 2012), *Fusarium* or *Trichoderma* (Fogliano et al., 2002; Yigit and Dikilitas, 2007). Similarly, EPN have been combined with other BeSO, with differing results. For example, the combination of *Steinernema kraussei* with the entomopathogenic fungus (EPF) *Metarhizium anisopliae* resulted in a synergistic effect in the control of *Otiorhynchus sulcatus* in strawberry (Ansari et al., 2010), while the combination of *Steinernema ichnusae* with the EPF *Beauveria bassiana* resulted in clear antagonism and competition for the host under controlled laboratory conditions (Tarasco et al., 2011).

Field applications of single BeSO have shown to greatly enhance plant growth and health in various crops (Jeffries et al., 2003; Berg, 2009; Kupferschmied et al., 2013; Campos-Herrera, 2015; Lacey et al., 2015), but the putative positive effect of combining various BeSO remains poorly predictable. The Swiss National Research Programme 68 (NRP 68) "Sustainable use of soil as a resource" (www.nrp68.ch) provided the framework for our multidisciplinary investigations into BeSO and their possible role in novel strategies for sustainable soil management. As part of this, we evaluated, for the first time, the simultaneous application of *Pseudomonas*, AMF, and EPN inoculants in field experiments, using wheat as the model crop. We hypothesized that the combined application of these BeSO would show greater benefits for the crop than their individual application.

MATERIALS AND METHODS

Beneficial Organisms

Selected species of BeSO, all known to naturally occur in Swiss soils (Campos-Herrera et al., 2015a; Jaffuel et al., 2016; Schlaeppi et al., 2016; Imperiali et al., 2017), were applied depending on the objective and design of each field experiment (Figure S1). The BeSO that were used included two species of the genus *Pseudomonas*, three AMF species and four EPN species and they were applied as inoculants either individually or in various combinations in the different experiments (**Tables 1**, **2**).

To monitor the bacteria following field application, the bacterial inoculants, i.e., *P. protegens* strain CHA0 (Stutz et al., 1986) and *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al., 1998) were tagged with a spontaneous resistance to rifampicin following previously described protocols (Natsch et al., 1994). Briefly, spontaneous rifampicin-resistant derivatives were obtained following plating concentrated cell suspensions of each parental strain on King's medium B agar (KMB) (King et al., 1954) supplemented with 100 μg/ml of rifampicin and incubated for 3 days. A CHA0-Rif derivative and a PCL1391-Rif derivative (**Table 1**), which stably maintained rifampicin resistance and displayed wild-type growth and antifungal and insecticidal activities, were selected. For the preparation of the bacterial field inocula, the selected rifampicin resistant strains were grown overnight at 25°C in lysogeny broth (LB) (Bertani, 1951) containing 100 μg/ml of rifampicin. Aliquots of 200 μl of each culture were spread on KMB plates without antibiotics. After incubation at 27°C for 16 h, bacterial cells were harvested and washed in sterile distilled water. The optical density at 600 nm (OD_{600}) of the bacterial cell suspensions was adjusted to 0.15 corresponding to a cell density of 8×10^7 CFU ml^{-1}. These bacterial stock suspensions were maintained on ice until final dilution and use on the field sites.

The AMF strains *Claroideoglomas claroideum* SAF12, *Funneliformis mosseae* SAF11 and *R. irregulare* SAF22 were selected from the Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF) at Agroscope (Reckenholz, Zurich, www.agroscope.

TABLE 2 | Details on the characteristics of the three field experiments used to assess effects of inoculation of beneficial soil organisms (pseudomonads, entomopathogenic nematodes, and arbuscular mycorrhizal fungi) on growth, health, and yield of spring wheat.

Field trials	COMBINATION	PERFORMANCE-1	PERFORMANCE-2
Coordinates SN/EW	46.397676/6.260763	46.397455/6.260166	46.39502/6.260444
Sowing/inoculation day	18th March 2014	18th March 2014	27th March 2015
Treatments (Treatments codes refer to **Table 1**)	Control (no inoculants) B1: *P. protegens* CHA0-Rif B2: *P. chlororaphis* PCL1391-Rif N1: *H. megidis* Andermatt N2: *H. bacteriophora* Andermatt N3: *S. carpocapsae* D83 N4: *S. feltiae* RS5 F1-H: *R. irregulare* INOQ Top (high dosage, 250 ml/row) F1-L: *R. irregulare* INOQ Top (low dosage, 50 ml/row) F2: *R. irregulare* SAF22 F3: *F. mosseae* SAF11 F4: *C. claroideum* SAF12 AMF control (substrate only) B1+ N2 B1+ F1 N2 + F1 B1+ N2 + F1	Control (no inoculants) B1: *P. protegens* CHA0-Rif B2: *P. chlororaphis* PCL1391-Rif N2: *H. bacteriophora* Andermatt BM: B1 + B2 B1 + N2 B2 + N2 BM + N2	Control (no inoculants) BM: B1 + B2 NM: N1 + N2 + N4 F1-L AMF control (substrate only) BM + NM NM + F1-L BM + F1-L BM + NM + F1-L
Number of treatments	17	8	9
Number of replicates (plots) per treatment	Four replicates in randomized complete block design	Four replicates in randomized complete block design	Nine replicates in randomized complete block design
Size of plots	1.5 m^2	6.75 m^2	9 m^2
Number of plant rows per plot	5	5	5
Wheat seeds per m of row	∼80	∼140	∼140
Bacterial inoculum (CFU in 400 ml H_2O per meter of row)	B1: 1.19 × 10^9 B2: 1.21 ×10^9	B1: 1.42 × 10^9 B2: 3.37 × 10^9	B1: 4.875 × 10^8 B2: 8.25 × 10^8
AMF inoculum	F1-H: 80 F1-L: 16 F2: 250 F3: 250 F4: 250 AMF control: 250	Not contributed	F1-L: 16 AMF control: 16
Nematode inoculum	50 infective juveniles/cm^2/4 L	50 infective juveniles/cm^2/8 L	50 infectice juveniles/cm^2/8 L
Pest insect stress	Heavy natural infestation with *Oscinella frit*	Heavy natural infestation with *Oscinella frit*	No relevant *Oscinella frit* infestation
Soil type and texture	Sandy loam (clay, 25.5%; silt, 34.3%; sand; 40.2%)	Sandy loam (clay, 25.5%; silt, 34.3%; sand, 40.2%)	Loam (clay, 14.5%; silt, 26.8%; sand, 58.7%)

ch/saf; **Table 1**). The inocula were prepared as described by Schlaeppi et al. (2016). Briefly, AMF were propagated over 6 months in the greenhouse in autoclaved sand:soil (85:15%; v/v) as substrate and using *Plantago lanceolata* as host. The final inoculum contained pieces of plant roots mixed with the substrate containing AMF hyphae and spores (SAF12, Propagation 0510, *P. lanceolata* roots were colonized by 28% and 763 spores could be washed from 25 g substrate; SAF#11, Propagation 0711, 27% and 29 spores; SAF#22, Propagation 0813; 97% and 475 spores). In addition, a "mock" inoculum consisting of *Plantago* roots and substrate free of AMF propagules was prepared following the same protocol and this mock treatment was termed "AMF control" (Quality inspection of the mock inoculum, Propagation 0711, roots were not colonized by AMF and no spores could be washed from 25 g of substrate). The COMBINATION experiment also comprised a treatment where nothing was applied, named "control". AMF inocula as well as the mock-inoculum were mixed in separate plastic bags and stored at room temperature until use. In addition, the commercial AMF inoculum *R. irregulare* TOP (INOQ GmbH, Schnega, Germany, www.inoq.de) was used as obtained from Otto Hauenstein Samen AG (Rafz, Switzerland, www.hauenstein.ch). For the second trial (PERFORMANCE-2), we utilized the lower dosage of the commercial inoculum based on the COMBINATION experiment results. For the PERFORMANCE-2 trial we utilized autoclaved commercial inoculum as AMF control treatment.

For the EPN, infective juveniles (IJs) of four species were prepared in adjusted suspensions. *Heterorhabiditis* species were obtained from a commercial source (Andermatt Biocontrol, Grossdietwil, Switzerland, www.andermattbiocontrol.com),

whereas *Steinernema* species were propagated from field collected populations under laboratory conditions following protocols described by Campos-Herrera et al. (2015a) (**Table 1**). All nematodes were received or harvested within 2 weeks prior to field application. The day before application, the EPN inoculant suspensions were prepared in sterile water. To this end, IJs were counted and their density was adjusted to deliver the required field concentration per experimental unit (**Table 2**) by using separate containers. Containers were kept at 5°C overnight and transported in coolers to the field. In addition, laboratory infections of *Galleria mellonella* larvae by the inoculant EPN at field concentrations were used to verify their infectivity for each experiment (Jaffuel et al., 2017).

Experimental Designs

From spring 2014 to summer 2015, three field experiments were conducted in wheat plots and the applications of beneficial soil organisms were adapted for each experiment. All the experiments were carried out with the commercial spring wheat variety "Rubli" in the experimental plots, whereas the commercial triticale variety "Trado" was seeded in the buffer zones. Fields were bordered by strips of non-managed grassland. The three experiments were named as follows: COMBINATION (2014), PERFORMANCE-1 (2014), and PERFORMANCE-2 (2015) (**Table 2**). The selection of the applied organisms and combinations of treatments were adapted on results of the preceding trial. The first experiment (COMBINATION) was set up to test various species of each group of beneficials and first combinatory treatments. The second experiment was designed to evaluate wheat yield effects after combining bacteria and EPN (PERFORMANCE-1). In this experiment, the AMF treatment was not included due to limitations in scaling the production of inoculum for the large plot size. Finally, the PERFORMANCE-2 experiment consisted of the full bacteria-AMF-EPN combinations during the subsequent season (**Table 2**).

All the experiments were conducted in neighboring experimental field sites located near Prangins, Switzerland (see **Table 2** for coordinates). The sites belong to Agroscope, research center of Changins, (Nyon, Switzerland) and have documented crop and management sequences for the last 30 years. The field sites chosen for the experiments had no overlapping areas to avoid cross-contaminations with inoculants. None of the experiments had irrigation systems. The soil type was sandy loam for the COMBINATION and PERFORMANCE-1 trials and loam for the PERFORMANCE-2 trial (**Table 2**). General agronomic preparations for all the experiments included tillage (15 cm deep) and harrowing about 4 days before seeding. The seeding machine "Hege Seedmatic" (Hege Maschinen, Eging am See, Germany) allowed the customized seeding for each plot size and arrangement (**Table 2**; Figure S1) and was modified to keep the seed furrows open after placing the seeds. After seeding, the plots were marked for the corresponding treatments (see Figure S1 for the exact field design of each of the three experiments) and inoculated on the same day with the beneficial soil organisms. In combination treatments, the application followed the order bacteria, EPN, and AMF.

Bacteria were applied as a cell suspension to the seed furrows (plant rows) using treatment-specific watering cans. Final cell suspensions were prepared directly on the field from bacterial stock suspensions (OD_{600} 0.15; i.e., 8×10^7 CFU ml^{-1}) by adjusting with water to obtain the required volumes (400 ml per meter of row) and bacterial cells (8×10^8 CFU per meter of row) (**Table 2**). Similarly, EPN were applied in variable volumes depending on plot size using treatment-specific watering cans. They were applied to entire plots (not just the rows), and in all the cases, the final concentration was 0.5 Mio. IJs/m^2 (equivalent to 50 IJs/cm^2, Grewal and Peters, 2005). Finally, AMF inocula were applied manually employing 250-ml glass beakers. The material was applied directly over the seeds in the furrows, thereby gently mixing seeds and inoculum with a small hoe. AMF control plots received the same quantity of AMF-free substrate. Control plots were treated with the same volumes of BeSO-free water. Immediately after treatment application, the seeds were covered with soil using hoes to close the seed furrows. All equipment used for inoculant application was thoroughly cleaned and disinfested between manipulations using 70% ethanol.

Weed control included the application of the herbicides Azur (Omya AG, Switzerland) against monocots 2 weeks after seeding and Apell (Syngenta AGRO SA) against dicots shortly before earing (BBCH 45-50). When necessary, some persistent weeds (*Galium* spp., *Setaria* spp.) were controlled manually. No fungicides nor nematicides were applied during any of the experiments. The insecticide Karate Zeon (Lambda-Cyhalotryne, Syngenta Agro GmbH) was applied in the PERFORMANCE-2 experiment against cereal mining dipterae such as the frit fly and hessian fly conducted in 2015, but not in the 2014 COMBINATION and PERFORMANCE-1 experiments. Plots were fertilized once by supplementing nitrogen in liquid (Lonza-sol N liquid, Basel, Switzerland) at 62 kg ha^{-1} of to reach 155 units N and potassium (K_2O) at 30.6 kg ha^{-1}. The PERFORMANCE-2 trial was covered with a black hail net during the first 2–3 weeks to protect the seeds and young plants from cold conditions and predation by birds and small mammals.

Sampling of Beneficial Organisms and Measuring of Plant Traits

Pseudomonas Bacteria

The presence of *P. protegens* CHA0-Rif and *P. chlororaphis* PCL1391-Rif was evaluated in both inoculated plots and non-inoculated control plots, as well as in the buffer zone around the experimental plots, and in the border zone (grassland) around the field site to control for possible cross contamination. This analysis was conducted four times during the growing season (i.e., at seeding, end of the winter, at earing, and maturity) in selected experiments (**Table 3**). For this, the root systems from four wheat plants per plot (triticale plants and grass for the buffer and border zones, respectively) were collected, pooled, washed, and gently dried with paper towels. Roots were weighed, cut into pieces (about 15 cm long), placed in 50-ml Falcon tubes (Greiner Bio One, Germany) containing 40 ml of sterile water and kept overnight at 4°C. All sampling equipment was cleaned with 70% ethanol between samples to avoid cross-contaminations. Samples

TABLE 3 | Description of the type of measurements and methods employed and timing in each of the field experiments.

Organisms	Type of measurement	Method	Reference	COMBINATION	PERFORMANCE-1	PERFORMANCE-2
Bacteria	Sample type	Composite samples of wheat roots	Authors	Wheat roots	Wheat roots	Wheat roots
	Quantification at seeding	CFU counting on selective media	Authors	28.03.2014	28.03.2014	27.03.2015
	Quantification during wheat growth	CFU counting on selective media	Authors	03.06.2014	03.06.2014 25.06.2014 22.07.2014	27.04.2015 18.05.2015 29.06.2015
	Tracing presence in buffer zones	CFU counting on selective media	Authors	Not done	03.06.2014	Not done
	Tracing in non-agricultural soil of border zone	CFU counting on selective media	Authors	Not done	25.06.2014 22.07.2014	Not done
AMF	Sample type	Composite samples of wheat roots	Authors	Wheat roots	Not contributed	Wheat roots
	Quantification of inoculum	Real-time qPCR using primers targeting the inocula	Authors	At harvest	Not contributed	At harvest
	Determination of AMF community	AMF community sequencing	Schlaeppi et al., 2016	At harvest	Not contributed	Not done
EPN	Sample type	Composite soil sample		12 soil cores/plot	15 soil cores/plot	15 soil cores/plot
	EPN presence: pre-inoculation (baseline)	Species-specific primers/probes and real time qPCR	Campos-Herrera et al., 2015a	27.03.2014 (Baseline)	27.03.2014 (Baseline)	27.03.2015 (Baseline)
	EPN presence: post-augmentation	Species-specific primers/probes and real time qPCR	Campos-Herrera et al., 2015a	25.06.2014	25.06.2014	17.06.2015
	EPN activity: insect-baits	*Galleria* bait	Bedding and Akhurst, 1975	25.06.2014	25.06.2014	17.06.2015
	Soil food web assemblage (nematophagous fungi, free-living nematodes and ectophoretic bacteria)	Species-specific primers/probes and real time qPCR	Atkins et al., 2005; Zhang et al., 2006; Campos-Herrera et al., 2011b, 2012, 2015b; Pathak et al., 2012	27.03.2014 (Baseline) 25.06.2014	27.03.2014 (Baseline) 25.06.2014	27.03.2015 (Baseline) 17.06.2015
Plants[a]	Height (average per plot)	Measured from shoot base to the upper growth	Authors	–	–	23.04.2015 05.06.2015 18.05.2015 01.06.2015 08.06.2015
	Weight	Eight plants	Authors	–	–	At harvest
	Density (% of plot surface covered by plants)/number of plants per linear meter	Visual scoring	Authors	–	14.05.2014 21.05.2014 11.06.2014	06.05.2015 18.05.2015 18.06.2015 29.06.2015
	Chlorophyll activity	N-tester (YARA)	Authors	–	–	08.06.2015
	Yield (g seeds/plot);	Weighing wheat seeds at dough developmental stage	Authors	–	At harvest	At harvest
	Thousand-seed weight (TSW)	Marvin seed analyzer	Gegas et al., 2010	–	–	At harvest
	Protein content (%)	Near-infrared spectroscopy	Authors	–	–	At harvest
	Insect pest and pathogen incidence	Visual counts	Authors	Weekly	Weekly	Weekly

[a]*Measurements were made for the three field experiments, but data are considered not representative due to the small size of the plots (COMBINATION assay) and/or the highly heterogeneous growth of the wheat plants within the plots following heavy frit fly damage in the 2014 COMBINATION and PERFORMANCE-1 assays.*

were vigorously agitated on a rotary shaker at 180 rpm for 20 min, and roots were removed and dried at 80°C for 3 days to obtain the dry weight. The remaining suspensions were transferred to fresh sterile Falcon tubes on ice and centrifuged at 8,500 rpm (9,300 g) at 4°C. The supernatant was discarded and the pellet was re-suspended in 1 ml of sterile water. Each sample was

then serially diluted and dilutions spread on KMB supplemented with 100 μg/ml of cycloheximide and 100 μg/ml of rifampicin (Scanferlato et al., 1990). The colonies were counted and the results were expressed as colony forming units (CFU) per gram of dry root weight.

Arbuscular Mycorrhizal Fungi

The inoculation success of the different AMF inocula was traced by quantitative PCR comparing their abundance in wheat roots sampled from inoculated, non-inoculated or mock-inoculated plots (**Table 3**). At harvest, the root systems of four plants per plot were pooled to become one sample. The fine roots (deeper than ca. 3 cm in soil) were cut from the root system using scissors, hackled into small pieces (1–2 cm long) with a scalpel and thereby homogenizing all root fragments of the four plants. The root samples were lyophilized and then ground to a fine powder using a Retsch Ball Mill (model MM301; settings 30 s at 30 Hz using one 1-cm steel ball). DNA was extracted from ∼200 mg of fine root powder utilizing the NucleoSpin® Plant II kit from Macherey-Nagel following the instructions. DNA concentrations were determined on a Varian Eclipse Fluorescence plate reader using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and Herring Sperm DNA (Invitrogen) as standard solution. The *R. irregulare* strains INOQ Top and SAF22 were quantified by qPCR utilizing primers developed by Alkan et al. (2006) and Bender et al. (unpublished), respectively. The AMF signals were expressed relative to a plant signal obtained with qPCR primers targeting the wheat ADP-ribosylation factor (Giménez et al., 2011). Triplicate amplifications were performed in 20 μl reactions using the HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) from Solis Biodyne (www.sbd.ee, Estonia) on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (www.bio-rad.com, USA). Reactions contained 4 μl qPCR mix (5X), 1 μl of each primer (10 μM), 9 μl distilled sterile water, and 5 μl template (5 ng DNA). The cycling program consisted of a 15 min initial denaturation step at 95°C followed by 40 cycles (95°C for 15 s, 63°C for 40 s for both *R. irregulare* primer sets or 60°C for 10 s for the wheat reference primers, 72°C for 20 s) and a 10 min final extension step at 72°C. Melting curve analysis consisted of a gradient from 65 to 95°C, increasing by half degrees/per 10 s to determine the uniformity of the amplicons. Raw data were imported from the qPCR cycler into the LinRegPCR program to determine the Ct and efficiency (E) values using a common fluorescence threshold for all samples (Ruijter et al., 2009). *F. mosseae* and *Claroideoglomus claroideum* were quantified with species-specific TaqMan probes following the protocols developed by Thonar et al. (2012). Template amounts were calculated for each reaction using the individual E, averaged among the replicates of each sample and expressed relative to the plant signal. Of note, for samples of the COMBINATION trial, we determined also the whole AMF community by amplicon sequencing (Schlaeppi et al., 2016).

Entomopathogenic Nematodes, Soil Food Web, and Post-Application Activity

A total of 18 soil organisms were identified and quantified before application (baseline) and post-augmentation (**Table 3**) to detect possible trophic cascade effects due to EPN augmentation (Campos-Herrera et al., 2013). These organisms comprised seven EPN species (all previously described for the area, Campos-Herrera et al., 2015a), four free-living nematodes (FLNs) that compete with EPN for the insect cadaver (Campos-Herrera et al., 2012, 2015b), six nematophagous fungi (NF) (Campos-Herrera et al., 2015a), and one nematode surface-associated bacterium (Enright and Griffin, 2005; Campos-Herrera et al., 2011a) (Table S1). Briefly, a composite soil sample composed of several cores (2.5 cm diameter, 20 cm depth, see **Table 3** for exact quantities per experiment) were taken per plot and kept on ice for transportation to the laboratory. The nematode community and other soil organisms were extracted from aliquots of 300–400 g of fresh soil by sucrose-centrifugation (Jenkins, 1964), concentrated in 1.5 ml tubes and stored at −80°C until processed, following Campos-Herrera et al. (2015a,b). Briefly, DNA was extracted from soil samples as well as from pure cultures for the generation of standard curves (when living material was available) with the Power Soil DNA Isolation Kit (MO BIO laboratories, Inc.). If no living material was available for a target organism, we employed plasmids with the whole sequence of interest to establish our positive control (Table S1; Campos-Herrera et al., 2015a). The quality and quantity of each DNA sample was analyzed prior to use (1 μl per duplicate, Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA).

Species-specific primers and probes were employed in real time qPCR assessment of the 18 soil organisms (Atkins et al., 2005; Zhang et al., 2006; Campos-Herrera et al., 2011a,b, 2012, 2015a,b; Pathak et al., 2012), following the MIQE procedures (Bustin et al., 2009). All samples were run in duplicates (unknown, positive, and negative controls) employing optical 100-well gene disc reaction plates (Biolabo, scientific instruments, Switzerland) on a Corbett Research real time PCR machine. Final reactions, concentrations, and protocols were used as previously described (Campos-Herrera et al., 2015a,b). Nematode quantification from the soil samples was done with a 10-fold dilution of the DNA, whereas the identification and quantification of NF and surface-associated bacteria required the use of total DNA without dilutions (see details in Campos-Herrera et al., 2015a). A correction factor was derived from the dilution series to transform qPCR data to numbers of IJs. Finally, a sub-sample of fresh soil was dried to allow quantification per 100 g of dry soil.

In addition to the EPN soil food web, we evaluated the EPN activity at post-application sampling times (**Table 3**), as previously described by Campos-Herrera et al. (2015a) and Jaffuel et al. (2016). Briefly, two aliquots of 200 g of fresh soil per sample were baited with larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) to test the suppressive potential of the soil. Following a modified procedure as described by Bedding and Akhurst (1975), each subsample (from augmented or not augmented plots) was baited with five final instar *G. mellonella* larvae (commercial stock, Au Pêcheur SARL Neuchâtel, Switzerland) in two independent rounds. After exposure for 4 days, the cadavers were recovered from the soil, thoroughly rinsed with tap water, and individually

placed in White traps (White, 1927). Under a stereoscope, we checked for nematode emergence every 2–3 days to determine the organisms responsible for larval mortality. We recovered the nematodes in tap water upon emergence. The cadavers for which no obvious cause of death could be determined after 1 month of incubation were discarded after dissection. The DNA of the progeny leaving the cadavers was extracted using the QIAamp DNA mini kit (Qiagen), purity checked (Nanodrop system), adjusted to the range of 0.5–1 ng/μl, and species identity assessed by qPCR as described above (Campos-Herrera et al., 2015a; Jaffuel et al., 2016).

Plant Traits

A total of eight measurements recorded the evolution of plant growth, productivity, and health. They were: average plant height per plot, plant density per plot, chlorophyll activity (N-tester), seed yield, thousand-seed weight, plant weight, plant protein content, and presence of pest insects and pathogens (**Table 3**). Regular monitoring of the experiments ensured the status of development into each phenostage. Most of the agronomical traits presented herein were measured at harvest (**Table 3**).

Statistical Analysis

All experimental field trials presented a Randomized Complete Block design (Figure S1). The data from each group of beneficial organisms were analyzed following standard procedures for their data presentation, transformation, standardization, and normalization whenever necessary. In the case of the EPN activity, data from the *G. mellonella* baits were expressed as the percentage of larval mortality per plot, averaged by treatment. The activity was determined with respect to the total mortality caused only by nematodes. For the EPN soil food web analysis, all the organisms (EPN, FLN, NF, and bacteria) quantified by using qPCR were expressed per 100 g of dry soil. The parasitism of nematodes by NF was expressed as "infection rate" (IR), which was calculated by dividing the DNA quantity of each species by the total amount of DNA (Campos-Herrera et al., 2012; Duncan et al., 2013). Similarly, to estimate the total FLN and NF, we divided all data within a species by the highest measurement for that species, which allowed the standardization of the units of measurement among species ranging from 0 to 1 (de Rooij-van der Goes et al., 1995).

Unless specified, all significant differences between treatments were assessed by one–way ANOVA, using Tukey's HSD test, considering block as co-variable (V 20.0, IBM SPSS Inc., Chicago, IL). In some cases, *t*-tests were employed to compare pre- and post-augmentation or control vs. a specific treatment. If necessary, data were transformed to conform the assumptions of normality and equal variances (transformation method is indicated with the respective statistics). The bacterial colonization data were statistically assessed with a non-parametric Kruskal-Wallis test, followed by a *post-hoc* test (Dunn's test). With the exception of the *Pseudomonas* root colonization data (presented as $\log_{10}$ of the obtained values ± SEM) all data are presented as mean ± SEM of untransformed values.

RESULTS

Survival and Persistence of *Pseudomonas* Inoculants

In the COMBINATION trial, *P. protegens* CHA0-Rif and *P. chlororaphis* PCL1391 reached similar population densities that surpassed the threshold of $\sim 10^5$ CFU per gram of roots, which is the level needed for a plant-beneficial effect (Haas and Défago, 2005) (**Figure 1A**; **Table 4**). However, in the PERFORMANCE-1 trial, the population density of the *P. chlororaphis* strain on wheat roots was significantly lower ($P < 0.05$) than the density of the *P. protegens* strain at all three monitoring times (**Figure 1B**; **Table 4**). In this trial, in general, for strain CHAO-Rif, alone or in the combinations, we observed a better progression of the population if compared with the strain PCL1391-Rif. If both strains were present in the same treatment, our agar plates almost only reported *P. protegens* CHA0-Rif colonies (personal observation). Moreover, in clear contrast to CHA0-Rif, PCL1391-Rif never approached the population threshold for plant-beneficial effects, neither when applied alone nor when combined with other BeSO, (**Figure 1B**; **Table 4**). In combination with a commercial population of the EPN *H. bacteriophora*, the density of CHA0-Rif was significant reduced for the June 2014 sample. July 2014, i.e., about 1 month later, CHA0-Rif still maintained its population density in presence of the nematode inoculant while PCL1391-Rif was no longer detectable (**Figure 1B**; **Table 4**). In the 2015 PERFORMANCE-2 trial, bacterial numbers approached or surpassed the threshold for plant-beneficial effects at all three sampling times (**Figure 1C**; **Table 4**). In general, no significant differences among treatments were observed, but there was a trend of higher bacterial population densities in the April 2015 and May 2015 samplings when bacterial inoculants where combined with the EPN inoculant mixture. In contrast, an opposite trend was observed for June 2015 samples. Finally, as already observed in the PERFORMANCE-1 trial, strain *P. protegens* CHA0-Rif dominated the colonization, while *P. chlororaphis* PCL1391 was hardly detected (Figure S2).

In all three field trials, no rifampicin-resistant bacteria were detected in the non-inoculated control treatments, in the buffer zones or in the grassland border zones at the experimental sites (data not shown), hence, the reported CFU data for the augmented bacteria required no baseline correction.

In general, the applied bacteria survived under field conditions until the end of the crop season (**Figure 1**), although the threshold required to provoke beneficial plant effects ($\sim 10^5$ CFE per g root) was not always attained in all the trials or treatments. Inoculation was successful in all, with good traceability of the different bacterial inocula without cross-contamination. Consistently, the *P. protegens* inoculant showed higher presence on wheat roots, but the effects of the combination with other BeSO were not conclusive and depended on the BeSO species, on the time of exposure to the field conditions and differed between trials.

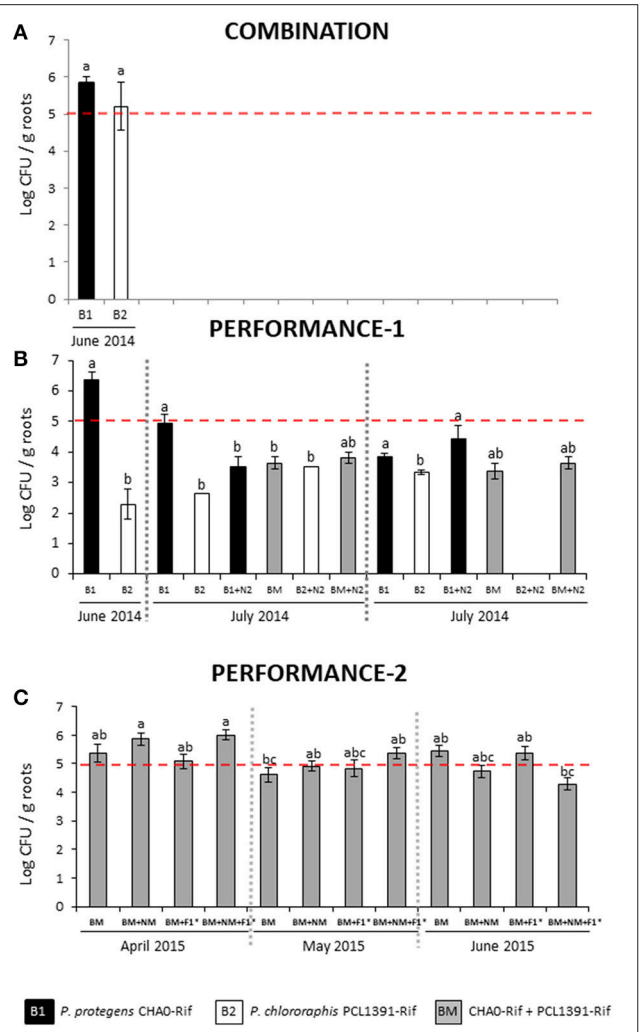


FIGURE 1 | Survival of *Pseudomonas protegens* strain CHA0-Rif (B1) and *Pseudomonas chlororaphis* strain PCL1391-Rif (B2) on wheat roots in the COMBINATION **(A)**, PERFORMANCE-1 **(B)**, and PERFORMANCE-2 **(C)** field trials. Bacterial strains were inoculated individually or in combinations with the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (N2), an EPN mixture (NM; comprising *Heterorhabditis megidis*, *H. bacteriophora*, and *Steinernema feltiae*) and the arbuscular mycorrhizal fungus *Rhizoglomus irregularis* (F1*). Inoculants were monitored by selective plating on KMB supplemented with rifampicin (100 μg/ml) and cycloheximide (100 μg/ml) at three different time points following seed furrow inoculation. The dashed red line indicates the generally agreed threshold (~10^5 CFU per g root) required to provoke beneficial plant effects with plant growth-promoting pseudomonads (Haas and Défago, 2005). Bar graphs show means of $\log_{10}$ transformed CFU values per gram of dry roots weight (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey *post-hoc* test, or with a non-parametric Kruskal-Wallis test (significance level $P < 0.05$), followed by Dunn's test for *post-hoc* comparisons. Different letters indicate statistical significance at $P < 0.05$. Inoculants were not detected in the buffer and border zones of the field assays. No Rifampicin-resistant background population was detected at the field sites.

AMF Inoculation Success

For the COMBINATION trial we mainly used the locally well-adapted AMF *R. irregulare* (Schlaeppi et al., 2016) (**Table 1**). We confirmed successful wheat root inoculation for both *R. irregulare* strains that we tested, as well as our custom strain SAF22 and the commercial inoculum INOQ Top (**Figure 2A**; **Table 4**). The higher dosage of the inoculum INOQ Top (80 g per row) corresponded approximately to the amount of SAF22 inoculum and it appeared that both *R. irregulare* strains colonized the wheat roots to a similar extent. The reduced dosage of the commercial inoculum (16 g per row) was reflected in lower levels of root colonization and only showed a minor tendency of augmentation. We also traced the inoculation of the *F. mosseae* strain SAF11 and the *C. claroideum* strain SAF12 using specific qPCR primers (Thonar et al., 2012). We did not detect these AMF species at the field site (data not shown) confirming the findings of a previous AMF community profiling (Schlaeppi et al., 2016). Hence, we concluded that these strains failed to establish at the tested field site in the wheat roots. In summary, *R. irregulare* could be augmented in wheat AMF communities using the strains SAF22 or INOQ Top, while this was not successful for *F. mosseae* SAF11 and *C. claroideum* SAF12.

Plots in which soil beneficial organisms showed low AMF colonization levels were not different from control (nothing applied) and mock (application of carrier substrate without AM fungus) plots (**Figure 2B**; **Table 4**). These measured abundances of *R. irregulare* correspond to the native strain in the field and indicated that the application of the carrier substrate on its own did not affect the root colonization by the AM fungus. Although, the level of root colonization by *R. irregulare* showed a slight tendency to increase in the combination treatments of the AM fungus with bacteria, nematodes or both, the AM fungus was not augmented to the same extent as in single application. These first insights on combining soil beneficial organisms suggest possible negative effects on the AMF inoculum if combined with bacteria or nematodes.

In the PERFORMANCE-2 experiment, the commercial *R. irregulare* strain INOQ Top was inoculated using a lower dosage level to larger plots compared to the previous experiments (**Table 2**). Again, there was a tendency of increased colonization of the wheat roots in the combined treatments, however, high inter-plot variation precluded statistic support for this effect (**Figure 2C**; **Table 4**). It remains to be validated whether the colonization by this *R. irregulare* strain is particularly facilitated if applied in combination with the *Pseudomonas* bacteria.

In summary, *R. irregulare* successfully colonized wheat roots if inoculated alone, and in the combination experiments, we found varying augmentation efficiencies for *R. irregulare* if combined with pseudomonads, EPN or both, indicating that interactions with these beneficial soil organisms are context dependent.

Nematode Survival, Activity, and Interactions with Soil Food Web Members

In all plots, very low numbers of background populations were detected as also found by Campos-Herrera et al. (2015a) and Jaffuel et al. (2017) in the same area. Five to seven species naturally occurred at the experimental field sites, and these species included the taxa that we augmented. Prior to inoculations (baseline; **Table 3**), there were no differences

TABLE 4 | Statistical analysis for beneficial soil organisms and plant traits in the three field experiments.

Organisms	Type of measurement	Statistical method	COMBINATION[a]	PERFORMANCE-1[a]	PERFORMANCE-2[a]
Bacteria	CFU quantification I	One–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	$F_{(1, 6)} = 0.1526$, n.s	$F_{(1, 2)} = 2.571$, **	$F_{(3, 15)} = 0.991$, n.s.
	CFU quantification II	One–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	–[b]	$F_{(5, 12)} = 3.675$, **	$F_{(3, 16)} = 0.656$, n.s.
	CFU quantification III	One–way ANOVA (Tukey's HSD test); Kruskal-Wallis (Dunn's test)	–	$F_{(4, 15)} = 2.311$, n.s.	$F_{(3, 16)} = 1.570$, n.s.
AMF	Quantification of INOQ Top[c]	One–way ANOVA	$F_{(2, 9)} = 10.42$, **	Not contributed	–
	Quantification of SAF22[c]	*T*-tests	$T = 10.377$ ***	Not contributed	–
	Quantification of INOQ Top in combination samples[c]	One–way ANOVA	$F_{(5, 18)} = 3.712$, *	Not contributed	$F_{(4, 36)} = 0.571$, n.s.
EPN	EPN presence: pre-inoculation (baseline)	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 1.273$, n.s.	$F_{(6, 21)} = 0.525$, n.s.	$F_{(4, 40)} = 0.281$, n.s.
	EPN presence: post-augmentation	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 4.604$, **	$F_{(6, 21)} = 2.194$, §	$F_{(6, 21)} = 2.888$, *
	EPN activity: insect-baits	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 3.317$, *	$F_{(6, 21)} = 1.243$, n.s.	$F_{(6, 21)} = 0.722$, n.s.
	Free-living nematodes: pre-inoculation	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 1.051$, n.s.	$F_{(6, 21)} = 0.498$, n.s.	$F_{(6, 21)} = 0.119$, n.s.
	Free-living nematodes: post-augmentation	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 0.395$, n.s.	$F_{(6, 21)} = 1.025$, n.s.	$F_{(6, 21)} = 0.341$, n.s.
	Nematophagous fungi: pre-inoculation	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 0.675$, n.s.	$F_{(6, 21)} = 0.288$, n.s.	$F_{(6, 21)} = 0.618$, n.s.
	Nematophagous fungi: post-augmentation	One–way ANOVA (Tukey's HSD test)	$F_{(7, 24)} = 5.820$, n.s.	$F_{(6, 21)} = 1.384$, n.s.	$F_{(6, 21)} = 0.582$, n.s.
Plants	Height (at harvest)	One–way ANOVA (Tukey's HSD test)	–	–	$F_{(8, 72)} = 1.009$, n.s.
	Density (% plot covered by plants at harvest)	One–way ANOVA (Tukey's HSD test) Kruskal-Wallis (Dunn's test)	–	$F_{(7, 88)} = 17.219$, ***	$F_{(8, 72)} = 0.756$, n.s.
	Chlorophyll activity (N-tester)	One–way ANOVA (Tukey's HSD test)	–	–	$F_{(8, 72)} = 0.161$, n.s.
	Yield (g seeds/plot)	One–way ANOVA (Tukey's HSD test)	–	$F_{(7, 23)} = 2.069$, **	$F_{(8, 72)} = 0.026$, n.s.
	Thousand-seed weight (TSW)	One–way ANOVA (Tukey's HSD test)	–	–	$F_{(8, 72)} = 0.129$, n.s.
	Protein content	One–way ANOVA (Tukey's HSD test)	–	–	$F_{(8, 72)} = 0.300$, n.s.

[a] *Data are presented as the statistical values, degree of freedom and probability levels: §P < 0.1, *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.*

[b] *For these variables, obtained data were not representative because of highly heterogeneous growth of the wheat plants within the plots following frit fly damage and thus were not considered for statistical analysis.*

[c] *Statistics corresponding to data from two sets of primers, i.e., by Alkan et al. (2006) for INOQ Top, and Bender et al. (unpublished) for SAF22.*

among treatments for any measured variable (EPN, free-living nematodes and nematophagous fungi; data not shown) in any of the three field trials. The evaluation of EPN soil food web members (free-living nematodes and nematophagous fungi) only revealed natural temporal fluctuations between baseline (pre-inoculation) and post EPN augmentation (data not shown), whereas their presence was not significantly affected by the EPN augmentation (alone or in combination) (Figure S3; **Table 4**). The nematophagous fungi and free-living nematodes species were in agreement with those already described by Campos-Herrera et al. (2015a,b) and Jaffuel et al. (2017). Finally, the ectophoretic bacterium *P. nematophilus* was not detected in any of plots (control or augmented).

In the COMBINATION trial, the EPN species *H. megidis* and *S. carpocapsae* were recovered in only 25% of the plots, 4 months after augmentation. In contrast, the species *S. feltiae* and *H. bacteriphora,* of which the latter was also combined with other BeSO, were detected in 100% of the plots, at the end of the

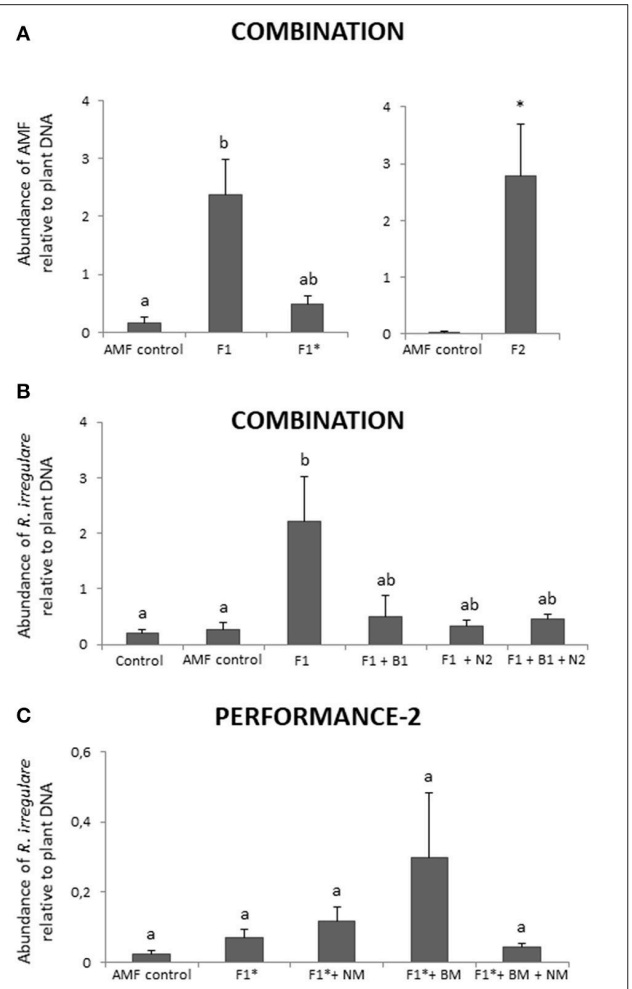


FIGURE 2 | Abundance of *Rhizoglomus irregulare* in wheat roots in the COMBINATION **(A,B)** and PERFORMANCE-2 **(C)** field trials. **(A)** In the COMBINATION experiment, *R. irregulare* strain INOQ TOP was inoculated comparing high (F1) vs. low (F1*) dosages, with one of the treatments including the AMF strain SAF22 (F2). **(B)** In the same experiment, *R. irregulare* INOQ TOP (F1) was quantified in combination with bacteria, i.e., *Pseudomonas protegens* CHA0-Rif (B1), and nematodes, i.e., *Heterorhabditis bacteriophora* Andermatt (N2). **(C)** In the PERFORMANCE-2 experiment, *R. irregulare* INOQ TOP at the lower dosage (F1*) was used for the combination treatments with bacterial mixture (BM; i.e., *P. protegens* + *Pseudomonas chlororaphis*) and nematode mixture (NM; i.e., *Heterorhabditis megidis* + *H. bacteriophora* + *Steinernema feltiae*; for details see Figure S1). Control, non-inoculated control; AMF control, substrate control for AMF inoculation. *R. irregulare* was measured with quantitative PCR employing species-specific primers developed by Alkan et al. (2006) for INOQ TOP or their modified variants with enhanced specificity for SAF22 (Bender et al., unpublished). Bar graphs report mean normalized (*R. irregulare* relative to plant DNA) abundance (± SEM; COMBINATION, $n = 4$; PERFORMANCE-2, $n =$ 7–9). Statistical analyses were performed on log-transformed data; asterisks and different letters indicate statistical significance at $P < 0.05$ for *t*-test and one-way ANOVA followed by the Tukey *post-hoc* test, respectively.

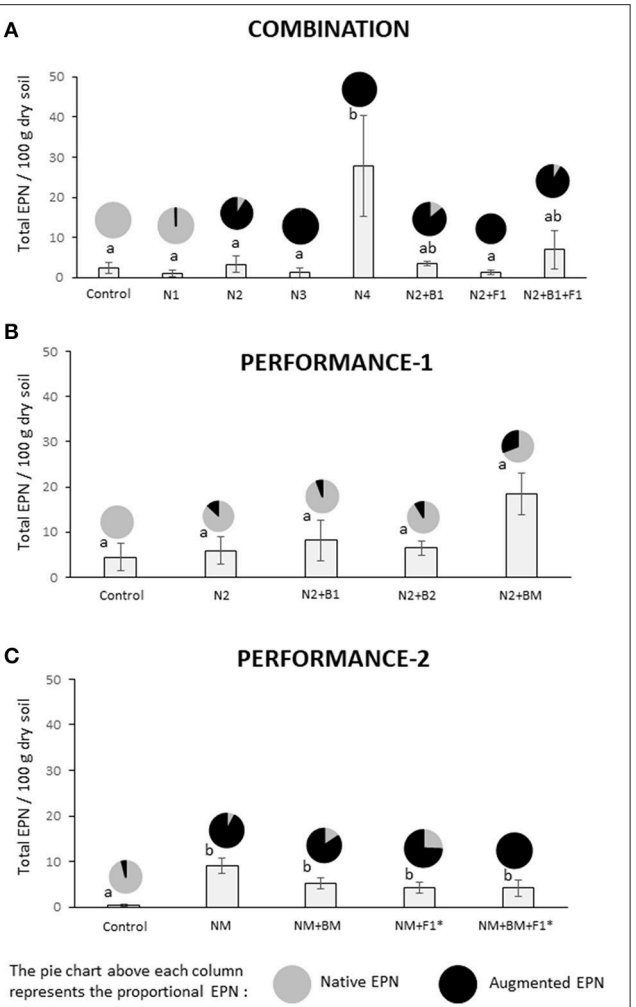


FIGURE 3 | End of the season presence of inoculant and resident entomopathogenic nematodes in the COMBINATION **(A)**, PERFORMANCE-1 **(B)**, and PERFORMANCE-2 **(C)** field trials. Four different EPN species *Heterorhabditis megidis* (N1), *Heterorhabditis bacteriphora* (N2), *Steinernema carpocapsae* (N3), and *Steinernema feltiae* (N4) were inoculated individually or in combination with *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2) and *Rhizoglomus irregularis* at two dosages (F1 and F1*). Mixtures of EPN (N1+N2+N4) or of the two bacteria (B1+B2) are indicated with NM and BM, respectively (for details see Figure S1). To determine the persistence of the EPN in soil of the different nematode inoculants as well as the impact of each treatment on the resident population of entompathogenic nematodes (EPN), a DNA extraction procedure followed by a qPCR approach was performed. Data are expressed as total EPN 100 g^{-1} of dry soil. Bar graphs report means (± SEM) and pie-charts show the proportion of native EPN vs. augmented EPN. Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey *post-hoc* test. Different letters indicate statistical significance at $P < 0.05$.

season. The augmentation with *S. feltiae* was the only treatment with a significant increase in total EPN numbers compared with the native populations (**Figure 3A**; **Table 4**). The remarkable persistence of *S. feltiae*, which was the only species detected in the soil in their corresponding plots, was in agreement with the nematode activity measured in the laboratory as % mortality of *G. mellonella* producing progeny. This was the only treatment with significantly higher activity than the control in the whole trial (**Figure 4A**; **Table 4**).

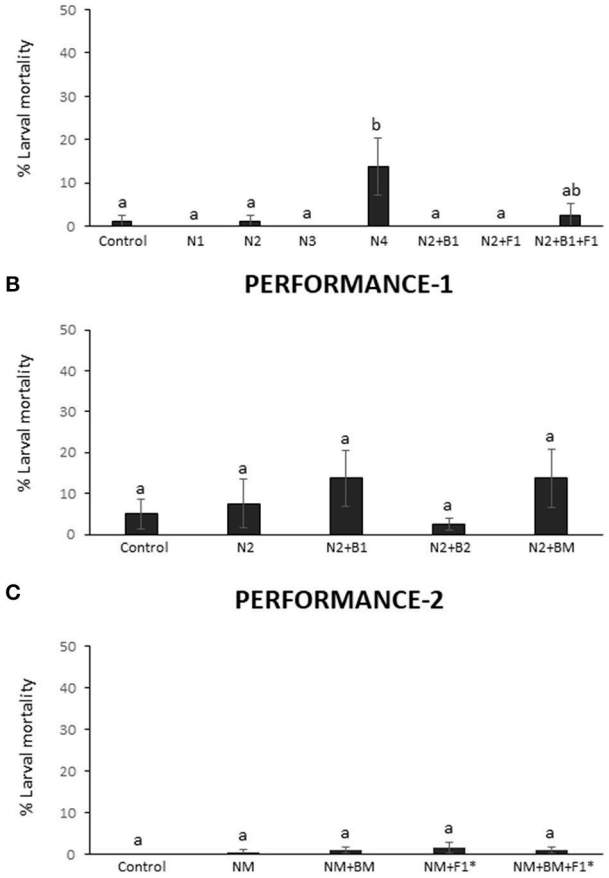


FIGURE 4 | Activity of entomopathogenic nematodes (EPN) post application in three field trials. EPN activity was quantified by a *Galleria mellonella* larvae infection assay in soil samples from the **(A)** COMBINATION, **(B)** PERFORMANCE-1 and **(C)** PERFORMANCE-2 trials. Inoculants were *Heterorhabditis megidis* (N1), *Heterorhabditis bacteriophora* (N2), *Steinernema carpocapsae* (N3), and *Steinernema feltiae* (N4), individually or in combination with *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2), and *Rhizoglomus irregularis* at two dosages (F1 and F1*). Mixtures of EPN or bacteria are indicated with NM and BM, respectively (for details see Figure S1). Bar graphs report means (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey *post-hoc* test. Different letters indicate statistical significance at $P < 0.05$.

In the PERFORMANCE-1 trial, we only augmented certain plots with *H. bacteriophora.* This EPN was detected in about 50% of the plots when applied alone or in combination with the bacterial inoculant *P. chlororaphis* and in about 75% of the plots when applied with the *P. protegens* in different combinations. No significant difference in EPN populations (qPCR measurements) and their activity (% larval mortality) was observed between plots where the EPN were applied alone or in combination with bacterial inoculants. Nor were they different from control plots (**Figures 3B**, **4B**; **Table 4**). In both trials, COMBINATION and PERFORMANCE-1, there was a slight trend to detect more *H. bacteriophora* in the combined treatment with AMF and/or bacterial inoculants (N2+B1+F1 and N2+BM, respectively) (**Figures 3A,B**). The same trend was also observed for nematode activity (**Figures 4A,B**). In the PERFORMANCE-1 field trials, *Steinernema affine* (**Table S1**) was the dominant native EPN species in the soil of the experimental plots as determined by qPCR (**Figure 3B**).

In the PERFORMANCE-2 trial, the augmented EPN species (a mix of *S. feltiae, H. megidis,* and *H. bacteriophora*) could be detected in 100% of the plots inoculated with the three EPN, alone or in combination with the *Pseudomonas* inoculants, in 91% of the plots where they were combined with AMF and in only 44.4% of the plots when combined with both bacterial and AMF inoculants. Again, the species *S. affine* was dominant among the native taxa as displayed in the proportional chart, although, contrary to the PERFORMANCE-1 trial, native species were largely displaced in all the treatments where EPN were applied (**Figure 3C**). All plots with EPN application showed significantly higher total numbers of EPN than the control plots (**Figure 3C**; **Table 4**). All the augmented EPN species were detected in each of the plots, but *S. feltiae* and *H. bacteriophora* dominated. The nematode activity was low and did not significantly vary among treatments (**Figure 4C**; **Table 4**). The progeny from the activity tests belonged mainly to *H. bacteriophora* (62.5%), followed by *S. feltiae* (34.5%), in all the cases we found mixed EPN-free-living nematodes emergence as observed in previous studies in Swiss soils (Jaffuel et al., 2016, 2017).

In general, inoculated EPN persisted during the crop season and remained active until the time for wheat harvest, but with limited pest suppressive potential as measured with a *Galleria* larvae infection assay. We observed that EPN application increased the total numbers of EPN only in specific treatments, displacing at least partially the native populations (**Figure 3**). No long-term effect was observed with respect to soil organisms that can be expected to be modulated by EPN augmentation, such as nematophagous fungi and free-living nematodes. The combined application of EPN with other BeSO indicated compatibility with respect to their persistence, prevalence, and activity, when compared with the single EPN application, but some differences depending on EPN species and co-inoculant identity were observed. As for the bacterial and AMF inoculants, the success of EPN inoculants appeared to be context dependent.

Agronomic Impact of the Applied Beneficial Soil Organisms

The 2014 trials (COMBINATION and PERFORMANCE-1) were intentionally not subjected to standard pesticide treatments and suffered from heavy attack by frit flies (*Oscinella frit*). For the small scale COMBINATION trial, insect damage was very patchy and therefore not agronomically representative and not included in the plant performance analyses. The larger plot sizes in the PERFORMANCE-1 trial permitted analysis of agronomically relevant plant density and seed yield data (**Table 4**). The % of plot surface covered with plants was significantly higher in augmentation plots than in the control

treatment when the two bacterial inoculants, *P. protegens* and *P. chlororaphis*, were applied individually (treatments B1 and B2, respectively) or as a mixture with and without the EPN (treatments BM and B1+B2+N2, respectively) (**Figure 5A**). Seed yield per plot followed a similar pattern, but only the combined treatment with both bacterial strains and the EPN showed significantly higher values than the control (**Figure 5C**). AMF effects could not be examined in the PERFORMANCE-1 trial due to limited inoculum production. Nevertheless, the neighboring COMBINATION experiment indicated that seedling survival after frit fly attack tended to be higher in plots inoculated with *R. irregulare* (Figure S4). In the 2015 PERFORMANCE-2 trial, plots were subjected to pesticide treatment, no pest damage was observed and all plant traits were considered in the analysis. However, none of these measures, including plant density and seed yield per plot (**Figures 5B,D**) nor the other plant performance traits (Figure S5; **Tables 3**, **4**) differed significantly from the control treatment.

In summary, when wheat was exposed to biotic stress (i.e., a heavy insect pest attack in 2014) a significant positive effect of the application of BeSO, notably *Pseudomonas* bacteria, on performance of the crop was observed. The presence of the EPN was only beneficial when combined with both bacteria together. In absence of a biotic stress conditions, as in the PERFORMANCE-2 trial in 2015, there was no measurable plant-beneficial effect of the presence of BeSO, highlighting the context dependence of their protective effect on the crops.

DISCUSSION

Overall, the three field experiments showed consistent results: (1) the inoculated BeSO persisted until the end of the crop season, although their prevalence gradually declined with time; (2) in most of cases, the introduced BeSO in augmented plots were consistently present at higher levels than the native populations, without cross-contamination between plots; (3) the augmented BeSO integrated with or displaced the natural community to varying degrees depending on the strain/population and dosage; and (4) the combined application of *Pseudomonas*, EPN, and AMF showed only beneficial effects under conditions with an insect outbreak. In particular and contrary to our expectations, our current tripartite BeSO inoculant system (bacteria + EPN + AMF) did not provide clear additive or synergistic positive effects to allow a better performance of wheat than the application of

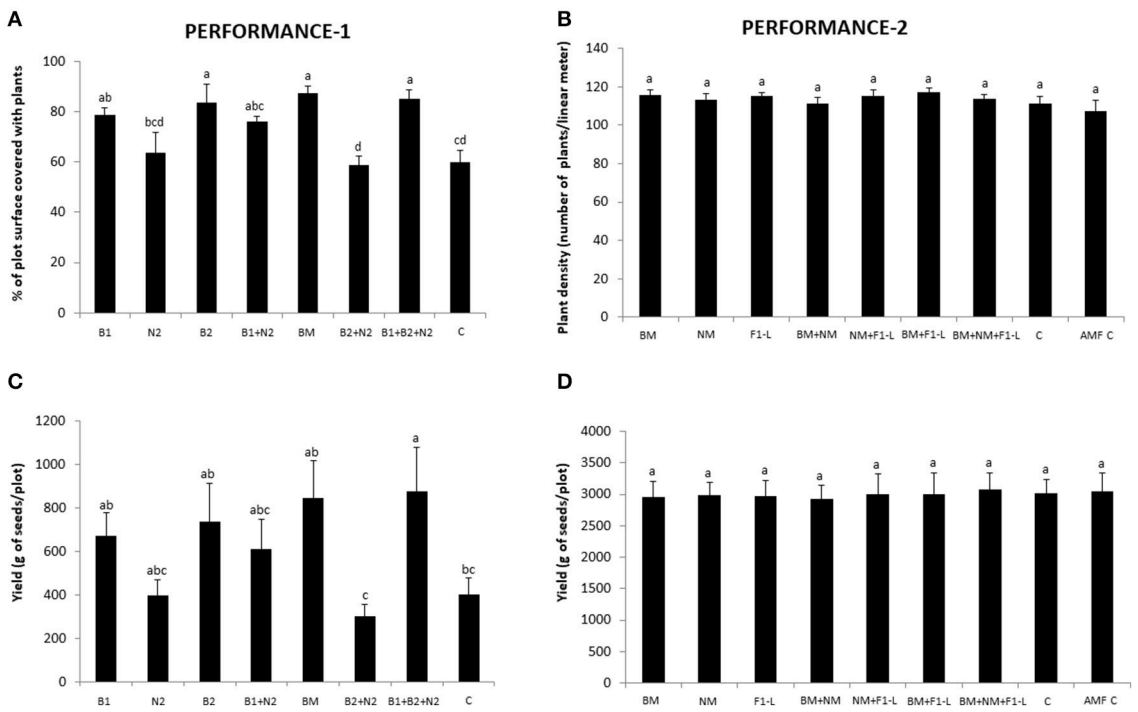


FIGURE 5 | Impact of field inoculations with beneficial organisms on plant performance in the PERFORMANCE-1 **(A,C)** and PERFORMANCE-2 **(B,D)** trials. Plant performance was evaluated in terms of plant density **(A,B)** and yield (weight of wheat seeds) **(C,D)** for each plot. The PERFORMANCE-1 experiment was exposed to heavy natural infestation with the firt fly (*Oscinella frit*) causing significant plant damage. Plant density in the PERFORMANCE-1 trial was therefore determined by visual scoring the percentage of plot area covered by wheat plants in this experiment while it was determined by counting the number of plants per linear meter in the PERFORMANCE-2 experiment, which had no measurable frit fly damage. Inoculants were *Pseudomonas protegens* (B1), *Pseudomonas chlororaphis* (B2), individually or in combination with *Heterorhabditis bacteriophora* (N2) and *Rhizoglomus irregularis* (F1*). Mixtures of the two bacteria or of the entomopathogenic nematodes (*Heterorhabditis megidis*, *Heterorhabditis bacteriophora*, and *Steinernema feltiae*) are indicated with NM and BM, respectively (for details see Figure S1). C, non-inoculated control; AMF-C, substrate control for AMF inoculation. Bar graphs report means (± SEM). Significant differences between treatments were calculated with one-way ANOVA (significance level $P < 0.05$) followed by the Tukey *post-hoc* test. Different letters indicate statistical significance at $P < 0.05$.

the individual BeSO. Overall, our results are in agreement with the previous observation that the combination of various BeSO can lead to a beneficial effect under certain conditions (Frey-Klett et al., 2007; Ansari et al., 2010; Walker et al., 2011; Couillerot et al., 2012), but mainly have similar effects as single applications (Tarasco et al., 2011; Glare, Hurst, and Narciso, personal communication). We can conclude that there is still a large gap between the promising results from BeSO applications under controlled experiments (laboratory and greenhouse settings) and their performance under field conditions.

Many factors can explain this difference between applications in laboratory/greenhouse and field settings. The characteristics of a particular agroecosystem (i.e., soil type, soil geochemistry, humidity, plant genotype, climate, etc.) play a decisive role in determining the success of augmented BeSO. From a biogeographic point of view, the selection of the BeSO should take in consideration the biology and ecology of the BeSO. The soil and environmental conditions in the target soils should match the conditions within the range of the natural occurrence of the BeSO, in order to obtain the desired activity. The soil is a complex medium, with physicochemical and biological interactions that vary over time and space (Ritz and van der Putten, 2012). In the three trial, the general characteristics of the soil were largely similar (**Table 2**), although unnoticed microhabitat differences might patchily occur and produce internal stochasticity, a factor that is better controlled in any greenhouse experiment where often soils are homogenize first and treatments are confined to smaller experimental units such as pots. In a field experiment, fundamental differences in soil chemistry (acid soils vs. basic soils, presence of micronutrients, etc.) and soil physical properties (texture, pore size, compaction, available water, etc.), should be considered to select the most appropriate BeSO (Schlaeppi and Bulgarelli, 2015). For example, AMF mostly perform better in low nutrient soils (Pellegrino et al., 2012, 2015). Also the effects of AMF on crop productivity are highly dependent on the plant species or genotypes investigated (Lekberg and Koide, 2005): plants and crops with fine roots such as wheat (as in this study) are usually less responsive to AMF compared to species with thicker roots such as red clover (Köhl et al., 2016). Similarly, EPN species have ecological and habitat preferences that are largely determined by texture and moisture of soils (Campos-Herrera et al., 2013, 2016; El-Borai et al., 2016). Soil physico-chemical characteristics can also impact persistence and activity of *Pseudomonas* species (Natsch et al., 1996; Troxler et al., 2012; Mascher et al., 2014; Imperiali et al., 2017). Hence, locally adapted species might have an advantage in persistence over exotic organisms that are not present in the target soil (Schlaeppi et al., 2016).

In addition to the abiotic soil conditions, BeSO inoculants are also subjected to interactions with the resident soil organism community. The diversity of soil organisms can contribute to buffering, masking and silencing beneficial effects of inoculations. Again, this is a major difference with controlled experiments in the growth chamber or greenhouse where conditions usually limit or simplify the interactions of inoculant BeSO with the naturally present soil organisms and the target crop. Often laboratory or greenhouse experiments are conducted with sterilized soils, with entirely or greatly reduced abundance of native soil organisms. Under field conditions, there are also spatial and temporal differences in these effects on the augmented BeSO. This is particular relevant when considering naturally occurring populations of the BeSO. In our experiments, we observed that the native populations of AMF and EPN were displaced to varying degrees, depending on the BeSO species/population inoculated in the field plots. In agreement with Schlaeppi et al. (2016) and Jaffuel et al. (2017), we also observed that augmented BeSO species that also occurred naturally in the area performed better than those that were not represented or only at low numbers. The fact that virtually no cross-contaminations with inoculants occurred between plots and in many cases the displacement of native populations was corrected by the time of harvest, i.e., returning to the original numbers/presence of native populations, underscore that these introductions have only low and transient impacts on the native populations. Yet, more studies are needed to evaluate the potential long-term impacts of implementing inoculation strategies of single or combined BeSO, especially if inoculants are not native or no present in the area of application (Abate et al., 2017; Hardt et al., 2017).

Here we introduce a comprehensive toolbox to trace Pseudomonads, AMF, and EPN after application. Some of the BeSO did not reach the numbers known to be required to reach beneficial plant effects (Haas and Défago, 2005), did not persist well-after application (i.e., the EPN species *H. megidis* and *S. carpocapsae*), or did not establish following field inoculation (i.e., the AMF species *F. mosseae* and *C. claroideum*). Nevertheless, results for some isolates and combinations were highly promising. Under the experimental field settings, the bacterium *P. protegens* CHA0, the AMF *R. irregularis* and the EPN *S. feltiae* established very well. Under conditions with high biotic stress (frit fly infestation in the PERFORMANCE-1 trial), the combination of bacterial and EPN inoculants produced the highest yields. Because such ecological conditions will change from one season to another, the development of a pre-application diagnosis tool may help the choice of an optimized BeSO (Schlaeppi and Bulgarelli, 2015; Schlaeppi et al., 2016). For example, areas strongly impacted by plant diseases and pests might benefit from the integration of various *Pseudomonas* bacteria. Whereas, the presence of insect pests will better support the development and persistence of native and augmented EPN, thereby enhancing their protective effects. Finally, selecting BeOS, in particular AMF, that are compatible with local soil conditions (e.g. low or high nutrient content) is highly advisable (Pellegrino et al., 2015; Schlaeppi et al., 2016).

Advancing our understanding of the soil-plant interface in its broadest sense is critical to achieve sustainable agriculture (Adl, 2016). We evaluated the simultaneous application of three types of BeSO (bacteria, EPN, and AMF) and its impact on wheat productivity under realistic field conditions. While we confirmed the prevalence and persistence of the three organisms throughout the season, their beneficial effects were variable and differed between inoculant strains. Clear beneficial effects on wheat growth were observed only when the plants were exposed to high insect infestation. We learned that there is still a major

gap in our understanding of the capacities of BeSO to enhance plant performance under well-controlled conditions and their performance and impacts when applied to the field. We believe that to close this gap and for the successful use of BeSO in agroecosystems there is an urgent need to unravel the context dependency of effective BeSO augmentations. Optimizations should go toward adapting and fine-tuning the selection of inoculant strains that are well-adapted to local abiotic and biotic soil conditions. Advancing such an integrative and context-dependent approach is vital before next-generation, sustainable agriculture, in which field crops are protected by applying beneficial soil organisms instead by agrochemicals becomes imaginable.

AUTHOR CONTRIBUTIONS

KS, MvdH, MM, FM, TT, CK, and RCH planned the experiments and supervised the study. NI, XC, KS, GJ, SB, FD, MF, RBP, DV, MvdH, MM, FM, CK, and RCH contributed in the field experiments and collected the data. NI, XC, KS, MF, CK, and RCH analyzed the data, discussed the main structure and wrote the manuscript. All authors contributed to revisions and commented on previous versions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01809/full#supplementary-material

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Screening and Characterization of Potentially Suppressive Soils against *Gaeumannomyces graminis* under Extensive Wheat Cropping by Chilean Indigenous Communities

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Wheat production around the world is severely compromised by the occurrence of "take-all" disease, which is caused by the soil-borne pathogen *Gaeumannomyces graminis* var. tritici (Ggt). In this context, suppressive soils are those environments in which plants comparatively suffer less soil-borne pathogen diseases than expected, owing to native soil microorganism activities. In southern Chile, where 85% of the national cereal production takes place, several studies have suggested the existence of suppressive soils under extensive wheat cropping. Thus, this study aimed to screen Ggt-suppressive soil occurrence in 16 locations managed by indigenous "Mapuche" communities, using extensive wheat cropping for more than 10 years. Ggt growth inhibition *in vitro* screenings allowed the identification of nine putative suppressive soils. Six of these soils, including Andisols and Ultisols, were confirmed to be suppressive, since they reduced take-all disease in wheat plants growing under greenhouse conditions. Suppressiveness was lost upon soil sterilization, and recovered by adding 1% of the natural soil, hence confirming that suppressiveness was closely associated to the soil microbiome community composition. Our results demonstrate that long-term extensive wheat cropping, established by small Mapuche communities, can generate suppressive soils that can be used as effective microorganism sources for take-all disease biocontrol. Accordingly, suppressive soil identification and characterization are key steps for the development of environmentally-friendly and efficient biotechnological applications for soil-borne disease control.

Keywords: ***Gaeumannomyces graminis,*** **Mapuche, microbial communities, suppressive soils,** ***Triticum aestivum, take-all disease,*** **biocontrol**

INTRODUCTION

Currently, the main concerns in modern agriculture are those associated with the effect of climate change on biotic and abiotic parameters in arable soils, and their impact on crop yields and food supply at global level (Soussana et al., 2010). In this context, several authors have pointed out an increase of soil-borne disease incidence in winter cereals as a consequence of climate change (French et al., 2009; Manici et al., 2014). Similarly, these studies have also described that climate change may contribute to certain soil-borne pathogen migration toward niches or regions previously uncolonized by these pathogens (French et al., 2009).

The southern region of Chile produces around 85% of cereals, where 40% is wheat (*Triticum aestivum* L.; ODEPA, 2016). However, wheat production is frequently reduced by the incidence of "take-all" disease, which is caused by the soil-borne pathogen *Gaeumannomyces graminis* var tritici (Ggt; Andrade et al., 2011), causing the highest wheat crop losses in Chile (Moya-Elizondo et al., 2015). Soil-borne pathogen incidence in cereal cropping is difficult to control due to their natural persistence in soils and the inefficiency of chemical controls (De Coninck et al., 2015); thus, biological control becomes a very promising alternative to prevent soil diseases. The incidence of take-all disease outbreak is favored under particular soil conditions, called "conducive" soils (Chng et al., 2015). In contrast, "suppressive" soils occur as a natural phenomena, preventing soil-borne pathogen establishment or reducing disease incidence (Jara et al., 2011) even in the presence of a susceptible host plant and favorable soil or climate conditions. In this context, several studies have shown that native soil microorganism activity can be pivotal in Ggt disease suppression (Weller et al., 2002; Cook, 2003; Mendes et al., 2011, 2013). Ggt (and other soil-borne pathogens) suppressive soils have been reported and characterized around the world (Bull et al., 1991; Bithell et al., 2012; Chng et al., 2013).

In the twentieth century, agrarian policies resulted in the establishment of numerous small farmers practicing extensive agriculture in southern Chile, particularly in the "Region de La Araucania" (38°54′00″S; 72°40′00″S) (Clapp, 1998). Most farmers belong to the "Mapuche" ethnic group, which represents 50% of the total population in La Araucania. The Mapuche community is characterized by the use of ancestral agronomic techniques to produce their own agricultural products without the application of chemicals such as, commercial fertilizers and pesticides. The extensive Mapuche agriculture is mainly directed to family group subsistence. Under this scenario, we hypothesize that this long-term land-use could represent a natural and effective source of suppressive soils against soil-borne pathogen diseases. Thus, suppressive soils may also be relevant in the context of alterations related to soil-borne pathogen incidence and migration that have been predicted by climate change, and the deleterious effect on intensive agro-chemical product use in arable soils (Meza and Silva, 2009; Neuenschwander, 2010). In fact, Andrade et al. (2011) detected five Ggt-suppressive soils located in La Araucania with a long history of monoculture and natural pasture. Similarly, Arismendi et al. (2012) reported the presence of *Pseudomonas fluorescens* strains, which are able to produce 2, 4-DAPG, a known biocontrol compound involved in soil Ggt suppression (Mavrodi et al., 2012; Weller et al., 2012), in 13 soils from La Araucania and Los Lagos regions. Moreover, we have recently isolated and characterized four endophytic bacteria from wheat plants in this area (*Acinetobacter* sp. E6.2, *Bacillus* sp. E8.1, *Bacillus* sp. E5 and *Klebsiella* sp. E1), which are able to inhibit Ggt mycelia growth *in vitro,* promote plant growth, and diminish take-all disease incidence under greenhouse experiments (Durán et al., 2014). Although most studies on Ggt suppression have focussed on bacteria, there are some reports showing that some fungal strains can also reduce Ggt incidence (Macia-Vicente et al., 2008). These examples illustrate the great potential of native microorganisms as soil inoculants able to increase plant growth and prevent soil-borne pathogen disease incidence in cereal cropping. Despite of this potential, studies focusing on Ggt-suppressive soils occurrence, and their characterization, are still very limited.

The main objective of our study was to screen Ggt-suppressive soils in 16 locations managed by indigenous Mapuche communities from La Araucania, southern Chile, where extensive wheat cropping was applied for more than 10 years. Soil chemical properties and microbial community composition were characterized. Furthermore, soils were categorized as Ggt suppressive or conducive based on their potential for pathogen inhibition *in vitro*, and their efficacy in controlling take-all disease under greenhouse conditions.

MATERIALS AND METHODS

Soil Sampling

In southern Chile, acidic volcanic soils (Andisols and Ultisols) are the predominant soil types supporting the bulk of agricultural and forestry production. Andisols include modern and recent ash deposits, and Ultisols correspond to ancient deposits (Andrade et al., 2011). Soil samples were collected from extensive wheat cropping areas managed by Mapuche communities in 16 locations from La Araucania (**Figure 1A**; **Table 1**). Nine samples (number 3, 4, 5, 7, 10, 12, 13, 14, 15, and 16) were taken from soils with a long rotation history between wheat monoculture and natural pasture for more than 10 years, and six samples (number 2, 6, 8, 9, 11, and 12) were taken from soils with wheat monoculture including oat rotation. An additional sample number 1 was taken from a Ggt-conducive soil with wheat, including clover rotation, and used as positive control. Parent material from soils 1 to 10 was classified as Andisol, whereas 11–16 were classified as Ultisols.

Samples were collected from rhizosphere and bulk soil at 0–20 cm depth, and then stored in a cold room at 5°C until usage. Soil sample chemical properties were determined as follow. Briefly, available P (P_{Olsen}) was extracted by using 0.5 M Na-bicarbonate and analyzed by using the molybdate method (Murphy and Riley, 1962). Organic matter contents were estimated by wet digestion (Walkley and Black, 1934). Soil pH was measured in 1:2.5 soil/deionized water suspensions. Exchangeable potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+) were extracted with 1 M ammonium acetate (CH_3COONH_4) at pH 7.0 and analyzed by flame atomic adsorption spectrophotometry

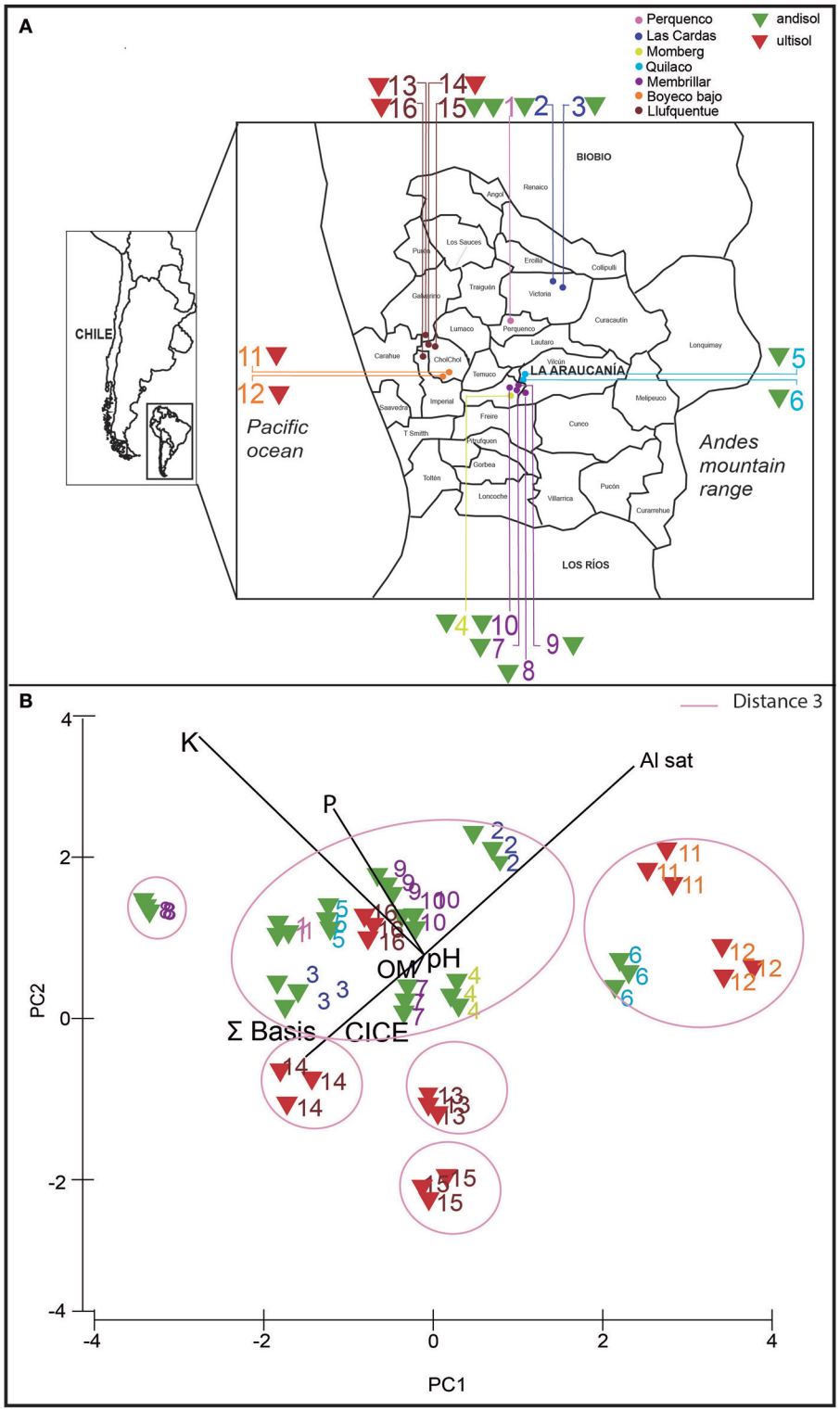


FIGURE 1 | Sampling locations **(A)** and principal component analysis (PCA) analysis **(B)** based on chemical properties of 16 rhizosphere soils used in this study. Triangle colors indicate type of soil: andisol (green) and ultisol (red).

TABLE 1 | Sample locations and cropping history of soils used in this study.

Soil	Parent material	Location	GPS coordinates	Cropping history
1	Andisol	Perquenco	38°22′25.8″S 72°23′07.5″W	Conducive soil (control)
2	Andisol	Las Cardas-1	38°19′09.3″S 72°17′03.9″W	Wheat- triticale- oat (more than 10 years)
3	Andisol	Las Cardas-2	38°19′32.7″S 72°17′18.6″W	Wheat (10 years)- natural pasture (10 years)-wheat
4	Andisol	Momberg	38°50′30.5″S 72°32′21.4″W	Natural pasture (10 years)-clover- wheat-wheat
5	Andisol	Quilaco-1	38°43′25.9″S 72°27′19.2″W	Natural pasture (10 years)-wheat
6	Andisol	Quilaco-2	38°43′35.7″S 72°27′34.5″W	Clover (5 years)-oat- wheat
7	Andisol	Membrillar-1	38°45′01.6″S 72°28′28.4″W	Natural pasture-wheat (15 years)
8	Andisol	Membrillar-2	38°45′15.1″S 72°26′63.2″W	Oat-wheat-wheat (30 years)
9	Andisol	Membrillar-3	38°44′57.9″S 72°28′58.7″W	Wheat-oat+natural pasture
10	Andisol	Membrillar-4	38°44′52.8″S 72°29′21.3″W	Natural pasture (10 years)-wheat (2 years)
11	Ultisol	Boyeco bajo-1	38°39′00.4″S 72°41′41.7″W	Wheat-oat-wheat-wheat (more than 20 years)
12	Ultisol	Boyeco bajo-2	38°39′48.8″S 72°42′08.8″W	Natural pasture (10 years)-wheat- oat- wheat
13	Ultisol	Llufquentue-1	38°25′44.5″S 72°46′12.3″W	Wheat- wheat- natural pasture- wheat (more than 10 years)
14	Ultisol	Llufquentue-2	38°28′55.1″S 72°43′25.6″W	Wheat (more than 20 years)
15	Ultisol	Llufquentue-3	38°28′53.1″S 72°43′27.6″W	Wheat (more than 20 years)
16	Ultisol	Llufquentue-4	38°29′38.2″S 72°45′26.2″W	Natural pasture (10 years)-wheat- wheat

(FAAS) (Warncke and Brown, 1998). Exchangeable aluminum (Al^{3+}) was extracted with 1 M KCl and analyzed by FAAS (Bertsch and Bloom, 1996). All samples analyses were made in triplicate. To group and determine significant differences between samples based on their chemical properties, data were imported into the PRIMER 7 software (PRIMER-E Ltd, Ivybridge, UK), transformed and normalized using square-root followed by a log (Xþ1) transformations (Lee et al., 2012). Then, a distance matrix was generated based on Euclidean distances and samples were grouped by hierarchical clustering (group average), and then visualized by principal component analysis (PCA).

Bacterial Community Composition and Ggt Detection in Soil Samples

Bacterial community compositions in rhizosphere and bulk soil samples were examined by denaturing gradient gel electrophoresis (DGGE), according to Iwamoto et al. (2000). Total genomic DNA was extracted from 0.5 to 1 g of soil samples using the PowerSoil® DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, CA, USA), according to manufacturer's instructions. The 16S rRNA gene fragments were amplified by touchdown PCR, using EUBf933-GC/EUBr1387 primer set (Iwamoto et al., 2000). DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc., USA). The PCR product (20 μL) was loaded onto a 6% (v/v) polyacrylamide gel with a 40–70% gradient (urea and formamide). Electrophoresis was run for 12 h at 100 V. Banding profiles were visualized by staining the gel 1:10.000 (v/v) with SYBR Gold (Molecular Probes, Invitrogen Co., USA) for 30 min, followed by image capture using GelDoc-It^{TS2} Imager (UVP, Upland, CA, USA). DGGE banding profile clustering, using a dendrogram, was also carried out by using Phoretix 1D analysis software (TotalLab Ltd., UK). The correlation between bacterial communities (biological parameters) and chemical soil properties (ecological parameters) was visualized by non-metric multidimensional scaling (MDS) analysis using Primer 7 + Permanova software (Primer-E Ltd., Ivybridge, UK) (Clarke, 1993). The *in silico* analysis was also used to estimate the bacterial diversity by richness (S), Shannon–Wiener, and dominance by Simpson Index (D), represented by 1-D or 1-λ (Sagar and Sharma, 2012).

Ggt occurrence in soil samples: DNA extracts from soil samples were subjected to PCR using the primer set NS5 (5′-AAC TTA AAG GAA TTG ACG GAA G-3′) and GGT-RP (5′-TGC AAT GGC TTC GTG AA-3′) designed by Fouly and Wilkinson (2000) specifically for Ggt. The PCR conditions were as follow: an initial denaturation at 93°C for 3 min, followed for 93°C for 1 min, 52°C for 1 min, and 72°C for 1 min to 35 cycles, and finally with 72°C for 5 min. All soil samples were tested in triplicate, and pure *G. graminis* var *tritici* (Andrade et al., 2011) and *Aspergillus niger* DNA (code CCT-UFRO 15.62), obtained from La Frontera University Type Strain Culture Collection (http://ccct.ufro.cl/), were used as positive and negative controls, respectively. The presence of Ggt in soil samples showing positive Ggt reaction was also confirmed by the presence of take-all disease symptoms, chlorosis, and blackening roots in wheat seedlings in the pot assays.

Putative Ggt-Suppressive Soil Screening

Putative Ggt-suppressive soil screening was performed by two *in vitro* inhibition tests using rhizosphere soil samples as follows:

In vitro Inhibition Test on Solid Media

A first screening was carried out in order to evaluate rhizosphere soil capability of inhibiting Ggt growth on agar plates (Supplementary Figure 1A). Briefly, the Ggt inoculum was produced by growing the fungus on potato dextrose agar (PDA) medium at 25°C for 1 week. Agar disks (4-mm diameter) containing Ggt were aseptically incised and transferred to the center of agar plates containing fresh Luria Bertani (LB) and PDA medium (proportion 1:1). A hole of 10 mm was performed in the agar medium at a distance of 3 cm from Ggt inoculum, and 0.05 g of rhizosphere soil were placed in the agar hole. Ggt mycelia growth was registered at 3, 5, and 7 days of incubation at 25°C in the darkness, as described by Liu et al. (2011). A fraction of all soil samples was sterilized and samples included in the agar as negative controls. All tests were carried out in triplicate.

In vitro Inhibition Test in Aqueous Soil Extracts

Because some soils contained elevated loads of microorganisms affecting fungal measurements on agar medium (categorized as un-determined in the *in vitro* test described in Section *In vitro* Inhibition Test on Solid Media), a second assay was performed in tubes with rhizosphere soil extracts (Supplementary Figure 1B). Briefly, 1 g of rhizosphere soil sample was suspended in 9 ml of sterile phosphate buffer saline (PBS; pH 7.4), and sonicated at 60% amplitude for 30 s. Then, 1 mL of supernatant was added to an eppendorf, and then inoculated with 1% Ggt inoculum. Soil extract tubes were incubated at room temperature for 3, 5, and 7 days, and fungal growth was estimated by quantification of fungal biomass by crystal violet (CV) staining as follows. After incubation, soil extract samples were washed with distilled water and fixed with 500 μl methanol for 15 min at room temperature. Later, they were centrifuged at 13,000 rpm × 1 min, supernatants were discarded, and tubes were air-dried; 400 μl of CV was added to each tube and incubated for 5 min. Tubes were washed three times with distilled water. Finally, 400 μl of acetic acid (33% v/v) were added and kept in the tubes for 5 min. The absorbance of the obtained solution was determined in triplicate in a multi-plate reader at 590 nm (Silva et al., 2009, Supplementary Figure 1B).

Nine putative suppressive soils from the inhibition tests, in both agar plates and soil extract tubes, were used for the greenhouse assay, using soil 1 as Ggt-conducivepositive control.

Take-All Disease Suppression Assay in Greenhouse

Inoculum Preparation

The characterization of the pathogen as Ggt was done based on the sequencing of ribosomal internal transcribed spacer 2 region (ITS2). ITS2 was amplified by touchdown polymerase chain reaction (PCR) with primer sets fITS9 (5′-GAACGCAG CRAAIIGYG-3′) and ITS4 (5-′TCCTCCGCTTATTGATATGC-3′), using the following conditions: an initial denaturation at 95°C for 3 min, followed by 25 cycles—each at 95°C for 30 s, with an annealing step with a 0.5°C decrease—each cycle from 65°C to 52.5°C, and extension at 72°C for 30 s. Twenty-five additional cycles were then carried out at 95°C denaturation for 30 sec, 55°C annealing, primer extension at 72°C for 30 s, and a final extension step of 7 min at 72°C. The PCR products were purified and sequenced by Austral-Omics (Universidad Austral of Valdivia-Chile). The sequence was compared with those in the GenBank database, fungal identity was confirmed (99%), and then deposited under accession no. KY689233.

The Ggt inoculum was prepared as follows: oat kernels were soaked in water for 24 h and sterilized for 3 consecutive days. Then, Ggt pathogenic isolate discs were grown on PDA for 7 days, put on the sterile oat, and maintained at room temperature for 20 days. Colonized oat kernels were blended, sieved to a particle size of 0.5–1.0 mm, and stored at 5°C until usage (Andrade et al., 2011).

Greenhouse Assay

Nine putative suppressive soils (number 2, 3, 4, 6, 11, 13, 14, 15, and 16), selected according to results obtained in the *in vitro* tests (Section Putative Ggt-Suppressive Soil Screening), were tested in their ability to suppress take-all disease *in planta* under greenhouse conditions. Plastic containers enclosing 200 g of soil were used in quintuplicate. Wheat seeds Otto cv were surface sterilized (15% ethanol plus 1% sodium hypochlorite for 2 min) and 5 seeds were used for each treatment. Plants were watered every 3 days, and Taylor and Foyd nutrient solution (Taylor and Foyd, 1985) was applied each 15 days.

The experimental design consisted in sterile (heat-treated) rhizosphere soil to determine disease level incidence, discarding, or diminishing the effect of soil microorganisms; untreated, air-dried soil (natural) to determine the effect of soil native microorganisms; and, sterile soil +1% untreated, natural soil (ster+1% nat) to assess suppression transferability to sterile soils. Suppression transferability has been shown to occur when natural soils are added in as low as 1% (v:v) to non-suppressive soils, as reported earlier (Shipton et al., 1973; Andrade et al., 1994). Ggt inocula were applied at 0.1% in relation to soil weight (2 g), and all treatments were also performed in soil without Ggt inocula as controls. After 40 days, plants were carefully removed from the soil, weighted and the root blackening percentage was determined.

Ggt Presence in Plant Tissues

The Ggt presence was evaluated in roots of PCR infected and non-infected plants. Roots were individually assessed for infection and root blackening percentage was evaluated against a white background. Shoots were carefully separated from the roots, placed into individual paper envelopes, and dried at 70°C for 72 h, to obtain shoot dry weight. In order to confirm Ggt infection, total DNA from wheat infected tissues was extracted with soil DNA Isolation Kit (Ultraclean, Mo-Bio Laboratories) according to manufacturer's instructions. Specific Ggt DNA fragments were amplified by using NS5 and GGT-RP primer sets, as described in Section Bacterial Community Composition and Ggt Detection in Soil Samples. Pure DNA extracts from Ggt and *A. niger* collection strains were used as positive and negative control, respectively.

Presence of 2,4-Diacetylphloroglucinol-Producing Bacteria

The presence of 2,4-DAPG-producing bacteria was also evaluated by PCR. Total DNA from rhizosphere soil was extracted with soil DNA Isolation Kit (Ultraclean, Mo-Bio Laboratories) according to manufacturer's instructions. Specific primer sets B2BF (5′ACC CAC CGC AGC ATC GTT TAT GAG C-3′) and BPR4 (5′CCG CCG GTA TGG AAG ATG AAA AAG TC-3′), which target the *phlD* gene (encoding a polyketide synthase that synthesizes monoacetylphloroglucinol, the precursor to 2,4-DAPG that is essential for the phloroglucinol biosynthesis) were used in the PCR reaction (Gardener et al., 2001). PCR conditions were: an initial denaturation at 95°C for 3 min, followed by 35 cycles each at 95°C denaturation for 1 min, 60° annealing for 1 min, 72°C extension for 1 min, and final extension step for 10′ at 72°C. Pure DNA extracts from *Pseudomonas* spp. (SA 32A) and *Enterobacter* spp. (RJAL6) strains were used as positive and negative controls, respectively (Mora et al., 2017).

Bacterial Community Structure in Suppressive Soils

The bacterial community composition in the rhizosphere suppressive soils from the greenhouse assay was examined by DGGE as described in Section Bacterial Community Composition and Ggt Detection in Soil Samples. The similarity between bacterial communities was visualized by non-metric multidimensional scaling analysis (MDS), using Primer 7 software (Primer-E Ltd., Ivybridge, UK), which showed a Bray–Curtis similarity index higher than 50% and 0.14 stress values (Clarke, 1993).

Statistical Analyses

Data normality was analyzed according to Kolmogorov's test. Data obtained in Section *In vitro* Inhibition Test on Solid Media (*in vitro* plate assay) were analyzed by a one-way analysis of variance (ANOVA) and compared by Tukey test, using SPSS software (SPSS, Inc.). Comparisons between inoculated and non-inoculated samples from screening 2 were made, and Student *t*-test was used for related samples with 95% confidence interval. For the greenhouse assay multivariate analysis of variance (MANOVA) and comparisons were carried out for each set with Tukey's test by SPSS software (SPSS, Inc.). Values were given as means ± standard errors. Differences were considered significant when the *P* value was lower than or equal to 0.01. The microbial diversity analysis was described above.

RESULTS

Collected Soils Grouped According to their Chemical Composition

In order to determine the chemical composition of the collected soils, chemical analyses were performed using triplicate samples of each soil. The main chemical parameters that were measured are shown in **Table 2**. In general, soil samples showed values of available P from 5.6 (soil 13) to 60 mg kg^{-1} (Soils 1 and 4). The pH ranged from 5.0 (soils 11 and 12) to 6.4 (soil 15). The OM contents varied from 6% (soil 14) to 15% (soils 6, 7, and 15). The higher values of S bases and Al saturation were observed in soil 15 (29.4 $cmol_{(+)}$ kg^{-1}) and soil 11 (14%), respectively; whereas lower values were observed in soil 6 (4.7 $cmol_{(+)}kg^{-1}$) and soil 15 (0 $cmol_{(+)}kg^{-1}$). In addition, the PCA analysis showed that soils were grouped based on their chemical composition and soil classification, Andisol and Ultisol (**Figure 1B**). Several soil groups were formed, soils collected from Perquenco, Las Cardas, Momberg, Quilaco, Membrillar, and Lufquentue clustered together (soils 1, 2, 3, 4, 5, 7, 9, 10, and 16, respectively), soils from Quilaco (6) and Boyeco (11 and 12), and three soils collected from Lufquentue (soils 13, 14, and 15) and Quilaco (soil 8) did not cluster with any other group.

Bacterial Community Composition is Related to Chemical Soil Composition and Differs in Bulk and Rhizosphere Soils in Terms of Dominance and Diversity

Rhizosphere community composition is highly related to soil classification as revealed by MDS, in which microbiology and soil chemical/environmental properties were analyzed (**Figure 2A**). Samples from Andisol and Ultisol were notably different from each other, with 55% similarity. According to the Spearman correlation, Ultisol soils were more significantly related with chemical parameters than Andisol soils ($r^2 = 0.90$ and $r^2 = 0.59$, respectively, **Table 3**). Bulk soils were also separated according to the chemical soil composition, but this difference was not significant, and all samples were grouped at 55% (**Figure 2B**). In

TABLE 2 | Average values for some chemical properties of rhizosphere soils used in this study.

Soil	P (mg kg^{-1})	K (cmol + kg^{-1})	pH (H_2O)	OM (%)	Al sat† (%)	CICE (cmol + kg^{-1})	Σ basis ($cmol_{(+)}$ kg^{-1})
1	$60.3 \pm 0.8^{a*}$	2.8 ± 0.1^{bc}	5.9 ± 0.1^{b}	12.2 ± 0.1^{d}	0.6 ± 0.0^{hij}	16.9 ± 0.0^{e}	16.9 ± 0.0^{e}
2	41.5 ± 0.9^{cd}	1.2 ± 0.0^{f}	5.3 ± 0.0^{f}	10.9 ± 0.2^{e}	9.9 ± 0.1^{c}	11.0 ± 0.1^{h}	9.9 ± 0.1^{i}
3	22.2 ± 0.6^{h}	3.0 ± 0.1^{b}	5.8 ± 0.0^{bc}	10.4 ± 0.3^{ef}	0.9 ± 0.1^{fgh}	18.5 ± 0.1^{d}	18.3 ± 0.1^{d}
4	59.8 ± 0.2^{a}	0.6 ± 0.0^{hi}	5.7 ± 0.0^{bc}	15.1 ± 0.1^{ab}	1.1 ± 0.0^{fg}	9.4 ± 0.0^{i}	9.3 ± 0.0^{i}
5	40.1 ± 1.0^{d}	2.6 ± 0.1^{c}	5.7 ± 0.1^{bc}	15.3 ± 0.3^{a}	0.7 ± 0.0^{fgh}	13.9 ± 0.0^{g}	13.8 ± 0.0^{g}
6	22.2 ± 0.1^{h}	0.4 ± 0.0^{ij}	5.4 ± 0.1^{def}	15.9 ± 0.2^{a}	3.3 ± 0.1^{d}	4.8 ± 0.1^{jk}	4.7 ± 0.1^{j}
7	33.1 ± 0.1^{f}	1.2 ± 0.0^{f}	5.7 ± 0.1^{bc}	15.4 ± 0.2^{a}	0.6 ± 0.1^{ghi}	10.9 ± 0.1^{h}	10.8 ± 0.1^{h}
8	53.4 ± 0.1^{b}	9.2 ± 0.1^{a}	6.3 ± 0.0^{a}	13.9 ± 0.2^{c}	0.0 ± 0.0^{j}	28.1 ± 0.2^{b}	28.1 ± 0.2^{b}
9	37.5 ± 0.2^{e}	2.2 ± 0.1^{d}	5.6 ± 0.0^{cde}	10.9 ± 0.3^{e}	2.3 ± 0.0^{e}	11.2 ± 0.2^{h}	11.0 ± 0.2^{h}
10	42.7 ± 0.2^{c}	2.1 ± 0.1^{de}	5.6 ± 0.1^{cde}	14.0 ± 0.3^{bc}	1.2 ± 0.0^{fg}	9.9 ± 0.1^{i}	9.8 ± 0.1^{i}
11	16.1 ± 0.3^{i}	0.8 ± 0.0^{gh}	5.0 ± 0.0^{g}	7.7 ± 0.1^{g}	14.0 ± 0.4^{a}	5.8 ± 0.1^{j}	5.0 ± 0.0^{j}
12	13.5 ± 0.2^{j}	0.2 ± 0.0^{j}	5.0 ± 0.0^{g}	9.6 ± 0.1^{f}	12.5 ± 0.1^{b}	6.3 ± 0.0^{j}	5.5 ± 0.0^{j}
13	5.6 ± 0.4^{k}	0.9 ± 0.0^{gh}	5.6 ± 0.0^{cd}	13.5 ± 0.2^{c}	0.5 ± 0.0^{hij}	15.7 ± 0.1^{f}	15.6 ± 0.1^{f}
14	30.0 ± 0.3^{g}	1.1 ± 0.0^{fg}	6.2 ± 0.1^{a}	6.1 ± 0.3^{h}	0.1 ± 0.0^{ij}	24.8 ± 0.4^{c}	24.7 ± 0.4^{c}
15	16.0 ± 0.3^{i}	0.3 ± 0.0^{j}	6.4 ± 0.1^{a}	15.2 ± 0.2^{a}	0.0 ± 0.0^{j}	29.4 ± 0.3^{a}	29.4 ± 0.3^{a}
16	54.2 ± 0.1^{b}	1.8 ± 0.0^{e}	5.3 ± 0.1^{ef}	7.9 ± 0.3^{g}	0.6 ± 0.0^{ghi}	11.6 ± 0.1^{h}	11.5 ± 0.1^{h}

†*Calculated as Al/cation exchange capacity [Σ (K, Ca, Mg, Na, and Al)] × 100, n = 3.*

**Different letters in same column denote significant differences (Tukey's test, P ≤ 0.05).*

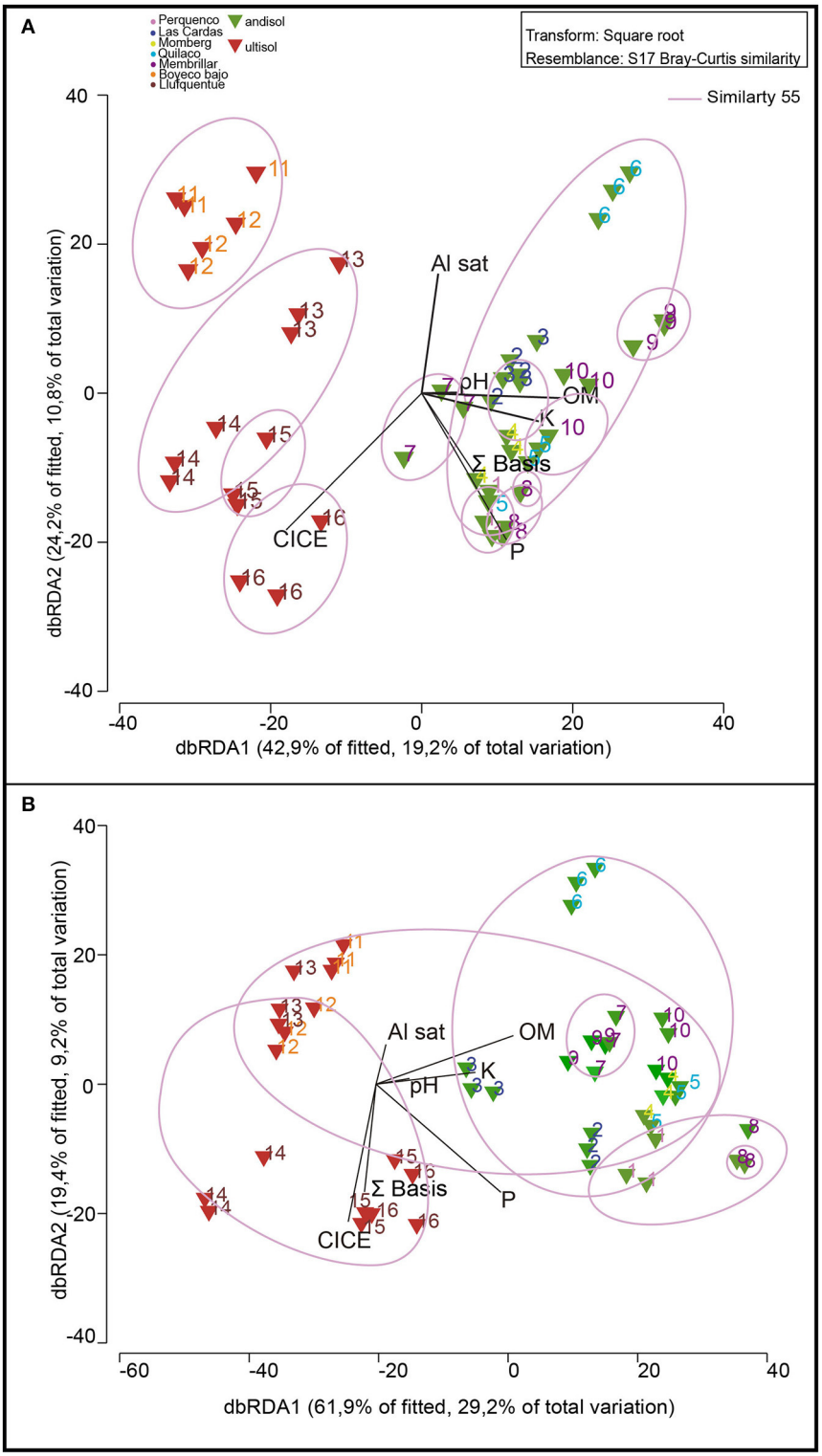


FIGURE 2 | Non-metric Multidimensional Scaling (NMDS) analysis of the 16 soils used in this study based in DGGE profiles of bacterial communities in relation with soil parameters (P, K, OM, Al sat, CICE, and Σ basis). Color of numbers represent sector of sampling and triangle colors indicate type of soil: andisol (green) and ultisol (red). The length and position of the black lines (soil parameters) indicate correlation strength and direction of significant variables ($P < 0.05$) with the microbial community of rhizosphere **(A)** and bulk soil **(B)**.

TABLE 3 | R^2 values and significance level for NMDS ordination and environmental variables.

	Rhizosphere soil			Bulk soil		
Soil parameters	**All samples**	**Andisol**	**Ultisol**	**All samples**	**Andisol**	**Ultisol**
P (mg kg^{-1})	0.001**	0.020*	0.001**	0.001**	0.003**	0.005**
pH (H_2O)	0.027**	0.037*	0.05*	0.257	0.513	0.508
K (mg kg^{-1})	0.028**	0.09	0.001**	0.128	0.001**	0.001**
OM (%)	0.003**	0.003**	0.001**	0.001**	0.014*	0.016*
Al saturation (%)	0.008**	0.006**	0.001**	0.167	0.066	0.08
CICE (cmol+ kg^{-1})	0.003**	0.001**	0.507	0.007	0.968	0.97
Σ basis ($cmol_{(+)}kg^{-1}$)	0.001**	0.012*	0.123	0.069	0.036*	0.023*
R^2 (Spearman)	0.45	0.59	0.90	0.47	0.83	0.83

*Represents statistically significant correlation ($P < 0.05$), **represent statistically significant correlation ($P < 0.01$).

this sense, both Andisol and Ultisol were equally related with soil chemical parameters ($r^2 = 0.83$, **Table 3**).

Differences in bacterial community structures between bulk and rhizosphere soil were revealed by MDS analysis, based on DGGE banding profiles. In relation to bulk soils, the nMDS analysis revealed the existence of major groups at 40% similarity formed by rhizosphere soils indistinctly grouped, all correlated among them. In general, bulk soils were separated into two main groups formed mainly by Andisol (soil 1, 2, 3, 4, 5, 6, 7) and Ultisol soils (11, 12, 13, 14, 15, 16) (**Figure 3**).

Regarding the microbial diversity in bulk and rhizosphere soils (Supplementary Figure 2), in general, the Shannon index (H') showed values <2.0 for bulk (except soils 5 and 6 from Quilaco) whereas it reached values ∼2.5 for rhizosphere soil, indicating a lower diversity in bulk than in rhizosphere soils. A similar trend was observed in the case of species number (S). The lowest diversities (<1.0) and species richness (S) were observed in bulk soils from Membrillar (soils 9 and 10) whereas highest diversity values were obtained in rhizosphere soils from Quilaco (soil 5 and 6). As for bulk soils, rhizosphere soils taken from Membrillar also showed lower diversity values (≤2.0). In contrast, less dominance values in rhizosphere soils compared with bulk soils were also observed by Simpsons (D) index represented by 1-D, in which the lower values indicate major dominance of species. Thus, samples 9 and 10, which showed less diversity according to the Shannon index, also showed major dominance of species. Therefore, bulk soils showed lower biodiversity and major dominance compared to rhizosphere soils (Supplementary Figure 2), and we observed a direct Pearson correlation between both indexes ($P < 0.01$, data not shown).

Screening for Putative Ggt-Suppressive Soils

According to the *in vitro* inhibition test on solid media (**Figure 4A** and Supplementary Figure 1A), four soils from Las Cardas (soils 2 and 3), Boyeco (soil 11), and Llufquentue (soil 16) were considered as suppressive against Ggt, since Ggt growth was significantly inhibited when compared with the positive control. However, several soils could not be properly evaluated based on this assay and were classified as undetermined. A second evaluation, using the *in vitro* inhibition test in aqueous soil extracts (**Figure 4B** and Supplementary Figure 1B) suggested the presence of nine Ggt-suppressive soils. They were collected from Las Cardas, Momberg, Quilaco, Boyeco, and Llufquentue (soils 2, 3, 4, 6, 11, 13, 14, 15, and 16, respectively). In line with the assay on solid agar, this test also showed that soils collected from Membrillar (soils 7, 9, 10, and 12) were Ggt conducive. This assay also revealed the presence of other Ggt-conducive soils (soils 5 and 8), including the positive control soil 1. In summary, based on *in vitro* experiments, 9 soils were determined as suppressive, and 7 as conducive (**Figure 4B**).

Suppressive Soil Greenhouse Assay

Incidence of Take-All Disease

Firstly, we aimed to establish a positive control for take all decline and soil conduciveness for our suppressive soil assays. Plants growing in soil 1 showed clear disease symptoms including leaf chlorosis and blackening root, distinctive take-all disease symptoms. We confirmed the presence and the identity of Ggt in the rhizospheric soil, extracting the total DNA and using Ggt specific primers. The PCR amplification confirmed that soil 1 was positive in terms of Ggt presence (Supplementary Figure 3). Then, to confirm the ability to suppress take-all disease in the putative nine rhizosphere soils selected through the *in vitro* assays (**Figure 4**), an assay with the pathosystem wheat-Ggt was performed under greenhouse conditions by growing wheat in Ggt-inoculated and non-inoculated soils. Take-all disease symptoms were evident in all inoculated soils, except in soil 11 (data not shown). The origin of the symptoms was confirmed by Ggt DNA amplification with Ggt specific primers of infected plants; the amplification band was present in all Ggt-inoculated rootswhereas it was absent in the non-inoculated plants (Supplementary Figure 4). In order to determine whether Ggt suppressiveness was due to microbial community or to soil physico-chemical characteristics, sterilization by heat treatment of the soils was performed. The plants grown in the greenhouse on heat-treated sterilized soils showed higher disease symptoms than in the corresponding natural soils (**Figure 5**): the percentage of blackening root for plants growing in sterile soils ranged between 10 and 40%, while only between 3 and 10% for natural

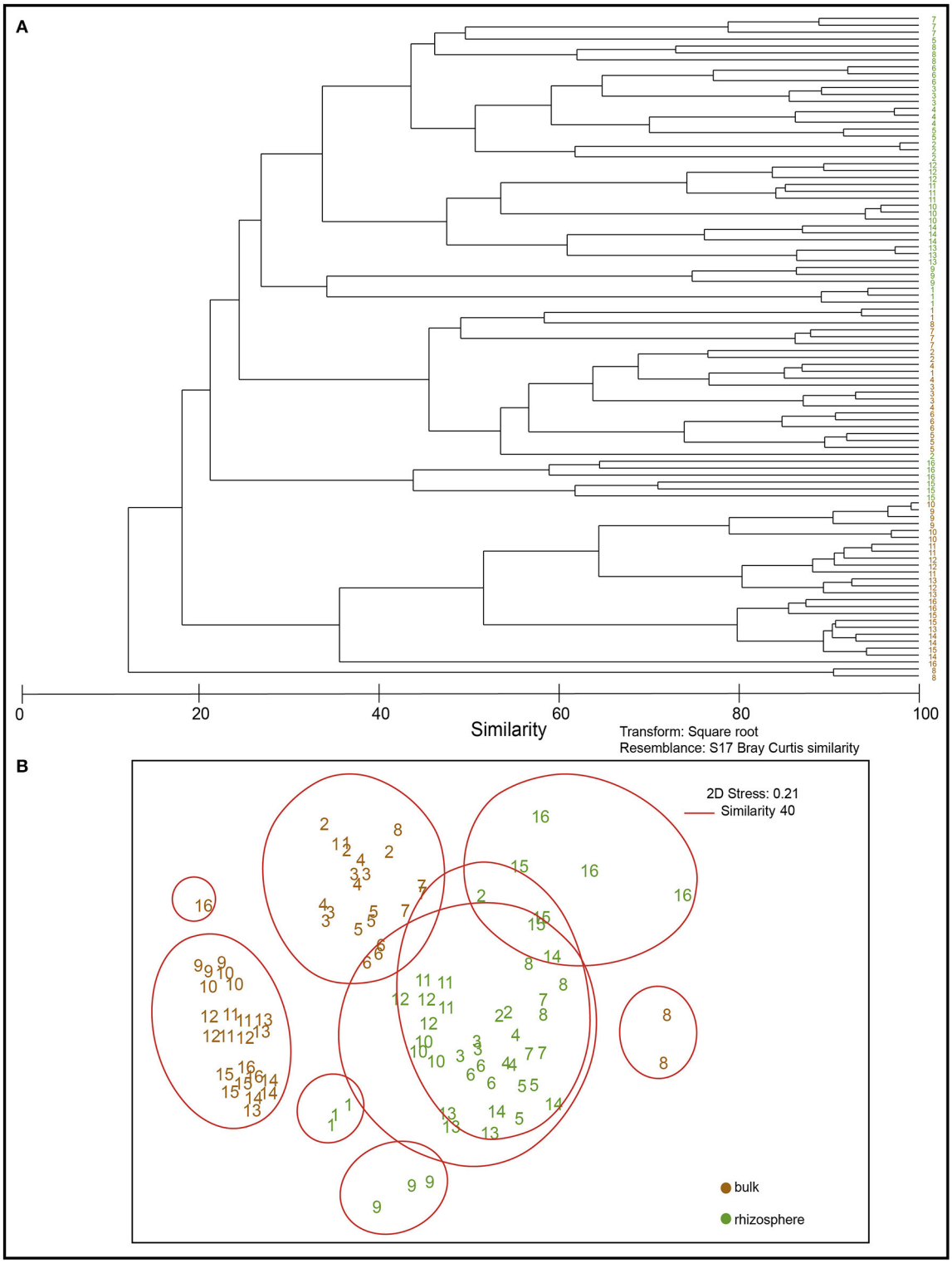


FIGURE 3 | Dendogram of DGGE profiles **(A)** and non-metric Multidimensional Scaling (NMDS) based on denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities present in bulk (brown) and rhizosphere (green) soil samples **(B)**.

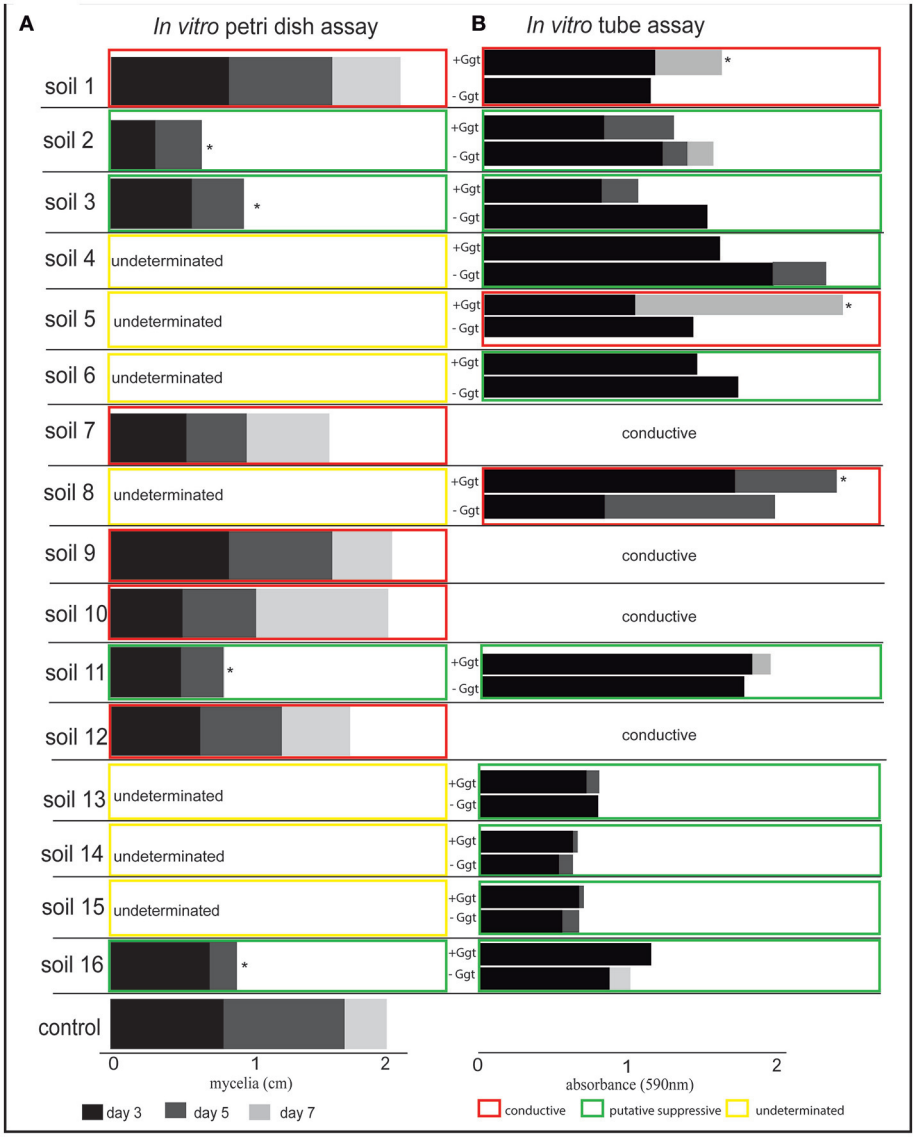


FIGURE 4 | *In vitro* assays to determine the inhibition of *Gaeumannomyces graminis* growth in the presence of rhizosphere soil samples. **(A)** Shows Ggt mycelial growth on solid media supplemented with the different rhizosphere soils after 3, 5, and 7 days of incubation. **(B)** Shows Ggt fungal biomass in liquid rhizosphere soil extracts after 3, 5, and 7 days of incubation. Asterisks indicate significant differences (Tukey's test, $p \leq 0.05$) in relation to the positive control (solid media assay) and negative control (liquid assay).

soils, except for soils 1, 6, and 14 (**Figure 5**). In fact, no significant differences were found between sterile and natural soil in the case of soils 1 and 14 (blackening roots levels ranging from 15 to 35%).

A negative correlation between blackening roots and biomass was also observed. Thus, the lower biomass was found in the sterile Ggt-inoculated soil treatments, where plants showed higher plant infection, with the exception, again, of soils 6 and 14, in which plant biomass was similar in sterile and natural soils (**Figure 6**). In treatments where a 1% of the natural soils was added to the sterile soils, biomass production increased significantly ($P \leq 0.05$). In fact, in soils 4, 13, and 15 the highest biomass for this treatment was found, being similar in the rest of the soils. Only in soil 1 (Ggt-conducive positive control) plant biomass was lower in the 1% natural soil supplemented treatment.

Presence of 2,4-DAPG Producing Bacteria

2,4-DAPG-producing bacteria have been reported as major take-all disease suppressors in soils (Kwak et al., 2012). Accordingly, we checked for their presence in our selected suppressive soils. However, we found that only soil 15 was positive for the phlD gene, essential in the DAPG biosynthetic pathway (Gardener et al., 2001). The rest of suppressive soil samples did not show the presence of amplicons for this gene (Supplementary Figure 5),

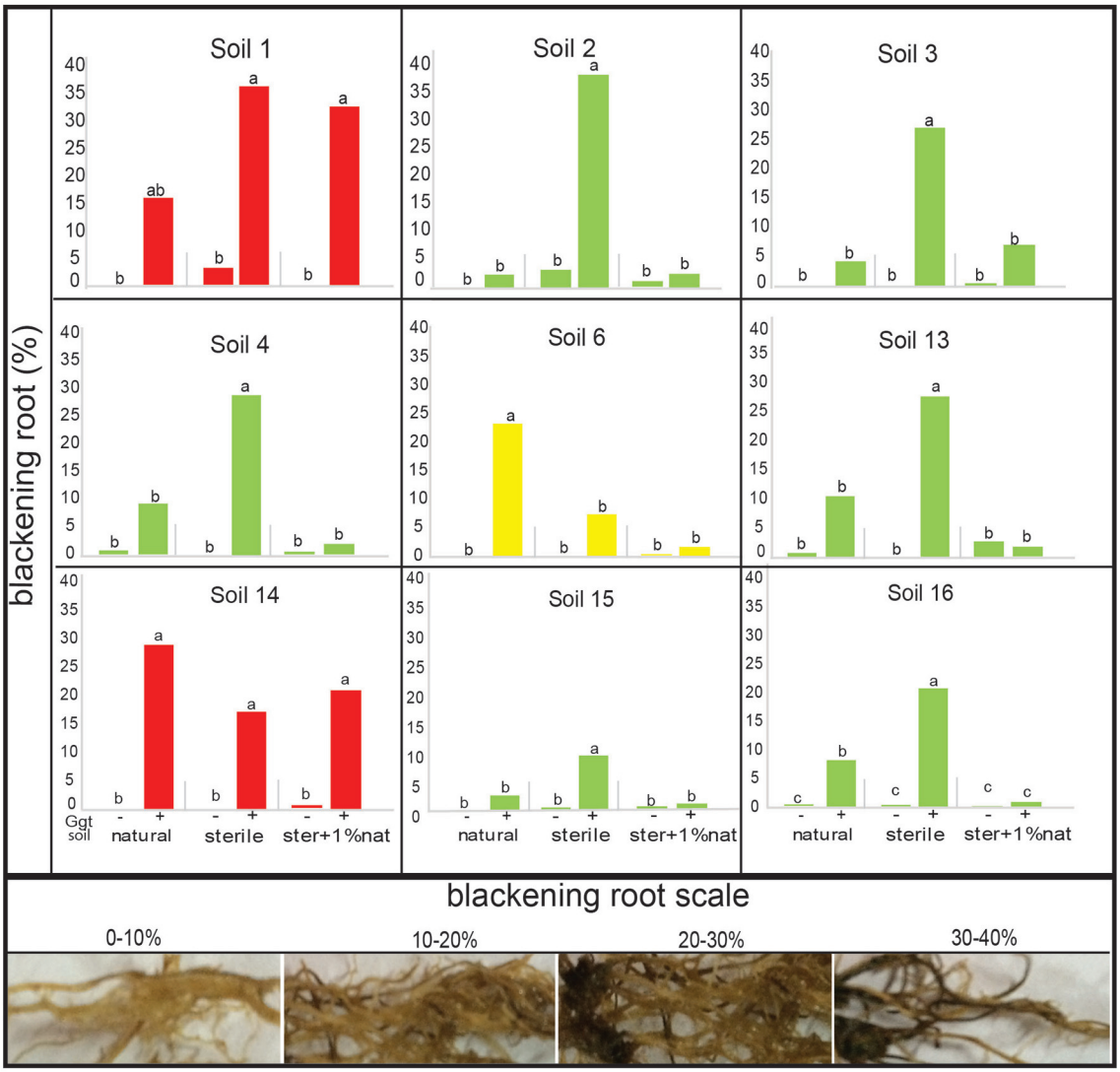


FIGURE 5 | Take all disease symptoms (root blackening) in wheat plants from the suppressive soil bioassay in greenhouse. Treatments were: natural soil (natural), sterile soil (sterile), and sterile soil supplemented with 1% of natural soil (ster+1% nat), inoculated (+) or not (–) by Ggt ($n = 5$). The disease index scale used is represented by the pictures in the bottom panel. Tukey's test was used to compare treatments means, values followed by the same letter do not differ at $P \leq 0.05$ ($n = 5$). Green bars represent suppressive soils, red bars conducive soils, and yellow bars represent undeterminated soil.

suggesting that other metabolic pathways should be responsible for suppressiveness in those soils.

Rhizosphere Bacterial Community Composition in Suppressive and Conducive Soils

Regarding the composition of the rhizosphere bacterial communities (as revealed by DGGE analysis), the dendrogram showed differences between suppressive and conducive soils in the case ofAndisols (**Figure 7**). These results were confirmed by using MDS analysis, showing clear differences between the conducive control soil (soil 1) and the rest of treatments in Andisol (55% similarity). However, in the case of Ultisols in which the bacterial communities were more significantly related with the soil chemical parameters than in Andisol soils (**Table 3**), this tendency was not observed (**Figure 7B**).

DISCUSSION

Several studies have described the importance of soil microorganisms as suppressive agents against phytopathogens worldwide, including soils with chemical properties similar to Chilean agricultural soils, such as, New Zealand soils (Bithell et al., 2013; Chng et al., 2013, 2015; Perez et al., 2016). However, despite of the great potential that Chilean suppressive soils offer in terms of microbial diversity and potential for the development of biocontrol strategies, their studies are extremely limited (Andrade et al., 2011). This topic acquires special relevance

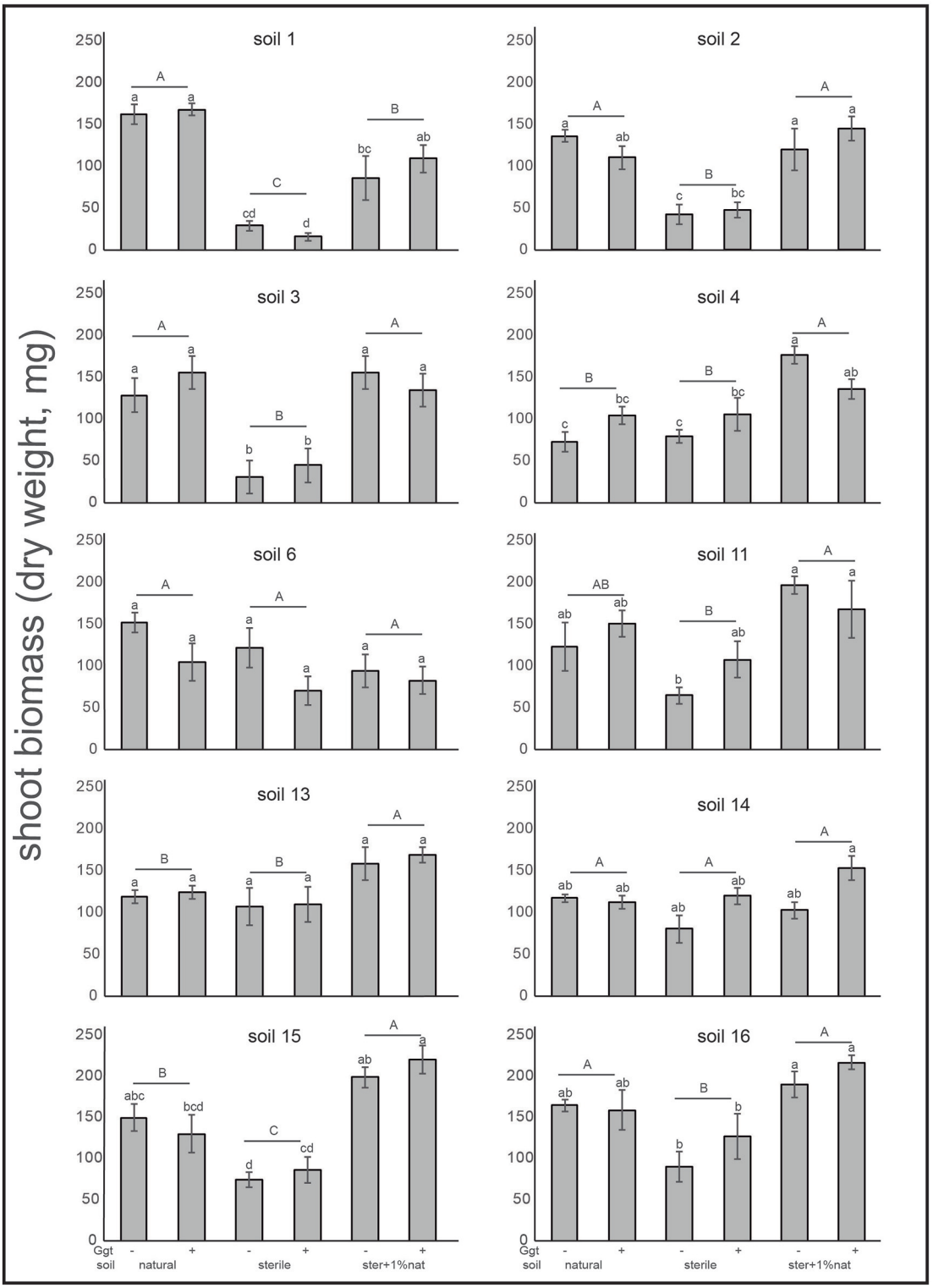


FIGURE 6 | Shoot biomass (dry weight) of wheat plants from suppressive soil assay in greenhouse. Treatments were: natural soil (natural), sterile soil (sterile), and sterile soil supplemented with 1% of natural soil (ster+1% nat)- and inoculated (+) or not (–) by Ggt ($n = 5$). Tukey's test was used to compare treatments means, values followed by the same letter do not differ at $P \leq 0.05$ ($n = 5$).

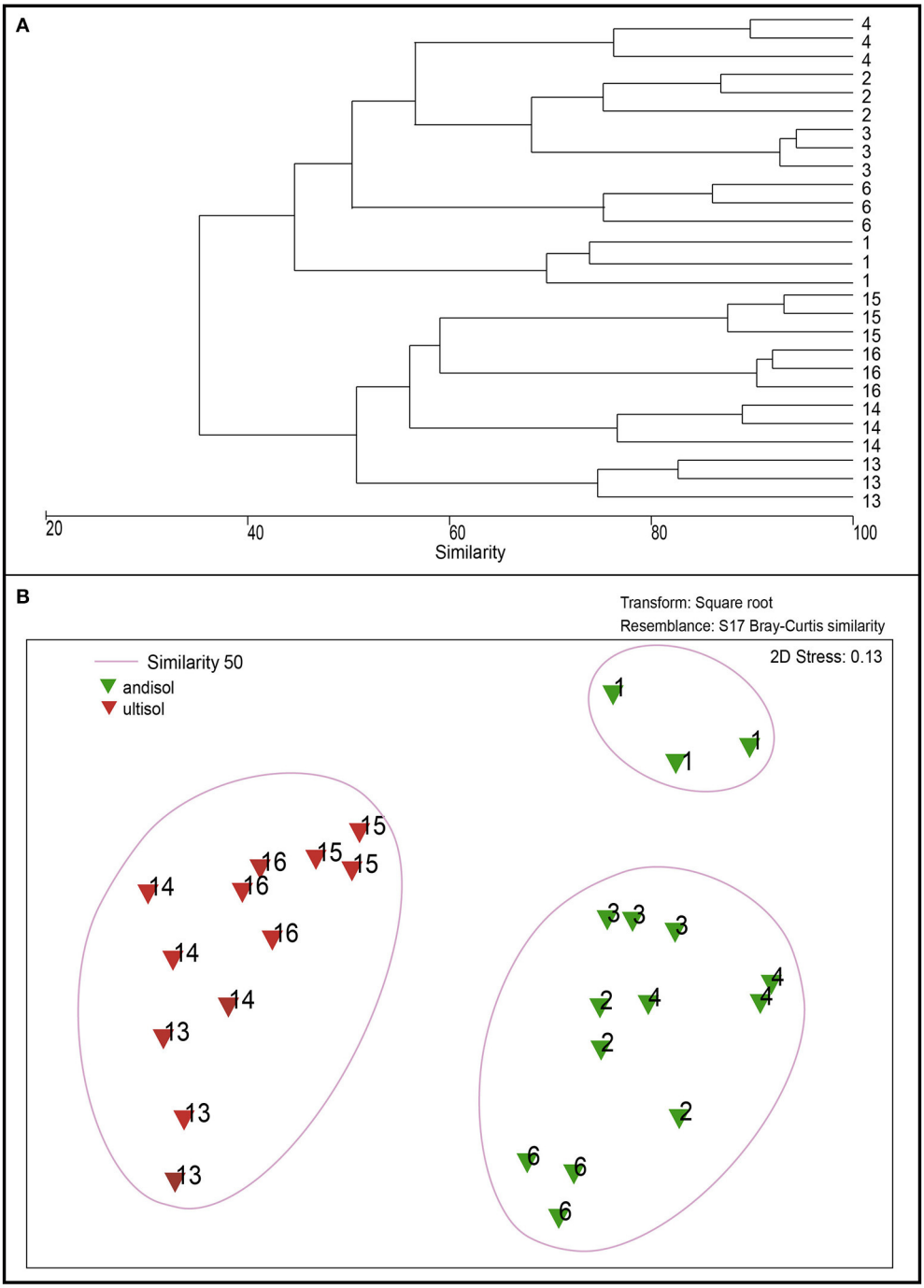


FIGURE 7 | Dendrogram of DGGE profiles **(A)** and non-metric Multidimensional Scaling (NMDS) analysis **(B)** of bacterial communities in the rhizosphere of soils from the greenhouse experiment (soils 2, 3, 4, 13, 14, 15, and 16) compared with Ggt-conducive soil (soil 1).

when ancestral extensive agriculture has been applied for a long term by native communities; Mapuche people cultivate in small areas to produce their own agricultural products using rustic metal tools and low inputs (Montalba-Navarro, 2004). This ancestral production could be replaced by more aggressive and intensive modern agriculture techniques, with the possible subsequent loss of diversity and suppressive potential of soil microbial communities. Under this scenario, it is essential to identify suppressive soils in these areas. In this work we screened the ability of different agricultural soils from southern Chile to suppress take-all disease in wheat plants. Suppressive soils were collected from little farms mainly belonging to indigenous Mapuche communities from Southern Chile, who practice monoculture and cultivate small subsistence wheat areas.

Soil Chemical Parameters and Their Relation with Microbial Community Composition

Our results on soil chemical properties showed that 25% of the studied soils presented a low content of available P (< 20 mg kg^{-1}), moderate acidity (pH < 5.5), and high Al saturation (>10%), which are main characteristics of agricultural soils from southern Chile (Mora et al., 2006). In general, Andisols grouped together when considering the chemical parameters, whereas Ultisols were more diverse. However, when we compared microbial diversity we observed a direct correlation with soil chemistry mainly in rhizospheric soils, and more significantly for Ultisols. Similar results were found by Smalla et al. (2007) despite the amplified fragments comprised different variable regions and lengths, DGGE, T-RFLP, and SSCP analyses led to clustering of fingerprints, which correlated with soil physico-chemical properties. In Chilean Andisol soils, Jorquera et al. (2014) also showed that soil chemistry influenced the composition of rhizobacterial communities, and in Ultisol soils from China, Li et al. (2017) found through nMDS analyses that microbial communities also correlated with soil chemical parameters and fertilization strategies. Although there is some similarity in microbial composition between rhizosphere and bulk microbial composition (de Ridder-Duine et al., 2005), in our study rhizosphere soils were more diverse in terms of richness and dominance than bulk soils. In fact, it is known that the amount of microorganism around the rhizosphere is 10- to 1,000-fold higher than that found in bulk soil due to rhizodeposition (Doughari, 2015; Glick, 2015).

Suppressive Soil Identification in Southern Chile

In terms of potential take-all disease suppression, *in vitro* Ggt-growth inhibition tests with 16 different soils allowed to identify 9 potentially suppressive soils, and 6 of them were later confirmed to be suppressive in plant bioassays under greenhouse conditions (soil 2, 3, 4, 13, 15, and 16). In general, these soils were cultivated with wheat monoculture and natural pasture for more than 10 years, a management similar to that described previously for other suppressive soils in South Chile (Andrade et al., 2011). Regarding the timing, take-all suppression appeared after 4–6 years of wheat monoculture (McSpadden Gardener and Weller, 2001), and even later, although they can also occur in soils with 3–4 years of monoculture under relatively high pathogen concentrations (Chng et al., 2015). In fact, early studies by Baker and Cook (1974) showed that 3 years of successive wheat cropping could be sufficient for the development of specific suppression. As an exception, soil 2, withrotation based in wheat-triticale and oat for more than 10 years, was also found to be Ggt-suppressive. It is well known that oat roots produce saponin avenacin, a glycosylated triterpenoid secondary metabolite with antifungal properties that has been involved in determining oat resistance to soil fungal pathogens (Osbourn et al., 1994; Freeman and Ward, 2004). On the other hand, wheat plants grown in soil 11, characterized by a very high humidity, showed no symptoms of Ggt infection in roots. This is in agreement with earlier reports by Nish (1973), who studied Ggt survival in the field under controlled conditions, and showed a significant reduction in Ggt incidence in wet cool soils.

In greenhouse assays, wheat plants grown on sterile Ggt-inoculated soils presented the highest disease incidence and the lowest biomass production, suggesting the relevance of the autochthonous soil microbial communities on plant growth promotion and disease suppression. The effect observed in sterile soils—increased susceptibility and reduced biomass—was improved when they were supplemented with 1% of the same natural soils, except in the case of the conducive soils 1 and 14. This improvement also confirms the role of the microbial communities originally present in the take-all disease suppressive soils and Ggt control. This characteristic is known as specific suppression (Cook, 2003; Andrade et al., 2011; Chng et al., 2015). It is noteworthy that we did not find any relation between suppressive soils and the presence of 2,4-DAPG-producing bacteria since its presence was only detected in one out of the six confirmed suppressive soils. The low occurrence of 2,4-DAPG determined by *phlD* gene, suggests that other mechanism(s) or antifungal compound(s) are synthetized by native soil microorganisms that could contribute to the effective biocontrol against Ggt. Thus, strains related to soil suppressiveness seem to be differentially shaped by multiple soil factors (Imperiali et al., 2017). Therefore, further studies are required to identify the mechanisms involved in Ggt disease suppression.

Suppressive Soils and Microbial Community Composition

As mentioned above, the selected suppressive soils did not group together in relation to their chemical properties and geographical origin. However, when bacterial communities were analyzed by DGGE, within Andisols, suppressive soils grouped separately with respect to the control conducive soil 1, but not for Ultisols, in which suppressive soils grouped together with soil 14, also classified as conducive. This could be attributed to the high relation between Ultisol soils and soil chemical parameters when compared to that relation in Andisol soils (r^2 = 0.90 and 0.59, respectively). Future research should explore which specific microbial groups act directly upon Ggt suppression and how rhizosphere microbial communities are selected and regulated by the plant rhizosphere, especially in the presence of the phytopathogens. Moreover, identifying the bacterial groups and their antagonist mechanisms, as well as exploring the potential stimulation of plant defense mechanisms are pivotal for the development of biocontrol strategies based on the use of suppressive soils. With this aim, future research should tackle the multifactor soil-microbiome-plant-pathogen systems, considering not only direct antifungal activities in the rhizosphere, but also potential stimulation of plant defense and microbe-selection mechanisms.

CONCLUSIONS

Suppressive soils represent an important microbial source for the biocontrol of soil-borne pathogens, and their identification and characterization is crucial since many of these soils may be lost

by the increase of intensive agriculture practices worldwide. Here, we identified six suppressive soils against take-all disease, which have been managed under ancestral and rudimental agronomic practices by Chilean indigenous Mapuche communities. Then, we showed that suppressive activity in the tested soils correlated with the microbial community composition and not with the chemical properties and geographical origin of the studied soils. The key role of the soil microbial communities in Ggt suppression was confirmed in assays with sterile (heat-treated) suppressive soils where Ggt suppresiveness was completely lost, and recovered again through the addition of 1% of the corresponding non sterile natural suppressive soil. Our understanding of microbial communities in suppressive soils as well as the mechanisms acting in disease suppression in the rhizosphere must be considered as a valuable tool for the development of sustainable control of soil-borne pathogen (such as, take-all disease) in agriculture.

AUTHOR CONTRIBUTIONS

PD, SV, MJ, MM designed the research. MP and VC supervised the study. PD organized the soil sampling and chemical soil analysis. SV performed the greenhouse experiments. VC analyzed the data. PD and MP wrote the manuscript and authors critically revised the manuscript and approved the final version.

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REVIEW
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Desirable Traits of a Good Biocontrol Agent against Verticillium Wilt

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The soil-borne fungus *Verticillium* causes serious vascular disease in a wide variety of annual crops and woody perennials. Verticillium wilt is notoriously difficult to control by conventional methods, so there is great potential for biocontrol to manage this disease. In this study we aimed to review the research about *Verticillium* biocontrol to get a better understanding of characteristics that are desirable in a biocontrol agent (BCA) against Verticillium wilt. We only considered studies in which the BCAs were tested on plants. Most biocontrol studies were focused on plants of the *Solanaceae, Malvaceae,* and *Brassicaceae* and within these families eggplant, cotton, and oilseed rape were the most studied crops. The list of bacterial BCAs with potential against *Verticillium* was dominated by endophytic *Bacillus* and *Pseudomonas* isolates, while non-pathogenic xylem-colonizing *Verticillium* and *Fusarium* isolates topped the fungal list. Predominant modes of action involved in biocontrol were inhibition of primary inoculum germination, plant growth promotion, competition and induced resistance. Many BCAs showed *in vitro* antibiosis and mycoparasitism but these traits were not correlated with activity *in vivo* and there is no evidence that they play a role *in planta*. Good BCAs were obtained from soils suppressive to Verticillium wilt, disease suppressive composts, and healthy plants in infested fields. Desirable characteristics in a BCA against *Verticillium* are the ability to (1) affect the survival or germination of microsclerotia, (2) colonize the xylem and/or cortex and compete with the pathogen for nutrients and/or space, (3) induce resistance responses in the plant and/or (4) promote plant growth. Potential BCAs should be screened in conditions that resemble the field situation to increase the chance of successful use in practice. Furthermore, issues such as large scale production, formulation, preservation conditions, shelf life, and application methods should be considered early in the process of selecting BCAs against *Verticillium*.

Keywords: biocontrol, biological control, cross-protection, endophytes, soil-borne pathogens, survival structures, vascular pathogen, Verticillium wilt

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INTRODUCTION

Vascular wilts caused by members of the genus *Verticillium* are among the most devastating fungal diseases worldwide. The genus *Verticillium* consists of a relatively small group of soil-borne ascomycete fungi and several of them cause wilt disease on a variety of plant hosts in many parts of the world. Causal agents of Verticillium wilt diseases are globally distributed, most prevalent in temperate and subtropical regions and rare in tropical regions. The consequences of

Verticillium infection can be far-reaching, leading to huge yield losses (Pegg and Brady, 2002). Currently, 10 species are defined within the *Verticillium* genus (**Table 1**) of which *Verticillium dahliae* has the broadest host range and infects over 200 plant species (Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014). *Verticillium* species produce long-lasting resting structures such as microsclerotia, chlamydospores, and resting mycelium in dead or dying plant tissues (**Table 1**). These resting structures serve as the primary inoculum from which hyphae are formed that directly penetrate the roots of host plants. Subsequently, the fungus reaches the vascular tissue and colonizes the xylem vessels (Puhalla and Bell, 1981; Schnathorst, 1981). Symptoms associated with Verticillium wilt are stunting, chlorosis, wilting, vascular discoloration, and early senescence. However, symptoms can differ considerably between hosts (Fradin and Thomma, 2006) and *Verticillium* species (**Figure 1**). For example, *Verticillium longisporum* causes wilting in cauliflower but necrosis on oilseed rape (Depotter et al., 2016). In addition, many plants can harbor endophytic populations of *Verticillium* without showing any symptoms and should be considered as "asymptomatic hosts" (Malcolm et al., 2013). Moreover, within the different *Verticillium* species non-pathogenic isolates can be found that do not cause symptoms upon inoculation of host plants. Several of these non-pathogenic *Verticillium* isolates show biocontrol efficacy against Verticillium wilt (Matta and Garibaldi, 1977; Davis et al., 2000; Robinson et al., 2007; Qin et al., 2008; García et al., 2011; França et al., 2013; Zhu et al., 2013; Tyvaert et al., 2014).

CURRENT CONTROL STRATEGIES FOR VERTICILLIUM WILT

Control of Verticillium disease is difficult due to the long persistence of the resting structures in the field and the broad host range of some species. Moreover, the pathogen is difficult to manage once it reaches the vascular plant tissue and fungicides appear to be ineffective. Reducing the primary inoculum in the soil has been considered as an important goal and can be accomplished by several management strategies. Chemical fumigants can reduce the inoculum of *Verticillium* in soil, however their use is restricted because of the detrimental effect on the environment. Disease management has been focusing on implementing integrated pest management (IPM). Different IPM strategies to reduce the primary inoculum were recently summarized by the EIP-AGRI focus group of soil-borne diseases (https://ec.europa.eu/eip/agriculture/en/content/focus-groups) and include crop rotation, the use of cover crops, green manures, and organic amendments, and non-chemical soil disinfestation (solarization, soil steaming, anaerobic disinfestation, inundation, and biofumigation). Those management strategies have been implemented into agricultural production and all of them have their specific concerns and limitations.

Another interesting approach is the protection of plants against *Verticillium* by genetic resistance. Resistance has been identified in a limited number of crops and has mainly been studied in tomato, potato and cotton. Grafting on resistant rootstocks is a common strategy to protect vegetables, such as tomato and pepper, against soil-borne pathogens, but is not always effective in controlling Verticillium wilt (Garibaldi et al., 2005; Geboloğlu et al., 2011). Resistance may break down under high disease pressure, leading to new races of the pathogen or a shift in the pathogen population (Lazarovits and Subbarao, 2009; Colla et al., 2012). For example, Verticillium wilt of tomato was effectively controlled by growing cultivars with resistance against *V. dahliae* race 1 (Schaible et al., 1951). Later on, a shift in the pathogen population occurred and race 2 became dominant (Grogan et al., 1979; Dobinson et al., 1996) for which no resistant cultivars are available.

Another tool for IPM is the use of biological control agents (BCAs), a promising strategy to control soil-borne diseases such as *Verticillium*. Although several microorganisms have shown efficacy against Verticillium wilt, hardly any of them

TABLE 1 | Species within *Verticillium* with their host range and survival structures.

Species	Host range	Survival structures
Verticillium albo-atrum	Pestilence wort, Potato, Stinging nettle	microsclerotia, resting mycelium
Verticillium alfalfae	Alfalfa	resting mycelium
Verticillium dahliae	wide	microsclerotia
Verticillium isaacii	Artichoke, Bear's breech, *Brassica* sp., Florist's daisy, Hairy nightshade, Lettuce, Potato, Spinach, Tomato	microsclerotia, resting mycelium, chlamydospores
Verticillium klebahnii	Artichoke, Lettuce	microsclerotia, resting mycelium, chlamydospores
Verticillium longisporum	Birdrape, Broccoli, Cabbage, Cauliflower, Field mustard, Horseradish, Oilseed rape, Sugar beet, Turnip, Wild radish	microsclerotia
Verticillium nonalfalfae	Alfalfa, Cotton, Hop, Petunia, Potato, Spinach, Tomato, Tree of heaven, Wild celery	resting mycelium
Verticillium nubilum	Potato	chlamydospores
Verticillium tricorpus	Carnation, Larkspur, Lettuce, Potato, Tomato	microsclerotia, resting mycellium, chlamydospores
Verrticillium zaregamsianum	Tomato, Potato, Lettuce, Ten weeks stock	microsclerotia, resting mycelium

Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014.



FIGURE 1 | Symptoms caused by *Verticillium* spp. Verticillium wilt of cauliflower **(A–C):** Asymmetric chlorosis of the leaves **(A)**; Vascular discoloration of the stem **(B)**; Wilting of cauliflower plants in the field **(C)**. Verticillium symptoms on oilseed rape **(D,E)**: Stunted growth and vein clearing in oilseed rape caused by artificial infection of *V. longisporum* **(D)**; Verticillium stem striping in oilseed rape caused by *V. longisporwn*, formation of microsclerotia in the stem cortex beneath the epidermis **(E)**. Pepper plants infected by *V. dahliae* showing wilted leaves **(F)**. Eggplant infected by *V. dahliae* showing chlorosis and necrosis of leaves **(G)**.

are available as biopesticide against *Verticillium* in Europe (http://ec.europa.eu/food/plant/pesticides/). To increase the use of BCAs in agriculture, some issues for successful practical implementation should be considered in the selection process of potential BCAs and good protocols of use are needed for farmers. In this review, we summarized the research about biocontrol against Verticillium wilt in various crops. The idea was to understand what makes a good BCA against *Verticillium* and how the development of these organisms into an effective biopesticide can be improved.

BIOLOGICAL CONTROL OF VERTICILLIUM WILT

We consulted the Web of Science database until February 28, 2017 using keywords such as "Verticillium," "Verticillium wilt," in combination with "biological control," "biocontrol," "cross-protection," and "endophytes" to search for relevant publications. Only studies in which the BCAs or their exudates were tested on plants were considered. **Tables 2, 3** give an overview of respectively the bacterial and fungal/oomycete isolates tested against Verticillium wilt. In the table of the fungal and oomycete BCAs all isolates tested against *Verticillium* were included regardless of their effect and their control efficacy is indicated. The taxonomy of the species belonging to the Glomeromycota was adjusted according to the classification proposed by Schüβler and Walker (2010). A different approach was used for bacterial BCAs. Only isolates that could control Verticillium wilt and were identified at least to the genus level were included in the table. For each antagonist, the studied host plant, the effect on growth with and without *Verticillium* and the (possible) mode of action are shown.

Studied Host Plants

Pathogenic *Verticillium* species affect a wide variety of plants and in particular *V. dahliae* has a broad host range, including important agricultural crops, woody species, and ornamentals (Pegg and Brady, 2002; Inderbitzin and Subbarao, 2014). Biological control of Verticillium wilt, however, has only been investigated for a few host plants. Studies with bacterial isolates were performed on nine different host plants belonging to six plant families, while studies with fungal and oomycete isolates were performed on 17 different host plants of 11 plant families (**Table 2**, **3**). Most biocontrol studies were focused on plants of the *Solanaceae*, *Malvaceae,* and *Brassicaceae.* In these families eggplant, cotton and oilseed rape were the most studied crops. Studies on economically important woody

TABLE 2 | Bacterial isolates with biocontrol activity against *Verticillium* in different host plants.

Antagonist	Host	Effect on growth(*)		Mode of action	References
		-Ve	+ Ve		
GRAM-POSITVE					
Arthrobacter					
Arthrobacter sp. FP15	Eggplant			Reduced MS germination, antibiosis (iv), IR	Papasotiriou et al., 2013
Bacillus					
B. amyloliquefaciens 41B-1	Cotton			Reduced MS germination, antibiosis (iv), IR	Han et al., 2015
B. amyloliquefaciens 5-127	Eggplant		+	Antibiosis (iv), mycoparasitism (iv)	Tjamos et al., 2004
	Potato			Antibiosis (iv), mycoparasitism (iv)	Tjamos et al., 2004
B. amyloliquefaciens UCMB-5033, UCMB-5036, UCMB-5113	Oilseed rape	+		Antibiosis (iv)	Danielsson et al., 2007
B. cereus CH2	Eggplant		+	Reduced spore germination (iv), antibiosis (iv), mycoparasitism (iv)	Li et al., 2008
B. cereus AR156	Cotton	+	+	Reduced spore germination (iv)	Yang et al., 2014
B. pumilus M1	Potato			Antibiosis (iv)	Uppal et al., 2007, 2008
B. subtilis B-26, B-121, B-135, B-136, B-150, B-181	Maple			Antibiosis (iv)	Hall et al., 1986
B. subtilis SM21	Cotton	+	+	Reduced spore germination (iv)	Yang et al., 2014
B. subtilis YUPP-2	Cotton			Antibiosis (iv)	Yang et al., 2013
B. subtilis Jaas ed1	Eggplant			Antibiosis (iv)	Lin et al., 2009
B. subtilis DF14	Cotton				Luo et al., 2010
B. subtilis TS06	Strawberry	+		Reduced spore germination, antibiosis (iv)	Zhang Y. et al., 2012
B. subtilis HJ5	Cotton			Antibiosis (iv), competition	Li et al., 2013
B. vallismortis HJ-5	Cotton		+		Zhang G. et al., 2012
Bacillus sp. K-160	Eggplant		+	Antibiosis (iv), mycoparasitism (iv)	Tjamos et al., 2004
Paenibacillus					
P. alvei K-165	Eggplant		+	Reduced MS germination, antibiosis (iv), mycoparasitism (iv), IR	Tjamos et al., 2004; Antonopoulos et al., 2008; Markakis et al., 2008; Angelopoulou et al., 2014
	Potato			Antibiosis (iv), mycoparasitism (iv)	Tjamos et al., 2004
	Arabidopsis			IR	Tjamos et al., 2005; Gkizi et al., 2016
	Olive				Markakis et al., 2016
P. polymyxa YUPP-8	Cotton			Antibiosis (iv)	Yang et al., 2013
P. xylanilyticus YUPP-1	Cotton			Antibiosis (iv)	Yang et al., 2013
Streptomyces					
S. albidoflavus S1	Strawberry			Antibiosis (iv), mycoparasitism (iv)	Berg et al., 2000
S. albidoflavus 1W1	Strawberry		+		Berg et al., 2001
S. cyaneofuscatus ZY-153	Cotton	+	0	Antibiosis (iv), mycoparasitism (iv), IR	Xue et al., 2013, 2016
S. diastatochromogenes S9	Strawberry			Antibiosis (iv), mycoparasitism (iv)	Berg et al., 2000
S. flavotricini Z-13	Cotton	+	0	Antibiosis (iv), mycoparasitism (iv), IR	Xue et al., 2013, 2016
S. kanamyceticu B-49	Cotton	+	0	Antibiosis (iv), mycoparasitism (iv), IR	Xue et al., 2013, 2016
S. lividans 66	Arabidopsis	+	+	Reduced spore germination, reduced MS formation, antibiosis (iv)	Meschke and Schrempf, 2010; Meschke et al., 2012
S. lydicus WYEC108 (wood chip-PAM cores)	Potato		+	Competition	Entry et al., 2000
S. rimosus 7W1	Strawberry		0		Berg et al., 2001
S. rochei X-4	Cotton	+	+	Antibiosis (iv), mycoparasitism (iv), IR	Xue et al., 2013, 2016
Streptomyces sp. DHV3-2	Tomato	+	+	Antibiosis (iv)	Cao et al., 2016

(Continued)

TABLE 2 | Continued

Antagonist	Host	Effect on growth(*)		Mode of action	References
		-Ve	+ Ve		
GRAM-NEGATIVE					
Acetobacter					
A. aceti VIN02	Olive			Reduced MS germination, mycoparasitism (iv)	Varo et al., 2016b
Enterobacter					
Enterobacter sp. AS09	Oilseed rape		+	Antibiosis (iv), mycoparasitism (iv)	Alström, 2001
Enterobacter sp. HA02	Cotton	+	+	Mycoparasitism (iv)	Li et al., 2010, 2012
Pseudomonas					
P. chlororaphis K15	Strawberry	+	+	Antibiosis (iv), mycoparasitism (iv)	Berg et al., 2001
P. chlororaphis MA342	Oilseed rape	+	+		Abuamsha et al., 2011
P. fluorescens M-4	Potato	0	+	Competition	Leben et al., 1987
P. fluorescens P6, P10	Strawberry			Antibiosis (iv), mycoparasitism (iv)	Berg et al., 2000
P. fluorescens B6, B41	Eggplant			Antibiosis (iv)	Malandraki et al., 2008
P. fluorescens DF37	Potato			Antibiosis (iv)	Uppal et al., 2007, 2008
P. fluorescens PICF4, PICF6, PICF8	Olive	0	+	Antibiosis (iv)	Mercado-Blanco et al., 2004; Varo et al., 2016b
P. fluorescens PICF7	Olive	0	+	Competition, IR	Mercado-Blanco et al., 2004; Prieto et al., 2009; Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2014; Maldonado-González et al., 2015b
	Arabidopsis				Maldonado-González et al., 2015a
P. putida B E2	Strawberry	+	+	Antibiosis (iv), mycoparasitism (iv)	Berg et al., 2001
P. putida PICP2	Olive	0	0	Antibiosis (iv)	Mercado-Blanco et al., 2004
P. putida PICP5	Olive	0	+	Antibiosis (iv)	Mercado-Blanco et al., 2004
Pseudomonas sp. FP22, FP23, FP30, FP35	Cotton	+	+	Antibiosis (iv)	Erdogan and Benlioglu, 2010
Serratia					
S. plymuthica HRO-C48	Strawberry	+	+	Mycoparasitism (iv)	Kalbe et al., 1996; Kurze et al., 2001
	Cotton		+	Mycoparasitism (iv)	Kalbe et al., 1996; Erdogan and Benlioglu, 2010
	Oilseed rape	+	+	Mycoparasitism (iv)	Kalbe et al., 1996; Müller and Berg, 2008; Abuamsha et al., 2011
Serratia sp. XY21	Cotton	+	+	Reduced spore germination (iv)	Yang et al., 2014
Stenotrophomonas					
S. maltophilia (isolate 1)	Oilseed rape			Antibiosis (iv), mycoparasitism (iv)	Berg et al., 1996
Stenotrophomonas AS10	Oilseed rape		+	Antibiosis (iv), mycoparasitism (iv)	Alström, 2001

**Plant growth promotion with or without Verticillium infection is represented by "+" and a negative effect on the growth by "−". No effect on the growth is indicated by "0". iv, in vitro; IR, Induced Resistance; PAM: polyacrylamide.*

species and ornamentals are limited to olive and *Acer* species. This may indicate that isolates controlling Verticillium wilt of woody plants are hard to find. A more likely explanation is that investigating biocontrol in these plants is time-consuming and labor-intensive. Moreover, except for maple and olive, *Verticillium* isolates of woody plants have not been studied extensively and information about their pathogenicity and genetic diversity is limited (Pegg and Brady, 2002; Chandelier et al., 2003; López-Escudero and Mercado-Blanco, 2011).

TABLE 3 | Fungal and oomycete isolates with potential biocontrol activity against *Verticillium* in different host plants.

Antagonist	Host	Control efficiency(*)	Effect on growth(**)		Mode of action	References
		Disease	−Ve	+Ve		
OOMYCOTA						
Pythium						
P. oligandrum (Polyversum®)	Pepper	+		+		Rekanovic et al., 2007
	Tomato			0		Giotis et al., 2009
P. oligandrum (mixture of 5 isolates)	Pepper		+	+	Reduced MS production, mycoparasitism (iv)	Al-Rawahi and Hancock, 1998
ASCOMYCOTA						
Acremonium						
Acremonium sp. CEF-193	Cotton	+	0	0	Antibiosis (iv)	Li et al., 2014; Yuan et al., 2017
Alternaria						
Alternaria sp. RF4	Oilseed rape			0	Mycoparasitism (iv)	Alström, 2000
Aspergillus						
A. alutaceus	Eggplant	0				Marois et al., 1982
Aureobasidium						
A. pullulans AP06	Olive	0			Antibiosis (iv)	Varo et al., 2016b
Blastobotrys						
Blastobotrys sp. FP12	Eggplant	+			Reduced MS germination, antibiosis (iv), IR	Papasotiriou et al., 2013
Chaetomium						
C. globosum B221, A354, *Chaetomium* sp.	Cotton	+	+		Antibiosis (iv), mycoparasitsm (iv)	Zheng et al., 2011
Fusarium						
F. culmorum	Tomato	+	0	+	Antibiosis (iv)	Dutta, 1981
F. lateritium BAFC2317 (ex)	Tomato		0	+	Antibiosis (iv), DAMP release	García et al., 2011
F. moniliforme FM01	Olive	0				Varo et al., 2016a
F. moniliforme FM02	Olive	+			Antibiosis (iv), IR	Varo et al., 2016b
F. oxysporum FO03, FO04	Olive	+			Reduced MS germination, antibiosis (iv)	Varo et al., 2016b
F. oxysporum FO12	Olive	+			Reduced MS germination, antibiosis (iv), IR	Varo et al., 2016a,b
F. oxysporum CanR-46	Cotton	+			Reduced germination of inoculum (VOCs, iv), antibiosis (VOCs, iv)	Zhang et al., 2015
F. oxysporum f. sp. *lycopersici* CECT 2715	Pepper	+	0	+	IR	Díaz et al., 2005
F. oxysporum f. sp. *lycopersici, F. oxysporum* f. sp. *dianthi*	Tomato	+				Matta and Garibaldi, 1977
F. oxysporum F2	Eggplant	+			Competition, IR	Malandraki et al., 2008; Pantelides et al., 2009; Gizi et al., 2011, Angelopoulou et al., 2014
F. oxysporum F4	Eggplant	+				Malandraki et al., 2008
F. oxysporum Fo47	Pepper	+	0	+	IR	Veloso and Díaz, 2012
	Olive	0				Varo et al., 2016b
F. oxysporum Fo47b10	Eggplant	(1)			mycoparasitism	Nagtzaam et al., 1998
	Potato	(2)				Nagtzaam et al., 1998
F. oxysporum By125, Ja127, *F. equiseti* By222, *F. solani* Bx 215	Cotton	+	+		Antibiosis (iv), mycoparasitism (iv)	Zheng et al., 2011
Fusarium sp. Bx144	Cotton	+	0		Mycoparasitism (iv)	Zheng et al., 2011

(Continued)

TABLE 3 | Continued

Antagonist	Host	Control efficiency(*)	Effect on growth(**)		Mode of action	References
		Disease	−Ve	+Ve		
Fusarium sp. MTB1, MNS1, MNB3	Eggplant	+				Narisawa et al., 2002
Fusarium sp. RF6	Oilseed rape			0	Parasitism (iv)	Alström, 2000
Gibellulopsis						
G. nigrescens CVn-WHg	Cotton	+	0	+		Zhu et al., 2013
G. nigrescens (formerly *V. nigrescens*)	Peppermint, spearmint	+				Melouk and Horner, 1975
G. nigrescens (formerly *V. nigrescens*)	Cotton	+	0	+		Vagelas and Leontopoulos, 2015
Gliocladium						
G. roseum GR01	Olive	0				Varo et al., 2016a
G. roseum GR02	Olive	0			Reduced MS germination, antibiosis (iv)	Varo et al., 2016b
Gliocladium sp. RF12	Oilseed rape	+		+	Antibiosis (iv), mycoparasitism (iv)	Alström, 2000
Gliocladium sp. RF15	Oilseed rape			0	Mycoparasitism (iv)	Alström, 2000
Gliocladium sp.	Tomato	+	+	+	Antibiosis (iv), mycoparasitism (iv)	Dutta, 1981
Heteroconium						
H. chaetospira H4007	Chinese cabbage	+		0		Narisawa et al., 2000, 2004
H. chaetospira MNB4	Eggplant	+				Narisawa et al., 2002
Leptosphaeria						
Leptosphaeria sp. CEF-714	Cotton	+	0	0	Antibiosis (iv)	Li et al., 2014; Yuan et al., 2017
Microsphaeropsis						
M. ochracea	Oilseed rape	0			Reduced MS germination, mycoparasitism	Stadler and von Tiedemann, 2014
Muscodor						
M. albus 620, *M. roseus* A3-5	Eggplant	+			Reduced MS germination	Stinson et al., 2003
Mycelium radicis atrovirens (MRA)						
MRA MTJ1, MRA MIB3, MRA MNB9	Eggplant	+				Narisawa et al., 2002
Myrothecium						
M. roridum A243	Cotton	+	+		Mycoparasitism (iv)	Zheng et al., 2011
Nectria						
N. haematococca Bx247	Cotton	+	+		Mycoparasitism (iv)	Zheng et al., 2011
Non sporulating fungus with white mycelium						
SWM MHB2	Eggplant	+				Narisawa et al., 2002
Paecilomyces						
P. lilacinus	Eggplant	+				Marois et al., 1982
Penicillium						
P. chrysogenum (dm)	Cotton	+			IR	Dong et al., 2003, 2006
P. chrysogenum	Cotton	+				Zhang et al., 2011
P. chrysogenum EEZ10 (ex)	Tomato		0	+	Antibiosis (iv), DAMP release	García et al., 2011
P. chrysogenum, *P. vermiculatum*, *Penicillium* sp.	Tomato	+	+	+	Antibiosis (iv), mycoparasitism (iv)	Dutta, 1981
P. oxalicum PO212	Tomato	+				Larena et al., 2003; Sabuquillo et al., 2005, 2006
P. simplicissimum CEF-818	Cotton	+	0	+	Antibiosis (iv), IR	Li et al., 2014; Yuan et al., 2017
Penicillium sp. MNT8	Eggplant	+				Narisawa et al., 2002

(Continued)

TABLE 3 | Continued

Antagonist	Host	Control efficiency(*)	Effect on growth(**)		Mode of action	References
		Disease	−Ve	+Ve		
Phialocephala						
P. fortinii J2PC2, LtPE2	Chinese cabbage	−		0		Narisawa et al., 2004
P. fortinii MNJ1	Eggplant	+				Narisawa et al., 2002
Phoma						
Phoma sp. PH01	Olive	+			Reduced MS germination, antibiosis (iv), IR	Varo et al., 2016b
Phoma sp. PH02	Olive	+				Varo et al., 2016a
Phomopsis						
Phomopsis sp. By231	Cotton	+	+		Antibiosis (iv), mycoparasitism (iv)	Zheng et al., 2011
Phomopsis sp. By254	Cotton	+	0/−		Antibiosis (iv)	Zheng et al., 2011
Talaromyces						
T. flavus	Eggplant	+				Marois et al., 1982
T. flavus Po-V-48, Po-V-49, Po-V-50, Po-V-51, Po-V-52	Potato	+			Antibiosis (iv)	Naraghi et al., 2010b
T. flavus Cu-V-55, Cu-V-57, Cu-V-58, Cu-V-59, Cu-V-60	Cucumber	+			Antibiosis (iv)	Naraghi et al., 2010a
T. flavus	Tomato, cucumber	0				Zeise and Kersten, 2000
	Oilseed rape	+				
	Strawberry	+				
T. flavus Tf-1	Hop	+	0	+		Solarska et al., 2000
T. flavus TN11 and TN41	Potato	(2)			Reduced MS germination	Nagtzaam et al., 1998
	Eggplant	(7)			Mycoparasitism	
T. flavus CEF-642	Cotton	+	0	0	Antibiosis (iv)	Li et al., 2014; Yuan et al., 2017
Trichoderma						
T. asperellum B35	Pepper	+				Ślusarski and Pietr, 2009
T. asperellum B35	Hop		+	0		Solarska et al., 2000
T. asperellum T-34	Strawberry	(3)				Martinez et al., 2009
T. asperellum Bt3	Olive	+	0	0	Antibiosis (iv)	Carrero-Carrón et al., 2016
T. asperellum T25	Olive	+	+	+	Antibiosis (iv)	Carrero-Carrón et al., 2016
T. asperellum + *T. gamsii* (BIOTEN®)	Olive	+			Reduced MS germination, antibiosis (iv)	Varo et al., 2016b
T. harzianum T-22 (GTG II®)	Spinach	(4)				Cummings et al., 2009
T. harzianum T-22 (Planter Box Biological Fungicide®)	Spinach	(5)				Cummings et al., 2009
T. harzianum T-35	Potato	+				Ordentlich et al., 1990
T. harzianum	Eggplant	0				Marois et al., 1982
T. harzianum (promot®)	Strawberry	0(6)	0	0(6)		Weissinger et al., 2009
T. harzianum T3, T94, T106, T108, T120, *T. viride* T9, T46, T67, T107, T117	Eggplant	+		+	Antibiosis (iv), mycoparasitism (iv)	D'Ercole et al., 2000
T. harzianum TU63, TU68, TU72, TU74, TU75, TU79, TU80	Strawberry	+			Antibiosis (iv)	Mirmajlessi et al., 2016
T. viride	Tomato	+	+	+	Antibiosis (iv), mycoparasitism (iv)	Dutta, 1981
T. viride	Eggplant	+				Marois et al., 1982
T. virens (formerly *Gliocladium virens*)	Eggplant	0				Marois et al., 1982

(Continued)

TABLE 3 | Continued

Antagonist	Host	Control efficiency(*)	Effect on growth(**)		Mode of action	References
		Disease	−Ve	+Ve		
Trichoderma sp. MNS11	Eggplant	+				Narisawa et al., 2002
Trichoderma sp. RF14, RF16	Oilseed rape			0	Mycoparasitism (iv)	Alström, 2000
Verticillium						
V. albo-atrum SS-4	Cotton	+				Schnathorst and Mathre, 1966
V. albo-atrum T-1	Tomato	0				Schnathorst and Mathre, 1966
V. albo-atrum, *V. tricorpus*	Potato	+				Robinson et al., 2007
V. alfalfae (formerly *V. albo-atrum*), *V. tricorpus*	Tomato	+				Matta and Garibaldi, 1977
V. dahliae Dvd-E6	Tomato	+	+	+	IR	Shittu et al., 2009
V. dahliae 2379 (ex)	Tomato		+	+	DAMP release	García et al., 2011
V. dahliae (CVd-WHw)	Cotton	+	+	+		Zhu et al., 2013
V. isaacii Ls. 432, Ls. 443 (formerly *V. tricorpus*),	Lettuce	+	0	0		Qin et al., 2008
V. isaacii Ls. 441, Ls 442, Ls. 183 (formerly *V. tricorpus*)	Lettuce	+				Qin et al., 2008
V. isaacii	Cauliflower	+				França et al., 2013
V. isaacii Vt305	Cauliflower	+				Tyvaert et al., 2014
V. tricorpus V-17, V-28, V-31	Potato	+				Davis et al., 2000
BASIDIOMYCOTA						
Coriolopsis						
C. rigida CECT20449 (ex)	Tomato		0	+	Antibiosis (iv), DAMP release	García et al., 2011
Dark septate endophytes						
isolate *LtVB3*	Chinese cabbage	+		+		Narisawa et al., 2004
DSE48	Tomato	0	0	0		Andrade-Linares et al., 2011
DSE49	Tomato	+	+	+		Andrade-Linares et al., 2011
Leptodontidium orchidicola	Tomato	+	0	+		Andrade-Linares et al., 2011
Piriformospora						
P. indica	Tomato	+	+	+		Fakhro et al., 2010
Trametes						
T. versicolor A136 (ex)	Tomato		0	+	Antibiosis (iv), DAMP release	García et al., 2011
GLOMEROMYCOTA						
Claroideoglomus						
C. claroideum (formerly *G. claroideum*)	Olive	0	+			Porras-Soriano et al., 2006
C. etunicatum (formerly *G. etunicatum*)	Eggplant	+	+		IR	Matsubara et al., 1995
Funneliformis						
F. mosseae (formerly *G. mosseae*)	Tomato, pepper	+	0	0		Demir et al., 2015
F. mosseae (formerly *G. mosseae*)	Tomato, eggplant		+	+		Karagiannidis et al., 2002
F. mosseae (formerly *G. mosseae*)	Alfalfa	+	+			Hwang et al., 1992
F. mosseae (formerly *G. mosseae*)	Pepper	0	0	0		Garmendia et al., 2004c
F. mosseae (formerly *G. mosseae*)	Olive	0	+			Porras-Soriano et al., 2006

(Continued)

TABLE 3 | Continued

Antagonist	Host	Control efficiency(*)	Effect on growth(**)		Mode of action	References
		Disease	−Ve	+Ve		
F. mosseae (formerly *G. mosseae*)	Cotton	+	+	+		Liu, 1995
F. mosseae (formerly *G. mosseae*) + *F. caledonium* (*G. caledonium*)	Tomato	0	0	0		Baath and Hayman, 1983
Gigaspora						
G. margarita	Eggplant	+	+		IR	Matsubara et al., 1995
Glomus						
G. deserticola	Pepper	+	0	0	IR	Garmendia et al., 2004a,b,c; Garmendia et al., 2006
G. hoi	Cotton	+	0	0		Liu, 1995
G. versiforme	Cotton	+	+	+		Liu, 1995
G. versiforme	Cotton	+		+		Zhang G. et al., 2012
Glomus sp.	Alfalfa	+	+			Hwang et al., 1992
Rhizophagus						
R. fasciculatus (formerly *G. fasciculatus)*	Alfalfa	+	+			Hwang et al., 1992
R. fasciculatus (formerly *G. fasciculatus)*	Cotton	0	+	0		Davis et al., 1979
*R. intraradices (*formerly *G. intraradices*)	Eggplant	+	0	0		Demir et al., 2015
R. intraradices (formerly *G. intraradices*)	Pepper	0	0	0		Garmendia et al., 2004c
R. intraradices (formerly *G. intraradices*)	Olive	0	+			Porras-Soriano et al., 2006
R. intraradices (formerly *G. intraradices*)	Olive	0	+			Kapulnik et al., 2010
Sclerocystis						
S. sinuosa	Cotton		0	0		Liu, 1995
ZYGOMYCOTA						
Mortierella sp. RF1, RF2	Oilseed rape			0	Mycoparasitism (iv)	Alström, 2000

**A reduction or increase of disease incidence or/and severity is indicated by respectively "+" and "−". No effect on the disease is indicated by "0". Isolates with biocontrol activity are also marked in green. ** Plant growth promotion with or without Verticillium infection is represented by "+" and a negative effect on the growth by "−". No effect on the growth is indicated by "0". (1) Reduced Verticillium colonization of the roots but not of the stem; (2) No reduced Verticillium colonization; (3) Trichoderma population was negatively affected by V. dahliae; (4) Reduced % of Verticillium infested seeds; (5) No reduced % of Verticillium infested seeds; (6) No Verticillium symptoms developed during experiments; (7) Reduced Verticillium colonization of the roots and stem. ex, exudates of the isolate were used to apply to the plants; dm, dry mycelium of the isolate was applied to the plants; iv, in vitro; IR, Induced Resistance.*

It should be noted that many of the potential BCAs were tested only once. The reasons can be that those isolates (1) were studied for scientific purposes only, (2) were not considered for further research or (3) insufficient control was established.

Bacterial Biocontrol Agents

The potential of bacterial endophytes as biocontrol agents of vascular wilts has recently been reviewed by Eljounaidi et al. (2016). In our study, we specifically focused on Verticillium wilt and included also non-endophytic bacterial BCAs. We divided bacterial biocontrol agents in Gram-positive and Gram-negative bacteria and further arranged them according to their genus (**Table 2**). Within the Gram-positive bacteria, strains belonging to the genera *Arthrobacter, Bacillus, Paenibacillus,* and *Streptomyces* have been studied. *Bacillus* species comprise the largest group within the Gram-positive bacteria, followed by *Streptomyces* and *Paenibacillus* species. The Gram-negative strains belong to the genera *Acetobacter, Enterobacter*, *Pseudomonas*, *Serratia,* and *Stenotrophomonas*, with *Pseudomonas* as the largest pool of potential BCAs of *Verticillium*.

The genus *Bacillus* is well-explored in the search of BCAs to control Verticillium wilt. Over two third of the *Bacillus* strains tested belong to the species *Bacillus amyloliquefaciens* and *Bacillus subtilis*. Remarkably, only the *Bacillus* strain *B. amyloliquefaciens* 5-127, isolated from tomato roots, was tested on different host plants. *B. amyloliquefaciens* 5–127 reduced the percentage of diseased leaves by 40–70% in eggplants challenged with *V. dahliae* in the greenhouse and could reduce disease incidence with more than 50% in a field experiment with potato (Tjamos et al., 2004). In one of the few studies regarding biological control of Verticillium wilt in trees, several *B. subtilis*

isolates were tested in the greenhouse against *V. dahliae* in maple tree. These isolates were obtained from healthy maple stem tissue and decreased disease incidence of *V. dahliae* in maple trees by 34–51% (Hall et al., 1986). *Bacillus* strains were also reported to protect cotton, strawberry and oilseed rape against Verticillium wilt (**Table 2**).

Paenibacillus isolates have recently gained interest as promising BCAs of plant diseases (Lal and Tabacchioni, 2009; Rybakova et al., 2016). *Paenibacillus alvei* K-165 was isolated from tomato root tips grown in solarized soil (Tjamos et al., 2004) and its biocontrol activity against *V. dahliae* in eggplant has repeatedly been shown in greenhouse experiments (Tjamos et al., 2004; Antonopoulos et al., 2008; Markakis et al., 2008; Angelopoulou et al., 2014). This strain also reduced the disease incidence in potato under field conditions and suppressed Verticillium wilt of olive tree under both greenhouse and field conditions (Tjamos et al., 2004; Markakis et al., 2016). In cotton, application of the *Paenibacillus* isolates *P. xylanilyticus* YUPP-1 and *Paenibacillus polymyxa* YUPP-8 resulted in a lower disease incidence and decreased severity of *Verticillium* (Yang et al., 2013).

Various species of *Streptomyces* have been studied in relation to their biological control effect against *Verticillium*. Xue et al. (2013) selected four *Streptomyces* strains isolated from the rhizosphere of different crops and evaluated their antagonistic potential against *V. dahliae* in cotton. Under greenhouse conditions the biocontrol efficacy ranged between 19 and 66%, while in field conditions the biocontrol efficacies of the four *Streptomyces* isolates were slightly lower and ranged between 14 and 51% depending on the application method. Co-inoculation of *Arabidopsis thaliana* seeds with *V. dahliae* and *Streptomyces lividans* 66 led to a strong suppression of the fungus within soil, which resulted in a strong reduction of Verticillium-induced disease symptoms (Meschke and Schrempf, 2010). In potato, tomato and strawberry, *Streptomyces* species reduced the disease incidence and/or severity in greenhouse experiments (Berg et al., 2000, 2001; Entry et al., 2000; Cao et al., 2016). However, the biofungicide Mycostop® based on *S. griseoviridis* K61 did not offer significant protection against *V. dahliae* in tomato (Minuto et al., 2006).

Pseudomonas spp. have been extensively studied as BCA of different pathogens including *Verticillium*. Most of the tested potential biocontrol strains belong to the fluorescent *Pseudomonas* group. Root treatment of olive plants with root-associated fluorescent pseudomonads during nursery propagation could suppress Verticillium wilt in olive caused by defoliating *V. dahliae* (Mercado-Blanco et al., 2004; Prieto et al., 2009). Other isolates of the fluorescent *Pseudomonas* group can be protective against *V. dahliae* in crops such as potato, strawberry, and eggplant (Leben et al., 1987; Berg et al., 2000, 2001; Malandraki et al., 2008; Uppal et al., 2008). Seed treatment with *P. chlororaphis* strain MA 342, the active organism in the biopesticides Cedomon® and Cerall® (BioAgri AB, Uppsala, Sweden), resulted in a lower infection of oilseed rape with *V. longisporum* (Abuamsha et al., 2011). The study of Erdogan and Benlioglu (2010) indicated that the *Pseudomonas* strains FP22, FP23, FP30 and FP35 are good biocontrol candidates against Verticillium wilt of cotton and moreover can improve the growth parameters in cotton fields.

Isolates of the Gram-negative genus *Serratia* have frequently been found associated with plant roots and possess antifungal properties (Grimont and Grimont, 1992; Kalbe et al., 1996). The biocontrol strain *Serratia plymuthica* HRO-C48 successfully controlled Verticillium wilt in strawberry fields (Kurze et al., 2001). Furthermore, treating the seeds of oilseed rape with *S. plymuthica* HRO-C48 via bio-priming, pelleting or seed coating suppressed Verticillium wilt in oilseed rape plants (Müller and Berg, 2008). Seed treatment with *S. plymuthica* HRO-C48 could also protect cotton plants against Verticillium wilt (Erdogan and Benlioglu, 2010).

The application of specific isolates belonging to the genera *Arthrobacter*, *Acetobacter, Enterobacter,* and *Stenotrophomonas* resulted in protection of eggplant, olive, cotton and oilseed rape against Verticillium wilt (Berg et al., 1996; Alström, 2001; Li et al., 2012; Papasotiriou et al., 2013; Varo et al., 2016b).

Fungal and Oomycete Biocontrol Agents

Fungal and oomycete isolates tested as BCA against *Verticillium* are listed in **Table 3**. The majority of isolates belong to the Ascomycota and a minor fraction of the isolates belong to the Basidiomycota and Glomeromycota. Only one Oomycete, *Pythium oligandrum*, has been investigated. Studies with *Trichoderma*, *Fusarium,* and *Verticillium* isolates as potential biocontrol agent were the most prevalent. Isolates of *Talaromyces*, *Funneliformis*, *Rhizophagus, Glomus,* and *Penicillium* have been studied more than three times. Isolates of other species were less frequently considered as BCA.

Talaromyces flavus reduced Verticillium disease of eggplant and potato with more than 75% in naturally infested soils (Marois et al., 1982; Naraghi et al., 2010b). Different formulations of *T. flavus* were tested (Nagtzaam et al., 1998; Zeise and Kersten, 2000), but up to date none of them have been registered in the European Union (http://ec.europa.eu/food/plant/pesticides).

Control of *Verticillium* by arbuscular mycorrhizal fungi (AMF) of the Glomeromycota is variable. Twelve of the tested strains could effectively protect plants against the disease with a maximum reduction of the disease incidence with 65%, while some of the AMF even worsened the disease (Davis et al., 1979; Porras-Soriano et al., 2006). Interestingly, *Glomus deserticola* influenced the plant phenology of pepper plants which contributed to more resistant or tolerant plants to pathogen attack (Garmendia et al., 2004c).

Some *Penicillium* isolates or their exudates or dry mycelium were tested for potential biocontrol. In cotton, the application of dry mycelium resulted in a control efficacy of 27–50% depending on the applied dose (Dong et al., 2006). Exudates of *Penicillium chrysogenum* EEZ10 decreased the negative effect of *Verticillium* on the plant growth of tomato (García et al., 2011). The formulation of *Penicillium oxalicum* PO-212 spores influenced the efficacy: mixing the conidia with the substrate gave better control compared to applying the conidial suspension immediately to the seedbed (Larena et al., 2003).

A lot of isolates belonging to *Trichoderma* have been evaluated for their capacity to control Verticillium wilt with variable successes. Ten *Trichoderma* isolates were tested by D'Ercole et al. (2000) and *Trichoderma viride* T46 and T117 resulted in the best protection with a reduction of the disease incidence of 30% in eggplant. Three strains reduced the disease with more than 80% in tomato, eggplant and pepper (Dutta, 1981; Narisawa et al., 2002; Ślusarski and Pietr, 2009). In the case of respectively *Trichoderma asperellum* B35 and *Trichoderma harzianum* T-35, the efficacy of control depended on several factors such as the field location of the experiments and the type of formulation (Ordentlich et al., 1990; Ślusarski and Pietr, 2009). In olive, *T. asperellum* isolates T25 and Bt3 and application of BIOTEN® (*T. asperellum* + *T. gamsii*) reduced the disease severity of Verticillium wilt but not the incidence (Carrero-Carrón et al., 2016; Varo et al., 2016b).

Recently, *Fusarium oxysporum* isolates have gained interest as BCA against Verticillium wilt. *F. oxysporum* is also a soil-borne fungi and able to colonize and penetrate the roots of host plants. *F. oxysporum* F2 has been extensively studied for its biocontrol capacity on eggplant and reduced disease severity and colonization by *V. dahliae* (Malandraki et al., 2008; Pantelides et al., 2009; Gizi et al., 2011; Angelopoulou et al., 2014). The strain was applied by seed treatment or amendment to the transplant soil plug. This last strategy gave the best results with a dose dependent response. Pepper and olive plants treated with *F. oxysporum* isolate Fo47 exhibited reduced symptoms (Veloso and Díaz, 2012; Varo et al., 2016b). In the case of olive, the *F. oxysporum* isolates FO04 and FO12 showed stronger biocontrol activity against Verticillium wilt than isolate Fo47 (Varo et al., 2016a,b). In cotton, *F. oxysporum* By125 and *F. oxysporum* CanR-46 reduced disease severity with respectively 69 and 92% (Zheng et al., 2011; Zhang et al., 2015). Applying exudates of *Fusarium lateritium* to tomato roots decreased the negative effect of *V. dahliae* on the growth of the plants (García et al., 2011).

Different isolates belonging to *V. dahliae*, *Verticillium albo-atrum*, *Verticillium isaacii*, *Verticillium tricorpus,* and *Gibellulopsis nigrescens* (formerly *Verticillium nigrescens*) protected plants against a virulent relative of *Verticillium* spp. The isolate *V. dahliae* Dvd-E6 was non-pathogenic on tomato and conferred protection to tomato plants challenged with the pathogen *V. dahliae*. The order of inoculation of both isolates influenced the level of protection (Shittu et al., 2009). Applying exudates of *V. dahliae* 2379 to tomato roots decreased plant growth reduction by a pathogenic *V. dahliae* isolate (García et al., 2011). In cotton, Verticillium wilt was reduced by *V. albo-atrum* SS-4 and *G. nigrescens* (Schnathorst and Mathre, 1966; Zhu et al., 2013; Vagelas and Leontopoulos, 2015). In all those studies, pre-inoculation of the protective isolate appeared to be more robust at reducing Verticillium symptoms relative to co-inoculation. The amount of inoculum applied also played a role for the level of protection by *V. albo-atrum* SS-4 (Schnathorst and Mathre, 1966). Two isolates, *V. dahliae* Dvd-E6 and *V. albo-atrum* SS-4, were able to reduce symptom development in respectively tomato and cotton, but were pathogenic on other host plants (Schnathorst and Mathre, 1966; Dobinson et al., 1998).

V. tricorpus and *V. isaacii* (formerly *V. tricorpus*) were both associated with soil suppressiveness of Verticillium wilt in respectively potato and cauliflower fields (Davis et al., 2000; França et al., 2013). *V. isaacii* Vt305, an isolate obtained from the suppressive cauliflower field, has shown to be able to reduce symptom development and colonization by *V. longisporum* of cauliflower (Tyvaert et al., 2014). The control was dependent on the applied dose of both the pathogen and the BCA. Robinson et al. (2007) found that *V. tricorpus* reduced Verticillium disease of potato with 74% in a field experiment and pre-inoculation resulted in the best protection. In the same study, protection by a *V. albo-atrum* isolate was comparable. Also the colonization of the different potato tissues by the pathogenic *V. albo-atrum* isolate was remarkably reduced by pre-inoculation with *V. tricorpus* or *V. albo-atrum*. Several *V. isaacii* isolates reduced Verticillium wilt of lettuce and pretreatment appeared to provide better protection than co-inoculation (Qin et al., 2008).

Modes of Action of the Studied BCAs

Several modes of action are known to be involved in biological disease control, but the underlying mechanisms of specific interactions with pathogenic *Verticillium* isolates are often unknown. The modes of action reported for the different genera of antagonists against Verticillium wilt are shown in **Table 4**. **Figure 2** shows how BCAs can interfere with different steps in the infection cycle of *Verticillium*. Direct microbial antagonism involves parasitism of the fungus and its surviving structures, competition for nutrients and infection sites or antibiosis. This leads to less inoculum present in the rhizosphere or a lower infection potential of the pathogen. Indirect mechanisms include plant growth promotion and induced resistance. Several bacterial and fungal BCAs promote plant growth and in this way the deleterious effects of Verticillium wilt are reduced. Induced resistance can also contribute to the protection against Verticillium wilt, particularly if this process is initiated in the root tissue which is primarily colonized by the pathogen. Often, several mechanisms are expressed by a single biocontrol agent and one mode of action does not necessarily excludes another.

Reducing Germination of Inoculum

Especially in the case of a monocyclic disease such as Verticillium wilt, reducing the germination of primary inoculum is an interesting mode of action of potential BCAs. Root application of the BCAs *P. alvei* K-165, *Arthrobacter* sp. FP15 and *Blastobotrys* sp. FP12 resulted in the reduction of microsclerotia germination of *V. dahliae* in the rhizosphere of eggplants (Antonopoulos et al., 2008; Papasotiriou et al., 2013). Al-Rawahi and Hancock (1998) furthermore demonstrated that *P. oligandrum* was able to parasitize *V. dahliae* and to impede its microsclerotia formation. Interestingly, the BCA *T. flavus* decreased the viability of *V. dahliae* microsclerotia on senescent potato stems, which eventually could limit the release of these surviving structures to the soil (Nagtzaam et al., 1998). Mycofumigation with the volatile organic compounds of *Muscodor albus, Muscodor roseus,* and *F. oxysporum* CanR-46 also effectively reduced inoculum density of *V. dahliae* in the soil, thereby suppressing Verticillium wilt in respectively eggplant and cotton (Stinson

TABLE 4 | Mode of action of selected biocontrol agents against Verticillium wilt.

Genus antagonist	Reduced germination of inoculum	Plant growth promotion	Competition for infection sites/space/nutrients	Induced resistance	Antibiosis *in vitro*	Mycoparasitism *in vitro*
BACTERIA						
Bacillus	x (iturins)	x	x	x (iturins)	x	x
Paenibacillus	x	x		x	x	x
Streptomyces	x (prodiginines)	x	x	x	x	x
Pseudomonas		x	x	x	x	x
Serratia	x	x				x
FUNGI						
Pythium	x	x				x
Fusarium	x (VOCs)[1]	x	x	x (DAMP release)[2]	x	x
Trichoderma	x	x			x	x
Verticillium		x	x	x (DAMP release)		
Talaromyces	x				x	x
Penicillium		x		x (DAMP release)	x	x
Muscodor	x (VOCs)					
Gliocladium	x	x			x	x
Mycorrhizae		x		x		

[1] *VOCs: volatile compounds.*
[2] *DAMP: damage associated molecular pattern.*

et al., 2003; Zhang et al., 2015). In addition, the iturins of the culture filtrate of *B. amyloliquefaciens* 41B-1 suppressed *V. dahliae* microsclerotial germination, while the prodiginines produced by *S. lividans* reduced the formation of *V. dahliae* microsclerotia (Meschke et al., 2012; Han et al., 2015). The importance of biosurfactant production in the suppression of *Verticillium* microsclerotia viability by *Pseudomonas* spp. has only been shown *in vitro* (Debode et al., 2007). The germination of *V. dahliae* microsclerotia was also reduced by several *Gliocladium roseum* strains (Keinath et al., 1991; Varo et al., 2016b). Remarkably, effects of BCAs on surviving mycelium and chlamydospores were not reported. A possible explanation is that almost all BCAs have been tested against *V. dahliae* and *V. longisporum*, which only form microsclerotia to survive in soil (**Table 1**).

Growth Promotion

BCAs of *Verticillium* often promote root and/or shoot growth and this has been reported for isolates of the bacterial genera *Bacillus*, *Paenibacillus, Streptomyces, Enterobacter, Pseudomonas,* and *Serratia,* and the fungal(-like) genera *Pythium, Fusarium, Nectria, Trichoderma, Verticillium, Penicillium, Phomopsis,* and AMF. The plant growth promoting effect of BCAs can counteract the adverse effect of pathogenic *Verticillium* species on the yield of crops as exemplified by the interaction of *S. plymuthica* R12 and *V. dahliae* in strawberry. Although treatment of strawberry with this *Serratia* strain resulted in a higher disease incidence of Verticillium wilt, a five-fold enhancement of the number of stolons and a yield enhancement of more than 70% was found (Berg et al., 2001). Production of plant growth hormones may be involved in improving plant growth mediated by the BCAs. Auxin production was demonstrated *in vitro* for some bacterial BCAs such as *B. amyloliquefaciens* 5-127, *P. alvei* K-165, and *S. plymuthica* HRO-C48 (Kalbe et al., 1996; Tjamos et al., 2004). Besides mechanisms involving phytohormones, enhanced growth may also be exerted by improved nutrient acquisition (Berg, 2009). Soil inoculation with a consortium of three plant-growth promoting rhizobacteria, active against *Verticillium* in cotton, improved soil properties in field experiments, including an increase in organic matter and the availability of nitrogen, phosphorus and potassium (Yang et al., 2014). AMF are known to promote plant growth and several of them reduce Verticillium wilt in solanaceous plants and alfalfa (Hwang et al., 1992; Liu, 1995; Matsubara et al., 1995; Karagiannidis et al., 2002; Garmendia et al., 2004a,b,c, 2006; Demir et al., 2015). Treatment with *Funneliformes mosseae* resulted in a higher phosphorus and nitrogen uptake in tomato and eggplant (Karagiannidis et al., 2002). Also pepper plants associated with *G. deserticola* had a higher phosphorus uptake (Garmendia et al., 2004b). This increased capacity for nutrient uptake could contribute to diminish the deleterious effect of the pathogen (Karagiannidis et al., 2002; Garmendia et al., 2004b).

Competition

Competition for space, infection sites and nutrients is well-established as working mechanism of BCAs and was suggested to be involved in the interaction between *Verticillium* and several biocontrol isolates of *Bacillus*, *Streptomyces, Pseudomonas, Verticillium,* and *Fusarium*. For *Verticillium,* particularly competition for nutrients and/or infection sites in the soil and

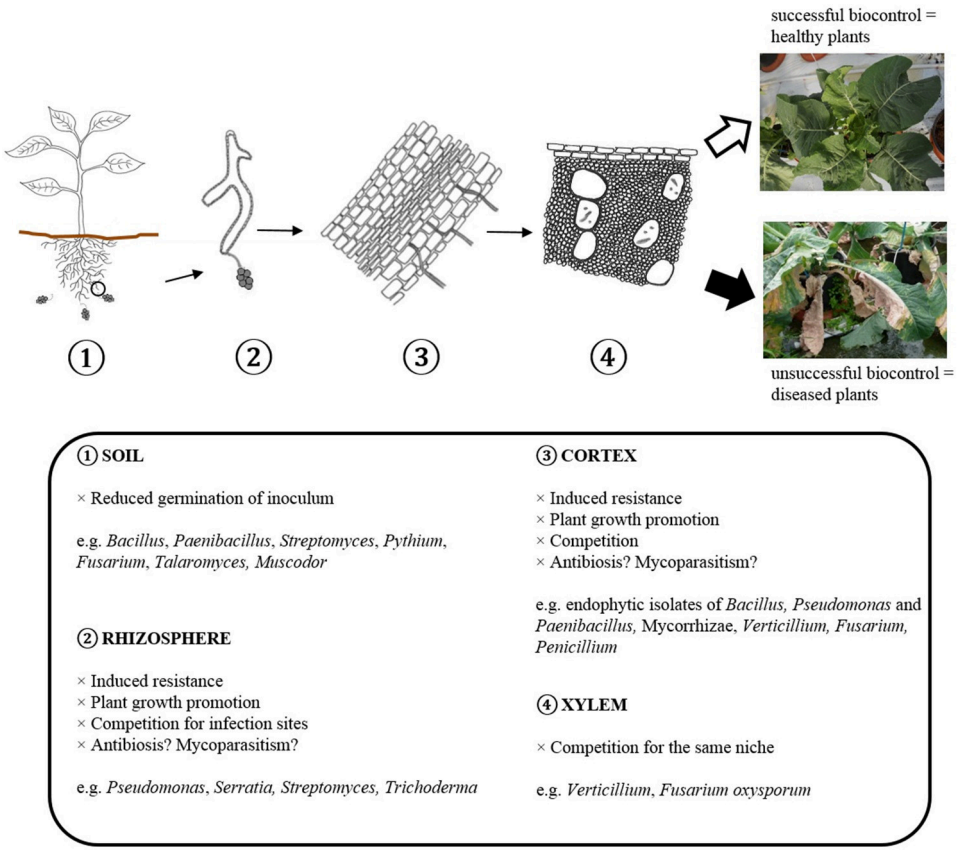


FIGURE 2 | Scheme showing how BCAs can interfere with different steps in the infection cycle of *Verticillium*.

in/on the roots may be an efficient mode of action in controlling the disease. It is expected that bacterial BCAs compete for nutrients and infection sites in the rhizosphere and cortex, while BCAs such as *Verticillium* and *Fusarium* can also colonize the xylem and occupy the same niche as *Verticillium*. A commonly cited example of competition is that for iron. Under iron-limiting conditions, bacteria produce siderophores with high affinity for ferric iron. By binding available iron these bacteria prevent the pathogens' access to the limited pool of soluble iron in the rhizosphere and in that way the growth of the pathogen is hindered (Loper and Buyer, 1991; Loper and Henkels, 1999). The *in vitro* production of siderophores was shown for a number of BCAs with antagonistic effect on *Verticillium* (Berg et al., 1996, 2000; Mercado-Blanco et al., 2004; Li et al., 2010; Xue et al., 2013). However, Maldonado-González et al. (2015a,b) showed that siderophore production is not required for biological control of Verticillium wilt by *Pseudomonas fluorescens* PICF7.

Induced Resistance

Induced resistance has frequently been proposed to be part of the working mechanism of the BCAs. Evidence of triggering plant defense responses was provided for antagonistic isolates of the bacterial genera *Arthrobacter, Bacillus, Paenibacillus, Streptomyces,* and *Pseudomonas,* and of the fungal genera *Fusarium, Verticillium, Penicillium*, *Blastobotrys, Coriolopsis,* and *Trametes.* Also AMF of the genera *Glomus*, *Gigaspora* and *Claroideoglomus* were able to induce resistance. *P. alvei* K-165 and *F. oxysporum* F2 induced the expression of defense-related genes *PR1* and *PR4* in eggplant. Moreover, the expression of these genes was positively correlated with the rhizosphere population of both BCAs (Angelopoulou et al., 2014). In *Arabidopsis*, it has been shown that the resistance induced by *P. alvei* K-165 against *V. dahliae* is dependent on both salicylate and jasmonate-dependent defense pathways (Tjamos et al., 2005; Gkizi et al., 2016). Results of a split-root experiment indicated the involvement of induced resistance in the protection of eggplant against *V. dahliae* by *Arthrobacter* sp. FP15 and *Blastobotrys* sp. FP12 (Papasotiriou et al., 2013). The endophytic BCA *P. fluorescens* PICF7 has been shown to activate an array of defense pathways in the roots and aerial tissues of olive upon colonization of the roots (Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2014). Recently, Gómez-Lama Cabanás et al. (2017) demonstrated that the expression of defense-related genes differed depending on whether or not *V. dahliae* and *P. fluorescens* PICF7 colonized the same sectors of the roots of olive plants. Interestingly, no biocontrol was observed when *V. dahliae* and *P. fluorescens* PICF7 were spatially separated. In the case of *B. amyloliquefaciens* 41B-1, iturins could induce plant defense responses and mediate pathogen-associated molecular pattern (PAMP)-triggered immunity against *V. dahliae* in cotton

(Han et al., 2015). Applying exudates of several saprobe fungi (*Coriolopsis rigida, Trametes versicolor, F. lateritium, P. chrysogenum,* and the non-pathogenic *V. dahliae*-2379) could control *V. dahliae* disease of tomato probably through hydrolyzing root cell wall components. This generates damage associated patterns (DAMPs) which could act as elicitors of plant defense (García et al., 2011). PAMPs and DAMPs can be recognized by specific membrane-bound receptors in the plant, leading to PAMP-triggered immunity (PTI; Boller and Felix, 2009; Zipfel, 2014). Induced resistance by AMF resulted in a more balanced antioxidant metabolism (Garmendia et al., 2004a), the induction of defense-related enzymes (Garmendia et al., 2006) and accumulation of lignin in the roots (Matsubara et al., 1995).

What about Cross-Protection?

The protection of plants against virulent *Verticillium* spp. by closely related isolates that are non-pathogenic on that specific host has often been described as cross-protection. Only in a few studies the underlying mechanisms of this phenomenon were elucidated (Shittu et al., 2009; García et al., 2011). Mechanisms involved include induced resistance, competition for space (including infection sites) and nutrients, and plant growth promotion. *In vitro*, it was often shown that neither isolate is inhibitory to the other. The best protection is accomplished if the protective isolates are applied to the plants before challenge treatment with the pathogen. Also the concentrations of inoculum of both the pathogen and the beneficial organism are of importance for the level of control (Shittu et al., 2009; Tyvaert et al., 2014). *Verticillium* species have proven to expand their host range and the stability of the interaction between non-pathogenic and pathogenic isolates remains an open question (Shittu et al., 2009).

What about Antibiosis and Mycoparasitism of Verticillium Mycelium?

The majority of BCAs included in this study showed *in vitro* antagonism against *Verticillium* mycelium (**Tables 2**, **3**, **4**) but a possible role of antibiosis in biocontrol *in planta* has not been demonstrated. Only when production at the site of biocontrol is demonstrated or when activity is proved by the use of non-producing or over-producing mutants, or reporter strains, the role of metabolites in disease biocontrol can be confirmed (Whipps and McQuilken, 2009). To our knowledge, these types of studies have not been reported for *Verticillium* biocontrol. Another type of direct antagonism is mycoparasitism and the associated production of extracellular lytic enzymes. Chitinases, proteases, and glucanases are produced *in vitro* by many of the studied BCAs of *Verticillium*, but clear evidence that these enzymes play a role in the direct interaction with the pathogen in the presence of plants is lacking. Regarding the life cycle of *Verticillium*, germination of survival structures such as microsclerotia is stimulated by the direct vicinity of germinating seeds or plant roots. Root penetration and subsequent colonization of the xylem vessels can be achieved within only 2–4 days (Heinz et al., 1998; Chen et al., 2004; Fradin and Thomma, 2006). Possibilities for reducing mycelial growth in the rhizosphere by direct antagonism may therefore be limited. Direct antagonism *in planta* is only possible for those BCAs that are able to colonize the cortex or xylem. The production of antibiotics and inhibitory metabolites is influenced by plant type and age, nutrient availability, environmental conditions, microorganisms present and the pathogen itself (Molina et al., 2003; Duffy et al., 2004; Maurhofer et al., 2004; Morello et al., 2004; Compant et al., 2005). It is not clear if conditions inside the plant are conducive for the production of antimicrobial compounds. *In planta* studies on the behavior of BCAs are limited but for *T. harzianum*, the interaction with *V. dahliae* in olive was investigated. Mycoparasitism of *V. dahliae* by *T. harzianum* occurred *in vitro*, although there was no evidence that this also happens *in planta* (Ruano-Rosa et al., 2016). In this context, it is interesting to notice that control of *Verticillium* by *Trichoderma*, for which the main modes of action include antibiosis and mycoparasitism, is limited. *Trichoderma* is one of the most studied and successful BCAs, with many commercial products that are used in practice to control a variety of soil-borne pathogens such as *Rhizoctonia*, *Fusarium*, *Sclerotinia*, *Botrytis,* and *Pythium*. Possibly, *Trichoderma* strains were originally selected for control of other soil-borne pathogens and were later on tested against *Verticillium*. Therefore, not the best strains for biocontrol of *Verticillium* might have been selected. Interestingly, it was shown by Carrero-Carrón et al. (2016) that *T. asperellum* T25 that was effective in controlling Verticillium disease in olive had the highest ability to grow endophytically in the roots. But in comparison with other isolates, it had the lowest inhibitory effect on the *in vitro* growth of *V. dahliae*. The capacity of a biocontrol strain to compete for the same ecological niche of *Verticillium* could be crucial, indicating that selection criteria should not focus on *in vitro* antagonism.

WHAT ARE THE KEY FACTORS IN THE PROCESS FROM SELECTION OF THE BCA TO SUCCESSFUL IMPLEMENTATION?

From our survey of biocontrol studies we can conclude that common BCAs such as *Trichoderma*, *Pythium*, *Gliocladium,* and AMF are not the best candidates for augmentative biological control of Verticillium wilt. Few studies reported the biocontrol effect of *Gliocladium* on Verticillium wilt. Some *Gliocladium* strains could reduce microsclerotia viability in soil conditions, but the number of reports about successful biocontrol *in planta* is limited (Keinath et al., 1991; Varo et al., 2016b). The biopesticide Polyversum®, containing *P. oligandrum*, showed no control of *Verticillium* in one study and in another study, it resulted in variable control (Al-Rawahi and Hancock, 1998; Rekanovic et al., 2007). Some of the *Trichoderma* strains (*T. asperellum* T34, *T. harzianum* T-22) were shown to be able to reduce Fusarium wilt (Cotxarrera et al., 2002; Gilardi et al., 2007; Sant et al., 2010) and are approved by the EU as biopesticide against *Fusarium* but not against *Verticillium.* It would be expected that *F. oxysporum* and *Verticillium* can be controlled by the same BCAs because they have apparently similar characteristics.

Both pathogens share the same ecological niche: they are soil-borne pathogens able to colonize the vascular system with the production of similar symptoms. A closer look to the infection and colonization process gives evidence for some important differences. *Verticillium* inhabits the lower parts of the plant for a longer time than *F. oxysporum* (Klimes et al., 2015). *F. oxysporum* has a higher degree of host specialization and produces symptoms faster (Klosterman et al., 2011). The *V. dahliae* enzyme VdThi4, required for biosynthesis of a thiamine (vitamin B1), has been shown to play a role in the colonization process. *VdThi4* deletion mutants are unable to colonize the upper portion of the plant. In *F. oxysporum*, however, the *VdThi4* homolog s*tri35* was not required for virulence (Hoppenau et al., 2014). Tomato plant cells respond differently to infection by both pathogens (Ferraris et al., 1974; Cooper and Wood, 1980; Bishop and Cooper, 1983a,b). Recently, genomic insights into both pathogens revealed some differences in the secretome. More specifically, a protein family involved in attachment to plant cell walls and increase of enzyme efficiency was expanded in *Verticillium* (Klosterman et al., 2011). These differences may explain why some BCAs are effective against *Fusarium* but not against *Verticillium*.

Where to Look for Potential BCAs?

Disease suppressive soils are an interesting source of BCAs with potential against soil-borne diseases (Cook, 1985). *Fusarium* suppressive soils have extensively been studied while soil suppressiveness for *Verticillium* is rarely reported. A strain of *F. oxysporum* (Fo47) originated from suppressive soils for Fusarium wilt of tomato and had also biocontrol activity against Verticillium wilt on pepper (Veloso and Díaz, 2012). Keinath and Fravel (1992) demonstrated that by successive croppings, some soils exhibit induced suppressiveness to Verticillium wilt of potato. Only a few studies were carried out with isolates from suppressive soils for Verticillium wilt of potato and cauliflower. From these soils non-pathogenic *Verticillium* isolates, belonging to *V. tricorpus* and *V. isaacii*, were obtained that could control Verticillium wilt in potato and cauliflower (Davis et al., 2000; França et al., 2013; Tyvaert et al., 2014).

Organic amendments have proven to be disease suppressive and are therefore interesting reservoirs of potential BCAs. Several isolates controlling Verticillium wilt were obtained from suppressive composts: two *F. oxysporum* and two *P. fluorescens* isolates originated from the rhizosphere of eggplants grown in soil amended with disease suppressive compost (Malandraki et al., 2008), while the isolates belonging to *Arthrobacter* and *Blastobotrys* were obtained from disease suppressive olive mill compost (Papasotiriou et al., 2013). Another strategy to look for successful BCAs is to identify healthy plants in infested fields. In this way a *Nectria* isolate and two *B. subtilis* isolates with biocontrol activity against *Verticillium* were recovered from healthy cotton roots in infested soil (Luo et al., 2010; Zheng et al., 2011; Li et al., 2013). Most of the other bacterial BCAs described in **Table 2** were obtained from the rhizosphere or roots of host plants. The origin of the fungal BCAs described in **Table 3** is not always indicated. Clearly, not a lot of the studied isolates were obtained from sources giving already some evidence for biological control. It does not necessarily mean that those isolates perform better but at least they are expected to establish better in field conditions, as they are able to colonize the soil or host plants.

Desirable Characteristics

The ability to affect surviving structures of *Verticillium* by antibiosis or mycoparasitism is a desirable trait of BCAs resulting in a reduction of the primary inoculum. Selection of BCAs sharing the same ecological niche as *Verticillium* is promising, since these organisms can compete with *Verticillium* for infection sites, space and nutrients. For instance in the tripartite interaction *V. dahliae*-olive-*P. fluorescens* PICF7, niche overlap between the BCA and the pathogen *in planta* was necessary for effective biocontrol (Gómez-Lama Cabanás et al., 2017). Efficient root colonizers can compete with *Verticillium* for infection sites. In addition, they may protect the plant by triggering induced resistance by secreting PAMPs or releasing DAMPs from plant cells. BCAs with an endophytic lifestyle that colonize the cortex and/or the xylem are protected against adverse environmental conditions, and can exclude *Verticillium* from the same niche by competition for space and nutrients, as exemplified by a non-pathogenic *F. oxysporum* (Pantelides et al., 2009), or by inducing resistance responses in the plant as shown for *Bacillus* spp. (Han et al., 2015). Often, non-pathogenic fungi that are closely related to the pathogen can successfully control disease in naturally infested soils (Herr, 1995; Gutteridge et al., 2007; Alabouvette et al., 2009). In the case of Verticillium wilt this has been demonstrated for non-pathogenic *Verticillium* isolates. However, it is important to confirm that these isolates are really non-pathogenic on a wide range of plants. Finally, the ability to promote plant growth can compensate for some of the deleterious effects caused by pathogenic *Verticillium* spp. *In vitro* screening for antimicrobial activity against *Verticillium* mycelium correlates poorly or not at all with biocontrol activity *in planta* and does not seem to be the best strategy to look for good *Verticillium* BCAs.

The ability to control *Verticillium* in several host plants or to control other soil-borne and/or vascular pathogens, is interesting to increase the market potential of the BCA. Several BCAs able to reduce Verticillium disease were also effective in controlling other diseases and examples are summarized hereafter. Non-pathogenic *F. oxysporum* isolates also controlled Fusarium wilt and *Phytophthora* root rot and blight of pepper plants (Díaz et al., 2005; Veloso and Díaz, 2012). Cotton plants treated with dry mycelium of *P. chrysogenum* exhibited reduced symptoms of *Verticillium* and Fusarium wilt (Dong et al., 2006; Zhang et al., 2011). Mycofumigation with *Muscodor* spp. could control seedling diseases of sugar beet next to Verticillium wilt of eggplant (Stinson et al., 2003). Besides its biocontrol effect on *V. dahliae* in eggplant and potato, the bacterial BCA *P. alvei* K-165 reduced root discoloration and hypocotyl lesions caused by the black root rot fungus *Thielaviopsis basicola* on cotton seedlings (Tjamos et al., 2004; Schoina et al., 2011). *Pseudomonas chlororaphis* MA 342, which suppressed *V. longisporum* in oilseed rape, furthermore controls a wide range of cereal seed-borne

diseases and is the active organism in the registered products Cedomon® and Cerall® (Johnsson et al., 1998; Abuamsha et al., 2011).

Omics technologies are an interesting tool for the selection of promising BCAs, as these technologies allow in-depth characterization of the strain. The modes of action of a BCA can be identified by characterization of genes, mRNAs, and proteins. Also the properties of strains with different control efficacy can be compared. This may lead to the selection of BCAs with the best control potential in terms of efficacy and consistency (Massart et al., 2015).

Evaluation of Biocontrol Activity

Experiments with BCAs are often carried out in sterile soils using plants that have been artificially inoculated with *Verticillium* via root dipping in a conidial suspension or via soil drench with a conidial suspension. These experimental conditions are quite different from natural infested field conditions. First of all, in sterile soils, the BCA can easily establish, while BCAs often fail to work in the field due to more complex conditions. Secondly, disease development in sterile soils is fast and often leads to severe symptoms. This can be a disadvantage for the BCA and possibly some effective BCAs are not selected because they seem of minor importance during the selection procedure in sterile conditions. Preferentially, experiments should be carried out with naturally infested soil, in field and greenhouse conditions, or by using microsclerotia as primary inoculum. In addition, the plants should be observed until the onset of flowering as the spread of *Verticillium* in the host tissue has been suggested to be induced by the initiation of flowering (Veronese et al., 2003; Zhou et al., 2006). Also screening for BCAs that target the primary inoculum should be done in conditions that mimic the natural situation. For instance, *Microsphaeropsis ochracea* reduced the microsclerotia viability in sterile soils but not in unsterile soils and failed to control Verticillium wilt of oilseed rape in the field (Stadler and von Tiedemann, 2014). It is therefore interesting to start screening for biocontrol strains from the field, to perform subsequently experiments in controlled conditions and to go back to the field finally.

Formulation and Application

In order to develop a promising BCA into a commercial product, large scale production, formulation, preservation conditions, shelf life, and application methods should be investigated. Nowadays, researchers interested in biocontrol are becoming more aware of the importance of these issues in product development.

Fungi and bacteria that produce surviving structures are interesting because these structures can be used as the active substance of the biocontrol product. Usually they are persistent to adverse environmental conditions and can be preserved and distributed without special requirements. Therefore, sporulating Gram-positive microorganisms, such as *Bacillus* and *Streptomyces*, are preferred rather than Gram-negative bacteria. Soil-borne fungi usually produce surviving structures such as chlamydospores in the case of *F. oxysporum* and microsclerotia in case of *Verticillium* species. A possible disadvantage of surviving structures is that the production process might be complex leading to a higher cost. Also the ability of those BCAs to become persistent in the new environment should be considered. The capacity of a strain to produce different structures is a desirable characteristic for application in different crop systems.

Application of the *Verticillium* BCAs close to the roots, where *Verticillium* initially infect the plants, could be the most effective strategy. The early introduction of the BCA by seed treatment and treatment of seedlings at the nursery stage could provide better relief from subsequent *Verticillium* infection than when the BCA is applied directly to the field. In the case of seed treatment, compatibility with standard seed treatments should be ensured. BCAs that can reduce germination of primary inoculum could be added to compost amendments or to the substrate.

Combining two or more BCAs is another interesting approach to improve the efficacy of biocontrol or to control different pathogens and even pests. Therefore, the application of the specific isolates should be compatible without reducing their single effect. Yang et al. (2013) showed that the combined application of three endophytic bacterial strains resulted in a better biocontrol efficacy of Verticillium wilt in cotton than their individual applications, which was probably linked to the fact that the different strains are predominant in different developmental stages of cotton. Also the application of a consortium of three rhizobacteria, *Bacillus cereus* AR156, *B. subtilis* SM21 and *Serratia* sp. XY21, resulted in higher biocontrol efficacy against Verticillium wilt in cotton compared to the individual strains (Yang et al., 2014). For other plant pathogens, it has been shown that mixtures of bacterial and fungal BCAs are more effective in controlling diseases such as *Rhizoctonia* and *Pythium* (Colla et al., 2012). The strength of a mixture is that BCAs can be combined that interact in a different way with the pathogen and/or the plant. Moreover, if conditions are not favorable for one of the BCAs, the other can take over. The drawback is that all isolates used in the mixture need to be registered.

The reliability of a product based on microbial BCAs is a crucial issue in ensuring long-term acceptance and sustained use by farmers. Standardized guidelines for quality control of the (potential) commercially available BCAs may help to avoid failures in their practical application and to prevent the application of organisms with detrimental effects. Parameters to be considered include content of fertilizers, presence of contaminants, traceability of the origin of the BCA, possible allelopathic effects of the BCA on the germination of some plant species and effectiveness under various conditions.

As Verticillium wilt is an emerging problem in different crops, some agricultural systems seem to promote Verticillium disease. Therefore, it could be difficult to reach satisfactory levels of control of Verticillium with a BCA in such a system. To implement biocontrol as a tool of IPM in agriculture, the current approach should be changed to a holistic management (van Lenteren et al., 2017).

CONCLUSION

The application of BCAs is an interesting building block of sustainable and environmentally sound management strategies of Verticillium wilt. A holistic management should be considered to reach satisfactory levels of control by a BCA. Based on the number of currently known isolates with biocontrol activity against *Verticillium* species, the predominant genera are *Pseudomonas, Bacillus, Fusarium,* and *Verticillium*. Particularly soils or organic amendments suppressive for *Verticillium* disease and healthy plants in infested fields are attractive spots to find (new) BCAs of *Verticillium*. The ability to affect survival structures, sharing the same ecological niche as *Verticillium,* inducing resistance responses in the plant and promoting plant growth are desirable characteristics of a competent BCA against Verticillium wilt. Evaluating the biocontrol efficacy of BCAs in conditions that mimic the field situation is expected to significantly improve the chance of successful application in practice. In order to facilitate the further commercialization of a promising BCA of *Verticillium*, potential bottlenecks such as large-scale production, formulation, preservation conditions, shelf life, and application methods, should be tackled early in the selection process.

AUTHOR CONTRIBUTIONS

SD wrote the part about bacteria involved in biocontrol against Verticillium and made the figures. LT wrote the part about fungi involved in biocontrol against Verticillium and helped in making the figures. SD and LT contributed equally. SF and MH revised the manuscript and helped in structuring and editing the work.

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Assessment of the Diversity of *Pseudomonas* spp. and *Fusarium* spp. in *Radix pseudostellariae* Rhizosphere under Monoculture by Combining DGGE and Quantitative PCR

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Radix pseudostellariae is a perennial tonic medicinal plant, with high medicinal value. However, consecutive monoculture of this plant in the same field results in serious decrease in both yield and quality. In this study, a 3-year field experiment was performed to identify the inhibitory effect of growth caused by prolonged monoculture of *R. pseudostellariae*. DGGE analysis was used to explore the shifts in the structure and diversity of soil *Fusarium* and *Pseudomonas* communities along a 3-year gradient of monoculture. The results demonstrated that extended monoculture significantly boosted the diversity of *Fusarium* spp., but declined *Pseudomonas* spp. diversity. Quantitative PCR analysis showed a significant increase in *Fusarium oxysporum*, but a decline in *Pseudomonas* spp. Furthermore, abundance of antagonistic *Pseudomonas* spp. possessing antagonistic ability toward *F. oxysporum* significantly decreased in consecutively monocultured soils. Phenolic acid mixture at the same ratio as detected in soil could boost mycelial and sporular growth of pathogenic *F. oxysporum* while inhibit the growth of antagonistic *Pseudomonas sp*. CJ313. Moreover, plant bioassays showed that *Pseudomonas* sp. CJ313 had a good performance that protected *R. pseudostellariae* from infection by *F. oxysporum.* In conclusion, this study demonstrated that extended monoculture of *R. pseudostellariae* could alter the *Fusarium* and *Pseudomonas* communities in the plant rhizosphere, leading to relatively low level of antagonistic microorganisms, but with relatively high level of pathogenic microorganisms.

Keywords: *Radix pseudostellariae*, DGGE, *Fusarium*, *Pseudomonas*, quantitative PCR

INTRODUCTION

As much as 70% of medicinal plants suffer from consecutive monoculture problem, also known as replant disease or soil sickness. These problems are commonly observed in the production of many Chinese medicinal herbs, including *Radix pseudostellariae*, *Rehmannia glutinosa*, *Panax notoginseng*, etc (Zhang and Lin, 2009). *R. pseudostellariae*, a perennial tonic medicinal plant, belongs to the family Caryophyllaceae with extremely high medicinal value (Zhao W.O. et al., 2015). Consecutive monoculture of this plant in the same field leads to a serious decrease in both quality and yield of roots along with poor plant performance, which severely limited production and utilization of its medicinal plant virtues (Lin et al., 2015). Therefore, it is necessary to explore the mechanism of consecutive monoculture problems affecting the plant and develop effective control strategies for *R. pseudostellariae.*

Fusarium species is one of the most abundant, prevalent, and important soil fungi (Damicone and Manning, 1985). It is notorious due to the ability of attacking diversity of host plants and bring upon them diseases like vascular wilts, seedling damping off and rots of stem (Pietro et al., 2003; Punja and Parker, 2009; Chakravarty and Hwang, 2010). Similarly, the soil-borne disease caused by *F. oxysporum* in *R. pseudostellariae* fields were reported (Zhao Y.P. et al., 2015), however, the other *Fusarium* species are often overlooked. Therefore, in order to develop the full potential of the disease-suppressive microbial community in the biological control, we need more information to unravel the different roles of this potentially important species.

In recent years, more attentions were paid to develop the environment friendly and good agriculture practices for disease control. It has become important to explore the nature of microbial diversities in the soil, particularly *Pseudomonas* in different cropping periods or regime (Mendes et al., 2011). *Pseudomonas* species were reported to have a wide range of functional groups, such as plant pathogens (Samson et al., 1998), xenobiotic degraders (Clausen et al., 2002) and plant growth promoters (Patten and Glick, 2002). In addition, *Pseudomonas* species can be used as biological control agents for soil-borne pathogens, including black rot of tobacco, disease of wheat and *Fusarium* wilt (Raaijmakers and Weller, 1998; Patten and Glick, 2002; Mendes et al., 2011).

Recently, the increasing evidences suggest that plant–microbial interactions play many pivotal roles in soil quality and plant health (Lakshmanan et al., 2014; Macdonald and Singh, 2014). Li et al. (2014) reported that the peanut root exudates can selectively inhibit certain communal bacteria, such as *Gelria glutamica*, *Mitsuaria chitosanitabida*, and *Burkholderia*, but stimulate the bacterial taxon of *Desulfotomaculum ruminis* and the fungal taxa *F. oxysporum* in soil. Wu et al. (2016c) found that the amount of two pathogenic fungi (*F. oxysporum* and *Aspergillus flavus*) in the rhizosphere significantly increased after *Rehmannia glutinosa* monoculture. Wu et al. (2015) indicated that long-term continuous cropping of black pepper (*Piper nigrum* L.) could lead to a significant decrease in soil bacterial content, especially the *Pseudomonas* spp., suggesting that the soil microbes might be responsible for soil health.

Denaturing gradient gel electrophoresis (DGGE) is considered as an effective technique to directly analyze the structural and diversity of microbial communities (Kozdrój and van Elsas, 2001). The traditional method of assessing the diversity of *Fusarium* is based on enumeration and isolation of strains which were grown on selective media (Vujanovic et al., 2002). However, morphological identification of *Fusarium* species is a time-consuming and formidable task. Yergeau et al. (2005) described a PCR-DGGE method to detect the presence of multiple *Fusarium* spp. from environmental samples. The method is based on the specific amplification and separation of the transcription elongation factor-1α (Ef1α) gene. Similarly, Widmer et al. (1998) designed a primer set (PsR and PsF) which was based on the 16S rDNA gene of *Pseudomonas* spp. in 1998. When combining the PsR and PsF primers, Evans et al. (2004) developed a semi-nested PCR and DGGE to rapidly study the diversity within the genus *Pseudomonas*. Therefore, the role of soil microbial ecology in the prevention and control of plant diseases has been given more attention (Philippot et al., 2013; Cha et al., 2015). However, few studies have been carried out to understand the relationship between *Pseudomonas* and *Fusarium* of *R. pseudostellariae*, and the approaches to overcome diseases associated with this plant.

In this study, DGGE combined with qPCR technique was used to analyze the shifts of *Pseudomonas* and *Fusarium* communities in rhizosphere soil under *R. pseudostellariae* monoculture. Several microorganisms closely related to the problem of prolonged monoculture were isolated and performed for plant-microbe interactions study. Our study can help to illustrate the effects of ecological environment and root exudates on the selection of soil microbes in rhizosphere soil, and provide useful information on potential indigenous microflora for soil remediation and improvement.

MATERIALS AND METHODS

Field Experiment

In this study, the *R. pseudostellariae* cultivar 'Zheshen 2' was used as the test material. The experiment was carried out at the experimental station of Fuding City, Fujian Province (27°26′ N, 120°04′ E). The experimental field which previously planted *Oryza sativa* was performed for this study with four treatments: (1) control with no *R. pseudostellariae* cultivation (CK), (2) the newly planted *R. pseudostellariae* cultivation (FP), (3) 2-year consecutive monoculture (SP), (4) 3-year consecutive monoculture (TP). The physical and chemical properties of the soil were detected before the experiment was initiated: total nitrogen of 1.83g kg^{-1}, available nitrogen of 26.23 mg kg^{-1}, total phosphorus of 0.47 g kg^{-1}, and available phosphorus of 96.34 mg kg^{-1}, total K of 8.46 g $kg^{-1,}$ and available K of 365.21 mg kg^{-1}. The station has a subtropical oceanic monsoon climate, annual

mean temperature at 18.4°C. All treatments were treated with the same fertilization and field management during the experiment.

Soil Sampling and DNA Extraction

The above ground or below ground biomass of *R. pseudostellariae* become significantly different after 5 months of planting (**Figure 1A**), according to our previously study (Wu et al., 2016b). Therefore, soil samples were randomly collected from five different points at each field on April 22nd, 2015. Additionally, we harvested the plants for yield determination on July 2nd, 2015 (**Figure 1B**).

Soil samples were collected after digging the plant samples. Firstly, the loosely adhering soil was shaken off, then scraping the soil that was still attached to the root as rhizosphere soil. DNA was immediately extracted from 0.5 g soil sample per treatment using Biofast Soil Genomic DNA Extraction Kit (BioFlux, Hangzhou, China) according to the manufacturer's protocols. We further determined the DNA concentration using Nanodrop 2000C Spectrophotometer (Thermo Scientific, United States) and then diluted it to 20 ng μL^{-1}.

PCR-DGGE and Analysis

Fusarium-specific PCR was performed according to the nested amplification of the Ef1α gene. The first round of PCR reactions was performed by the Ef-1 and Ef-2 primers (O'Donnell et al., 1998). It was carried out in 50 μl volumes containing 25 μl of 2 × EasyTaq PCR SuperMix (Transgen Biotech, Beijing, China), 1 μl of each primer and 40 ng template soil DNA. The program of PCR was performed by the following protocol: 95°C for 5 min, 30 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min), extension (72°C for 1 min), and 1 cycle of final extension (72°C for 10 min). The amplicons were subsequently diluted (1:20) and used for the second PCR reaction via Alfie1-GC and Alfie2 (Yergeau et al., 2005) primers. Second round PCR protocol was similar to the method of the first reaction, except for the annealing (57°C for 50 s) and extension (72°C for 50 s).

Pseudomonas-specific PCR was based on the nested amplification of the V6/V7 region of *Pseudomonas* spp. The first round of PCR reactions was used the PsF and PsR primers (Tan and Ji, 2010). PCR reaction was carried out in 50 μl volumes containing 25 μl of 2× EasyTaq PCR SuperMix (Transgen Biotech, Beijing, China), 1 μl of each primer and 20 ng template soil DNA. The program of PCR was performed by the following protocol: 95°C for 5 min, 30 cycles of denaturation (95°C for 1 min), annealing (64°C for 1 min), extension (72°C for 1 min), and 1 cycle of final extension (72°C for 10 min). The PCR products of first round was used to perform the second PCR reaction and the primers F968-GC1 and PsR were used (Garbeva et al., 2004). The following cycling protocol was performed for the second PCR: 1 cycle of initial denaturation at 94°C for 5 min, 10 cycle of denaturation (94°C for1 min), 1 min at 60°C (every subsequent one using a 0.5°C lower annealing temperature), and 2 min at 72°C, 1 cycle of 95°C for 5 min, 30 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min), and 1 cycle of final extension (72°C for 10 min). All PCR products were detected using 1.2% agarose gel and purified using a Gel Extraction Kit (OMEGA Bio-Tek, United States) according to the manufacturer's instructions. The purified PCR products were used to perform DGGE experiments.

DGGE Analysis

We performed DGGE by using an 8% (w/v) polyacrylamide gel with 35–55% and 45–60% denaturant gradients for *Fusarium*-specific and *Pseudomonas*-specific communities, respectively, using the Junyi JY-TD331A system (JUNYI, Beijing, China). DGGE was carried out at 80 V and 60°C for 12 h and 15 h in 1x TAE buffer. After electrophoresis, gels were stained with silver stain. For analysis of the molecular community profiles, gels were digitized by using the Quantity One 4.0 software (BioRad). When bands were identified, they were excised from the DGGE gel by using a sterile scalpel. After incubation overnight at 4°C, DNA was eluted from the gel. The amplicons were amplified by using the Alfie1-GC/Alfie2 and F968-GC/Psr primer sets (as mentioned before). PCR amplicons were cloned into the pEASY-T1 Cloning vector (Transgen Biotech, Beijing, China) by using manufacturer's instructions. Sequences were compared to the sequences on GenBank of NCBI using the BlastN search method.

Quantitative PCR for *Fusarium oxysporum* and *Pseudomonas* spp.

The fragments of *F. oxysporum* and *Pseudomonas* were cloned into the pEASY-T1 Cloning vector (TransGen Biotech Co., Beijing, China). Two plasmids were purified as described above. After determining DNA concentration, it was immediately diluted into 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 ng ml^{-1}. The reaction of standard curve was performed following the qPCR amplification protocol as described in Supplementary Table S1. In addition, the standard curve was generated by log10 value against the threshold cycle (Ct) value.

We further performed real-time PCR quantifications of *F. oxysporum* (primer sets ITS1F and AFP308R) and *Pseudomonas* (primer sets PsF and PsR) in four soil samples, and amplification protocol as described in Supplementary Table S1. Reaction of qPCR was performed in 15 μl mixture, containing 7.5 μl TransStart Green qPCR SuperMix (Transgen Biotech, Beijing, China), 0.6 μl of each primer (10 μ M) and 20 ng DNA.

Isolation of *Fusarium* spp.

For isolation of *Fusarium* spp., potato dextrose agar (PDA) was used to isolate and subculture the fungus. Soil suspensions were prepared by adding 10 g of fresh soil in a flask containing 90 ml of sterile water (10^{-1} g l^{-1}), 100 μl soil suspensions were plated onto PDA. Plates were incubated at 30°C for 18 h, and then each single colony was isolated and purified. *Fusarium* genomic DNA extraction was done by using CTAB-based method as described by Rogers and Bendich (1985). The primer sets ITS1F and ITS4 (Supplementary Table S2) were used for ITS amplification. PCR amplicons were sent to Shanghai BoShang for sequencing. We

further used BlastN search method to compare sequences to the GenBank database.

Isolation and Counting of *Pseudomonas* spp. with Antagonistic Activity toward *Fusarium oxysporum*

For isolation of *Pseudomonas* spp., *Pseudomonas* selective isolation agar (PSIA) (Krueger and Sheikh, 1987) was used. As described above, each soil suspensions was prepared (10^{-1} g l^{-1}), after serial dilution, 60 μl soil suspensions (10^{-3} g l^{-1}) were plated onto PSIA, incubated at 30°C for 30 h, and then each single colony was purified. Results were descripted as the numbers of CFU per g^{-1} (dry weight) soil.

For *in vitro* antagonism assays, we inoculated *F. oxysporum* to the center of the PDA plates and *Pseudomonas* isolates to the side of the plates at the same time. The results of antagonistic activity against *F. oxysporum* were recorded after 5 days of incubation at 30°C.

After incubation, we selected *Pseudomonas* isolates that had antagonistic activity against *F. oxysporum* for DNA extraction. *Pseudomonas* genomic DNA was extracted using the Bacteria Genomic DNA kit (CWbiotech, Beijing, China). The primer sets 27F and 1522R were used for 16S rRNA amplification. The thermal conditions are listed in the Supplementary Table S2. PCR amplicons were sent to Shanghai BoShang for sequencing. Finally, we used BlastN search method to compare sequences to the GenBank database for the identification purpose. Sequences were used Clustal X to align, and then phylogenetic trees were constructed with MEGA6.06 using a neighbor joining approach.

Evaluation of the Pathogenicity of *Fusarium oxysporum* and Biocontrol Effects of *Pseudomonas* sp. CJ313

Radix pseudostellariae were planted in plastic pots and placed in a green house on December 15, 2015. The spore suspension of isolated *F. oxysporum* was added to the soil through pipette for observing the effects of *Fusarium* wilt in *R. pseudostellariae* after 5 months of planting. In order to assess biocontrol potential of *Pseudomonas* spp., the effect of isolated strain CJ313 was examined after 15 days of its exogenous addition. We added equal amount of LB as a control (CK) at the same time. Each treatment has three replicates. After 16 days, we collected rhizospheric soil from two treatments, then soil samples were immediately used to extract DNA and qPCR of *F. oxysporum* and *Pseudomonas* spp. as described above.

The Effect of Phenolic Acids on the Growth of Isolated *Fusarium oxysporum*, *Pseudomonas* sp. CJ313 and *Pseudomonas* sp. CJ361

Based on our previous HPLC results of phenolic acids in the *R. pseudostellariae* rhizosphere (Wu et al., 2016a), we prepared the solutions of eight phenolic acids (*p*-hydroxybenzoic acid, gallic acid, coumaric acid, syringic acid, vanillic acid, ferulic acid, vanillin and benzoic acid) and their mixture to assess its effect on the growth of isolated *F. oxysporum*. The ratio of their mixtures was the same as detected in the soil. We prepared the 10-fold dilution of soil extract agar medium (SEM), and added the phenolic acids into the SEM to reach final concentrations 30, 60, 120, 240, 480, 960 μ mol L^{-1}. We inoculated isolated *F. oxysporum* onto the SEM plates to assess the mycelium growth mediated by phenolic acids. There were three replicates for each treatment. After incubation at 28°C for 8 days, we recorded the mycelium diameter. Likewise, isolated *F. oxysporum* was inoculated into 10-fold dilution of SEM by adding the phenolic acid mixtures, and solution was incubated at 200 rpm and 30°C for 7 days. *F. oxysporum* spores were counted by a hemocytometer.

We also detected the effects of eight phenolic acids and their mixtures on the growth of isolated *Pseudomonas* sp. CJ313 and CJ361. Specifically, the isolated *Pseudomonas* sp. CJ313 and CJ361 were determined by adding the phenolic acids to a LB medium with 8-fold dilution. After 8–10 h incubation at 200 rpm and 30°C, we determined the bacterial density at 600 nm using a microplate reader (Thermo Scientific Multiskan MK3, Shanghai, China).

Statistical Analyses

For all parameters, multiple comparison was carried out by one-way analysis of variance (ANOVA) followed by LSD's test

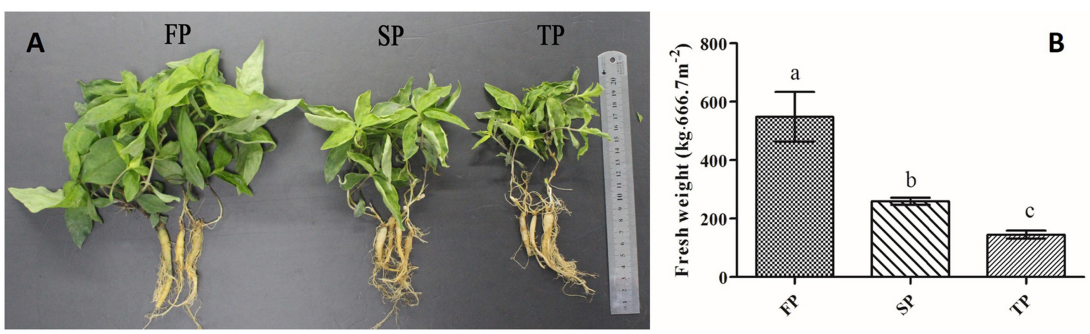


FIGURE 1 | (A) Photographs of above and below ground components of *R. pseudostellariae* under 1-year, 2-year, and 3-year consecutive monoculture. **(B)** Yield of *R. pseudostellariae* under 1-year (FP), 2-year (SP) and 3-year consecutive monoculture (TP).

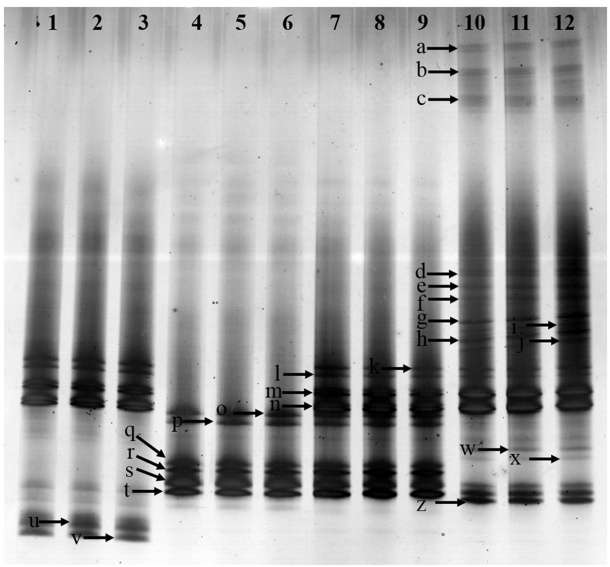


FIGURE 2 | Denaturing gradient gel electrophoresis (DGGE) gel showing community structures of *Fusarium* elongation factor genotypes (EF1α) amplified from soil extracted DNA. Lanes 1, 2 and 3-control with no *Radix pseudostellariae* cultivation (CK), lanes 4, 5 and 6- the newly planted *R. pseudostellariae* cultivation(FP), lanes 7, 8, and 9- 2-year consecutive monoculture(SP), lanes 10, 11, and 12- 3-year consecutive monoculture (TP). Band positions are marked with an arrow and correspond to data in **Table 2**.

($P \leq 0.05$) using DPS 7.05 software. PCA analysis was performed by SPSS 20.0 software. DGGE for detecting the band was performed with the Quantity one v4.6.2 software.

RESULTS

The Morphology and Yield of *R. pseudostellariae* under Consecutive Monoculture

We observed that plants of FP displayed more aboveground biomass and less adventitious roots relative to continuously monocultured plants of SP and TP (**Figure 1A**). Moreover, our results revealed that the yield of newly planted *R. pseudostellariae* roots (FP) was significantly ($P \leq 0.05$) higher that of 2-year consecutive monoculture (SP) and 3-year consecutive monoculture (TP) (**Figure 1B**).

Fusarium-Specific DGGE

Fusarium-specific PCR-DGGE analysis showed that the rhizosphere *Fusarium* community structures changed with the increasing years of monoculture (**Figure 2**). The principal component analysis (PCA) of the DGGE profile was performed to demonstrate the relative position of four soil samples. In PCA, first principal component explained 57.70% of variance and second principal component 28.44% of total variance (**Figure 3A**). Furthermore, PCA showed that the *Fusarium* community in CK, FP and SP was separated from TP by the first principal component, and FP was separated from SP by the second principal component (**Figure 3A**).

The diversity of *Fusarium*-specific DGGE was also determined. The study revealed that Simpson, Shannon, evenness and Brillouin's index of *Fusarium* communities significantly increased with prolonged or increasing years of monoculture ($P \leq 0.05$) (**Table 1**).

Analysis of the DGGE Bands of *Fusarium* spp.

In order to further extract more detailed information from the DGGE bands in this study, excised bands from DGGE were sequenced. A total of 17 bands were identified in rhizospheric soil (**Table 2**). The *Fusarium* spp. belonged to 5 species, e.g., *F. oxysproum* (band a, b, c, g, h, k, l, m and n), *F. solani* (band p, q, r, s and t), *F. asiaticum* (band d), *F. falciforme* (band e), *F. foetens* (band f). Specifically, the bands of *F. oxysporum* significantly increased along with years of continuous cropping.

Pseudomonas-Specific DGGE

Pseudomonas-specific PCR-DGGE analyses showed significantly changed *Pseudomonas* community structures in the rhizosphere with increasing years of monoculture (**Figure 4**). Likewise, we performed PCA to demonstrate the relative position of four soil samples. In PCA, the first principal component explained 83.30% of variance and second principal component 9.0% of total variance (**Figure 3B**). Furthermore, PCA showed the *Pseudomonas* community in CK, FP and SP were separated from the microbial community in TP by principal component 1, and the community in FP and CK was separated from the microbial communities in SP and TP by principle component 2 (**Figure 3B**).

The diversity of visible bands, Shannon and Brillouin' index of *Pseudomonas* community significantly decreased with increasing years of monoculture ($P \leq 0.05$). However, the opposite was true for the Simpson' index of the *Pseudomonas* community. There was no significant difference in evenness index among the four samples (**Table 3**).

Analysis of the DGGE Bands of *Pseudomonas* spp.

To further extract more detailed information from the DGGE bands, we excised and sequenced bands from DGGE. A total of 15 bands were identified in rhizospheric soil (**Table 4**). *Pseudomonas* spp. could be further divided into five species, e.g., *Pseudomonas lutea* (band c and k), *Pseudomonas fluorescens* (band d), *Pseudomonas aeruginosa* (band l), *Pseudomonas knackmussii* (band o and r), *Pseudomonas* sp. (band j) and uncultured bacterium (a, b, f, g, i, m, p and q).

Abundance of *Pseudomonas* and *Fusarium oxysporum* by Quantitative PCR

First, standard curves of $y = -0.2487x + 9.898$ ($R^2 = 0.997$) and $y = -0.271x + 9.8309$ ($R^2 = 0.990$) were developed for *Pseudomonas* and *F. oxysporum* qPCR analyses respectively. The

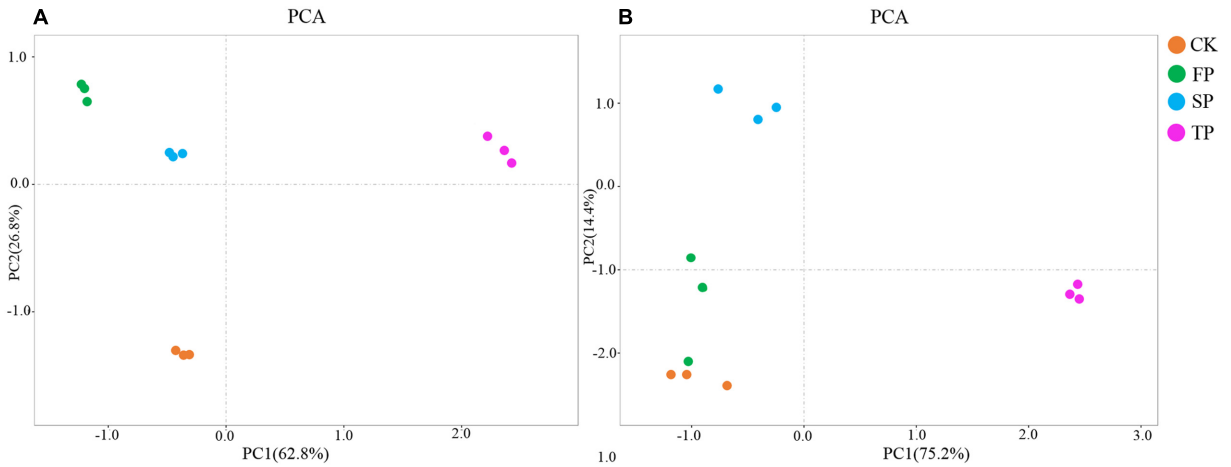


FIGURE 3 | Principal component analysis of *Fusarium* DGGE results **(A)** *Pseudomonas* DGGE results **(B)**. CK, control with no *R. pseudostellariae* cultivation. FP, the newly planted *Radix. pseudostellariae* cultivation. SP, 2-year consecutive monoculture. TP, 3-year consecutive monoculture.

TABLE 1 | Estimated Simpson, Shannon, Evenness and Brillouin's indices for all the samples using *Fusarium*-specific DGGE.

Treatments	Simpson	Shannon	Evenness	Brillouin
CK	0.8632 ± 0.0041c	2.9324 ± 0.0218c	2.9324 ± 0.0218c	2.9011 + 0.0216c
FP	0.8497 ± 0.002d	2.762 ± 0.0099d	2.762 ± 0.0099d	2.7279 + 0.0097d
SP	0.8801 ± 0.0016b	3.1098 ± 0.013b	3.1098 ± 0.013b	3.0784 + 0.0146b
TP	0.9364 ± 0.0018a	4.0264 ± 0.0199a	4.0264 ± 0.0199a	3.9928 ± 0.0188a

Different letters within a column indicate significant differences according to LSD ($P \leq 0.05$). CK, control with no Radix pseudostellariae cultivation. FP, the newly planted R. pseudostellariae cultivation. SP, 2-year consecutive monoculture. TP, 3-year consecutive monoculture).

amount of *F. oxysporum* was significantly ($P \leq 0.05$) higher in continuous monoculture soils (SP and TP) than in control (CK) and the newly planted soils (FP) (**Figure 5A**). The result of qPCR was consistent with the *Fusarium*-specific DGGE results (**Table 1**). However, the opposite was true for the qPCR result of *Pseudomonas* (**Figure 5B**).

Isolation and Screening for *F. oxysporum* with High Pathogenicity

In our study, we separated and sequenced one strain of *F. oxysporum*. We found that the isolated *F. oxysporum* quickly led to wilt disease on the tissue culture of *R. pseudostellariae* (**Figure 7A**), and it also occurred in pots with *F. oxysporum* (**Figure 7B**). These results demonstrated that isolated *F. oxysporum* had the high pathogenicity on *R. pseudostellariae*.

Screening for *Pseudomonas* Isolates with Antagonistic Activity toward *F. oxysporum*

For *in vitro* antagonism assays, we screened a total of 317 *Pseudomonas* isolates from four different soils. The results showed that the isolation frequencies of *Pseudomonas* were significantly higher in FP than SP and TP. The highest isolation frequencies were found in the newly planted (FP) soil (**Figure 6A**). *In vitro* antagonism assays, the number of *Pseudomonas* spp. with antagonistic activity toward *F. oxysporum* significantly declined with prolonged monoculture (**Figure 6B**). These isolation frequencies were similar to results of *Pseudomonas* obtained by qPCR. Approximately 17.4% (87 of 317) of all isolates showed the antagonistic activity. Strain

TABLE 2 | Sequencing of the identified bands in the *Fusarium*–specific DGGE gel.

Bands	Species	Identity	NCBI accession numbers
a	*F. oxysporum*	99%	KT224070.1
b	*F. oxysporum*	94%	KT224065.1
c	*F. oxysporum*	96%	KF728239.1
g	*F. oxysporum*	99%	LC177322.1
h	*F. oxysporum*	99%	KT224113.1
k	*F. oxysporum*	99%	AB674272.1
l	*F. oxysporum*	99%	AB674271.1
m	*F. oxysporum*	97%	JF430176.1
n	*F. oxysporum*	99%	AB674272.1
p	*F. solani*	99%	KU361412.1
q	*F. solani*	99%	KT224163.1
r	*F. solani*	99%	KP267291.1
s	*F. solani*	99%	KR108757.1
t	*F. solani*	99%	KU361425.1
d	*F. falciforme*	99%	LC177299.1
e	*F. foetens*	95%	JX298790.1
f	*F. asiaticum*	99%	KY283868.1

Note: Identification letters refer to the identified bands in ***Figure 2****.*

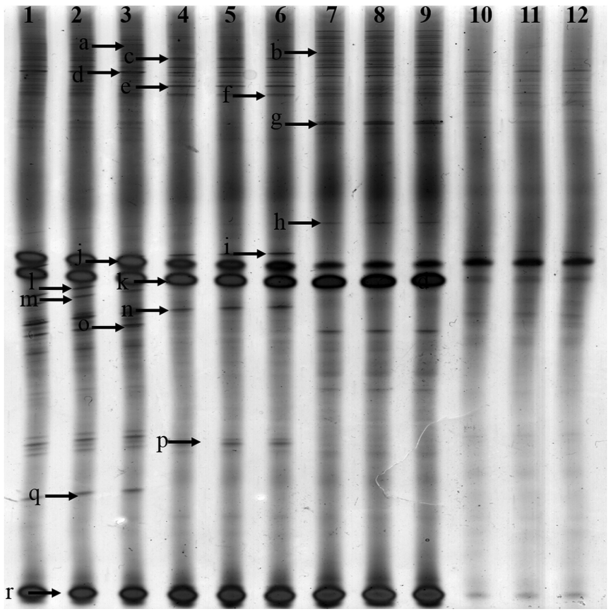


FIGURE 4 | Denaturing gradient gel electrophoresis gel showing community structures of *Pseudomonas* amplified from soil extracted DNA. Lanes 1, 2, and 3-control with no *Radix pseudostellariae* cultivation (CK), lanes 4, 5, and 6- the newly planted *R. pseudostellariae* cultivation(FP), lanes 7, 8, and 9-2-year consecutive monoculture(SP), lanes 10, 11, and 12- 3-year consecutive monoculture(TP). Band positions are marked with an arrow and correspond to data in **Table 4**.

TABLE 4 | Sequencing of the identified bands in the *Pseudomonas* –specific DGGE gel.

Bands	Species	Identity	NCBI accession numbers
a	Uncultured bacterium	96%	KM318828.1
b	Uncultured bacterium	98%	EU408015.1
c	*Pseudomonas lutea*	99%	NZ_JRMB010000003.1
d	*Pseudomonas fluorescens*	95%	KX503978.1
e	Uncultured bacterium	94%	JQ054733.1
f	Uncultured bacterium	94%	KM318828.1
g	Uncultured bacterium	97%	KR309072.1
i	Uncultured bacterium	97%	DQ468062.1
j	*Pseudomonas* spp.	88%	AY365077.1
k	*Pseudomonas lutea*	99%	NZ_JRMB010000004.1
l	*Pseudomonas aeruginosa*	93%	AB793685.1
m	Uncultured bacterium	93%	JQ054733.1
o	*Pseudomonas knackmussii*	93%	NZ_GH322950.1
p	Uncultured bacterium	93%	DQ129211.2
q	Uncultured bacterium	93%	KM323906.1
r	*Pseudomonas knackmussii*	82%	NZ_GH322950.1

*Identification letters refer to the identified bands in **Figure 4**.*

313 and 361 but not 117 had antagonistic activity against *F. oxysporum* (**Figure 6C**). The sequences of *Pseudomonas* sp. CJ313 and *Pseudomonas* sp. CJ361 isolates were obtained to perform phylogenetic tree analysis. The neighbor-joining method generated a dendrogram with two main branches, where the first branch included *Pseudomonas* sp. CJ361 and the second branch comprised *Pseudomonas* sp. CJ313 (Supplementary Figure S1).

Biocontrol Effects of *Pseudomonas* sp. CJ313

We further evaluated the antagonism of *Pseudomonas* CJ313 to *F. oxysporum*. In the pot experiment, we found that the isolated *Pseudomonas* CJ313 significantly inhibited the growth of *F. oxysporum*, and the *R. pseudostellariae* grew well without disease symptoms during the period of experiment (**Figure 7B**). Moreover, qPCR indicated that the abundance of *Pseudomonas* was significantly higher in *Pseudomonas*. CJ313 treatment than in control (CK), whereas *F. oxysporum* showed the opposite trend (**Figure 7C**). The results clearly showed that strain *Pseudomonas*. CJ313 has the potential of biological control. The results further suggested that exogenous antagonism of *Pseudomonas* could be effective against *F. oxysporum* infection. In addition, the results also demonstrated that the imbalance of these two strains (*Pseudomonas* sp. CJ313 and *F. oxysporum*) could be an important cause of the continuous cropping related diseases.

The Effect of Phenolic Acids on the Growth of Isolated *Fusarium oxysporum*, *Pseudomonas* spp.

The results showed that mycelial and sporular growth of *F. oxysporum* was significantly promoted by phenolic acid mixture (**Figures 8A,B**). Further analysis showed that *p*-hydroxybenzoic acid, vanillin, coumaric acid and ferulic acid could significantly promoted mycelial growth of *F. oxysporum* among the eight phenolic compounds (Supplementary Figure S2). The results also indicated that

TABLE 3 | Estimated Simpson, Shannon, Evenness and Brillouin's indices for all the samples using *Pseudomonas*-specific DGGE.

Treatments	Simpson	Shannon	Evenness	Brillouin
CK	0.9394 ± 0.0036b	3.4111 ± 0.0373a	0.7473 ± 0.0057a	2.0966 ± 0.131a
FP	0.9018 ± 0.0087c	2.8775 ± 0.0376c	0.7365 ± 0.0096a	1.707 ± 0.0874b
SP	0.932 ± 0.0131bc	3.1293 ± 0.0467b	0.7367 ± 0.011a	1.8585 ± 0.0217b
TP	1.1522 ± 0.0303a	2.6477 ± 0.0278d	0.7385 ± 0.0078a	0.5653 ± 0.0914c

Different letters within a column indicate significant differences according to LSD ($P \leq 0.05$). CK, control with no Radix pseudostellariae cultivation. FP, the newly planted R. pseudostellariae cultivation. SP, 2-year consecutive monoculture. TP, 3-year consecutive monoculture.

the growth promotion by mixture was more than that of single phenolic acid on *F. oxysporum* (**Figure 8A**). However, the mixture significantly inhibited *Pseudomonas* sp. CJ313 (**Figure 8C**) and *Pseudomonas* sp. CJ361 (**Figure 8D**) growth. Among them, vanillic acid and syringic acid has the more inhibitory effects on *Pseudomonas* sp. CJ313 than others (Supplementary Figure S3). Likewise, coumaric acid, ferulic acid syringic acid had the greatest inhibitory effect on *Pseudomonas* sp. CJ361 (Supplementary Figure S4). The results indicated that certain allelochemicals of *R. pseudostellariae* root exudates possessed the selective effects on rhizosphere microbes.

DISCUSSION

Our studies presented a significant decline in the yield of *R. pseudostellariae* along with less aboveground biomass in consecutive monoculture field (**Figure 1**). Recently, researchers have focused on the biological relationships between plants and rhizosphere microorganisms, which are essential for plant growth and health (Haney and Ausubel, 2015; Lebeis et al., 2015). The study of *F. oxysporum* has become common due to its ability to cause diseases of important economic crops (Gordon et al., 1989; Gordon and Martyn, 1997). DGGE results revealed significant changes in *Pseudomonas* and *Fusarium* communities in the rhizosphere of *R. pseudostellariae* with prolonged monoculture (**Figures 2**, **4**). Based on the DGGE analysis of *Fusarium*, we indicated that prolonged monoculture of *R. pseudostellariae* led to a significant increase in *Fusarium* species, especially *F. oxysporum* (**Table 1**). Quantitative PCR assay confirmed the increase in *F. oxysporum* with the increasing years of monoculture (**Figure 3**). These results are supported by the work of different researchers as stated that *F. oxysporum* is one of main pathogenic species to plants under monoculture regime (Wu et al., 2016b,c).

Due to an extensive distribution of *Pseudomonas* species in the environment, several studies reported an abundance of antagonistic *Pseudomonas* species, which controls specialized pathogens that are responsible for disease suppression in soils (Gorlach-Lira and Stefaniak, 2009; Mendes et al., 2011). Our study of *Pseudomonas*-DGGE revealed that the diversity of *Pseudomonas* spp. significantly declined with the prolonged monoculture. More importantly, it was found that the relative abundances of antagonistic *Pseudomonas* spp. declined in soils under consecutive monoculture, and a similar tendency was recorded for other *Pseudomonas* species studied in the selective medium assay. Similar effects of plants on the abundance of antagonistic *Pseudomonas* spp. under monoculture were found by Gorlach-Lira and Stefaniak (2009). Hence, the abundance of the *Pseudomonas* populations in soil of *R. pseudostellariae* were seriously affected by monoculture. In addition, it was also found that the abundance of *Pseudomonas* spp. having antagonistic activities against *F. oxysporum* significantly decreased with the increasing years of monoculture, and this was confirmed by the *in vitro* antagonism assays. This important antagonistic interaction effects between *Pseudomonas* and *F. oxysporum* need particular attention in disease management under a clear cropping system of *R. pseudostellariae*. Therefore, it is necessary to make robust inferences about balance between *Pseudomonas* communities and *Fusarium* of *R. pseudostellariae*.

Our previous study revealed that most phenolic acids of *R. pseudostellariae* from rhizosphere soil indicated no direct autotoxicity toward tissue culture seedlings of *R. pseudostellariae* (Wu et al., 2016a). Besides, many researchers did not support the assumption that the concentrations of allelochemicals in the soil were sufficient to directly influence the development of host plants or neighboring plants (Ehlers, 2011; Weidenhamer et al., 2013). A growing number of researchers reported that the microflora disorder mediated by plant root exudates was the crucial factor leading to plant consecutive monoculture problems (Wu et al., 2014). Root exudates have selective effects on certain microorganisms in the soil and can promote or inhibit the growth of a certain population (Haichar et al., 2008; Hartmann et al., 2009). In this study, the results indicated that the phenolic acid mixture had a significant improvement on the growth of mycelial and spore of pathogenic *F. oxysporum* (**Figures 8A,B**). However, phenolic acid mixture could greatly inhibit the growth of antagonistic *Pseudomonas* sp. CJ313 and CJ361

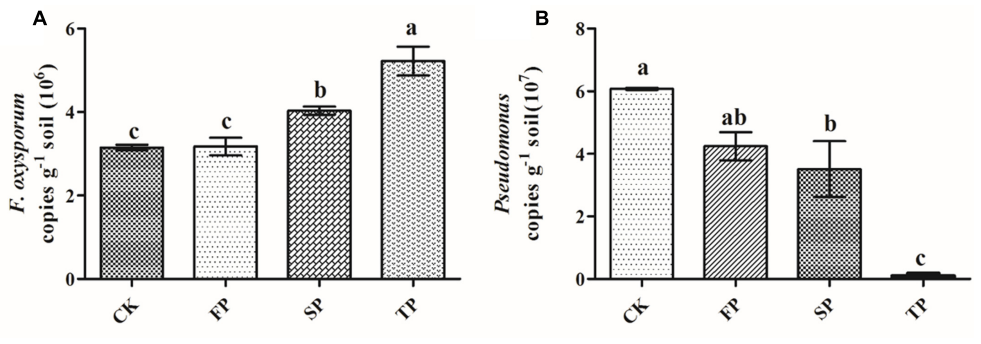


FIGURE 5 | Quantification of *Fusarium oxysporum* **(A)** and *Pseudomonas* **(B)** in control (CK), the newly planted *Radix pseudostellariae* cultivation (FP), 2-year consecutive monoculture (SP), and 3-year consecutive monoculture (TP) by quantitative PCR. Data are means ± standard errors (one-way analysis of variance, $n = 4$).

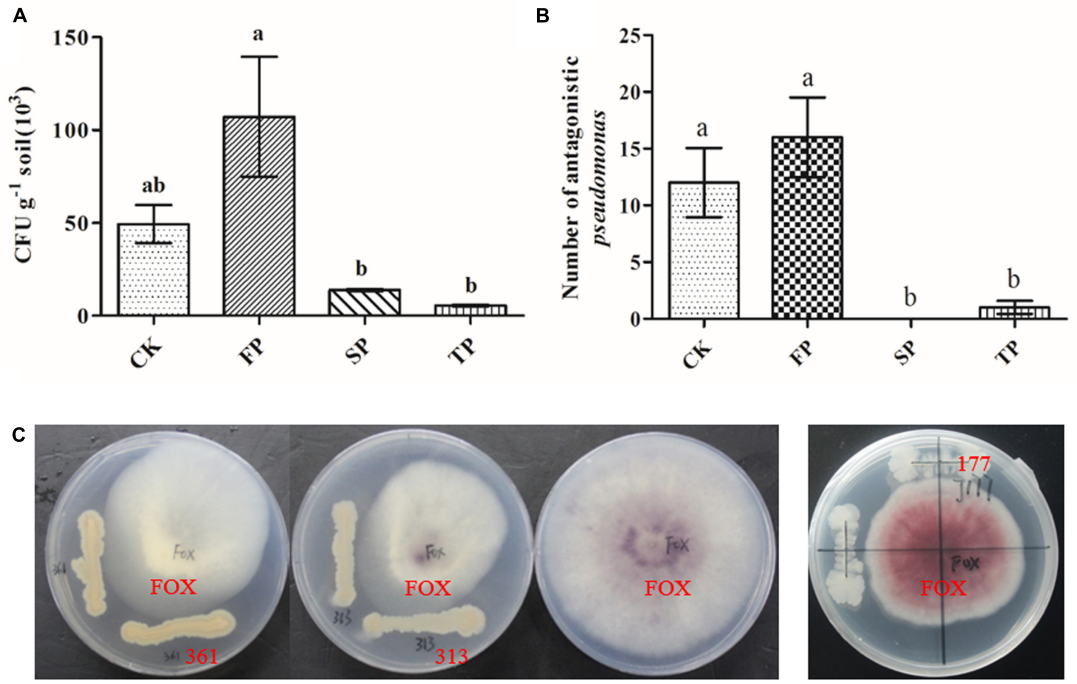


FIGURE 6 | *Pseudomonas* populations (CFU g^{-1} of dry soil) in rhizosphere soils under four different samples **(A)**, Number of *Pseudomonas* spp. with antagonistic activity against *F. oxysporum* in four different samples **(B)**. Petri plates used for evaluation of antagonistic activity of *Pseudomonas* strains 313, 361 and 117 against *F. oxysporum* **(C)**. CK, FP, SP and TP represent the control, newly planted, 2-year, and 3-year consecutively monoculture soils, respectively. Data are means ± standard errors (one-way analysis of variance, $n = 3$).

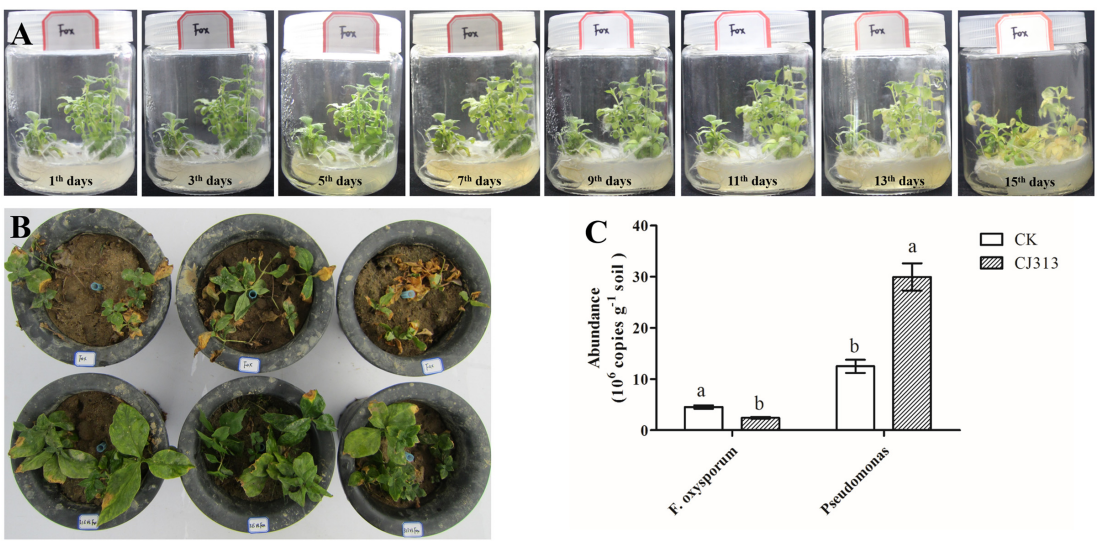


FIGURE 7 | Assessment of the pathogenic potential of isolated *F. oxysporum* **(A)**, the biocontrol potential of *Pseudomonas* sp. CJ313 against *F. oxysporum* (FOX) **(B)**, Quantification of *F. oxysporum* and *Pseudomonas* spp. from two samples. Data are means ± standard errors (one-way analysis of variance, $n = 4$).

(**Figures 8C,D**). Zhou and Wu (2012) observed that the abundance of *F. oxysporum* in soil was significantly increased by *p*-coumaric acid, which led to the severity of *Fusarium* wilt in field conditions. Wu et al. (2016a) reported that phenolic acid, such as syringic acid, significantly promoted the growth of *Talaromyces helicus* and *Kosakonia sacchari*, and inhibited growth of *Bacillus pumilus*. Bais et al. (2002) found that rosmarinic acid had a significant and deleterious effect on *Pseudomonas aeruginosa*. Furthermore, plant bioassays with representative isolates of *Pseudomonas* showed that *Pseudomonas*

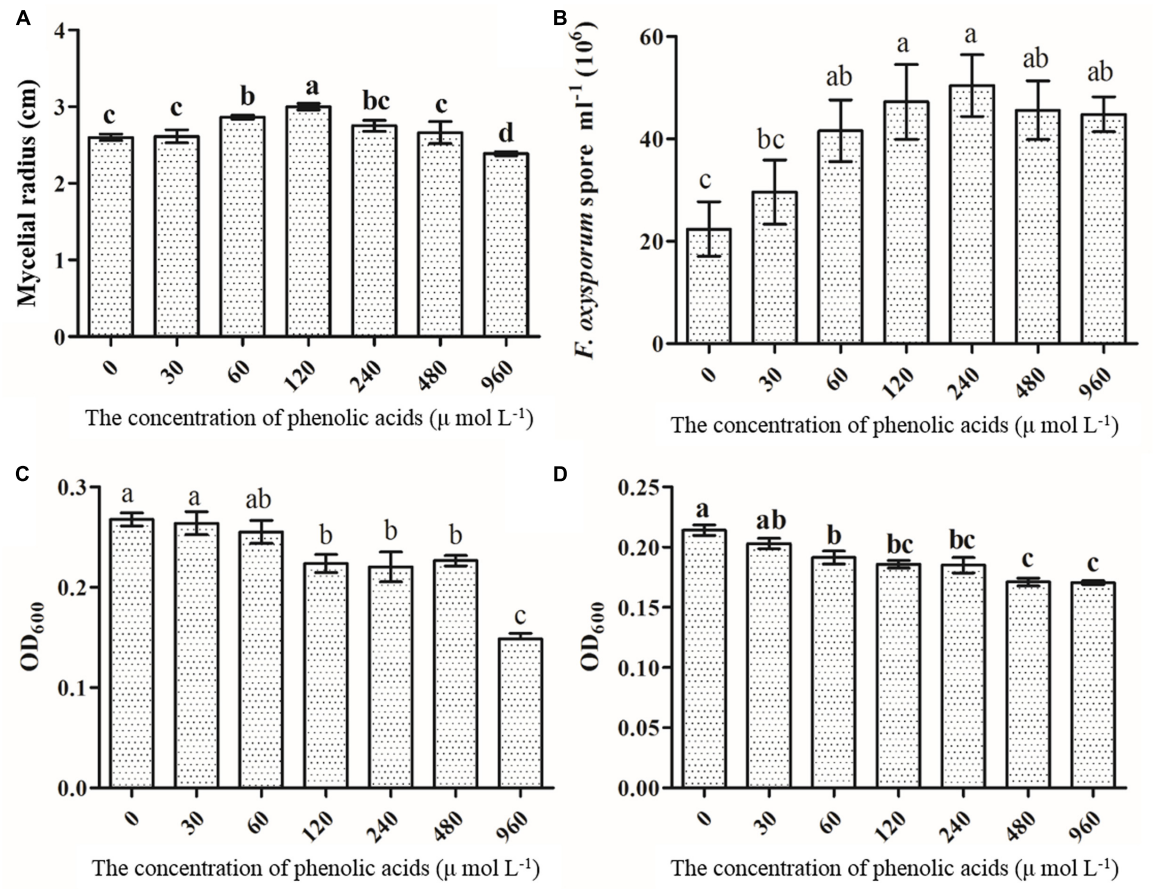


FIGURE 8 | The effects of phenolic acid mixture on the growth of *F. oxysporum* **(A)**, sporulation of *F. oxysporum* **(B)**, *Pseudomonas* sp. CJ313 **(C)**, *Pseudomonas* sp. CJ361 **(D)**. The proportion of phenolic acids was the same as the ratio detected in the rhizosphere soil of *Radix pseudostellariae*. CK, FP, SP and TP represent the control, newly planted, 2-year, and 3-year consecutively monoculture soils, respectively. Data are means ± standard errors (one-way analysis of variance, $n = 4$).

CJ313 had a good performance that protected *R. pseudostellariae* from infection by *F. oxysporum* (**Figure 7B**). Combined with above-mentioned results, we can draw robust inferences that the imbalance of belowground microbial community resulted in the poor growth of monocultured *R. pseudostellariae* by root exudates.

CONCLUSION

Based on multifaceted approaches, such as cultural-independent and culture-dependent analyses, this study indicated that *R. pseudostellariae* biomass decreased under 3-year extended monoculture resulted from two important factors: (i) the decrease of antagonistic microorganisms (*Pseudomonas* sp. CJ313 and CJ361) against pathogens (*F. oxysporum*) might be due to selective inhibitory effect of root exudates, especially phenolic compounds and (ii) an increase of *F. oxysporum* which significantly induced the poor growth of *R. pseudostellariae* at a time when pathogenic microbes (*F. oxysporum*) have become dominant. (iii) Isolated *Pseudomonas* CJ313 of its exogenous addition could protected *R. pseudostellariae* from infection by *F. oxysporum*. These results are very important in the development of potential management approaches to solve the *R. pseudostellariae* problems under consecutive monoculture. However, additional works are still needed to explore the relationship between aboveground plant performance and belowground microbial diversity.

AUTHOR CONTRIBUTIONS

WL and JC conceived the study; JC wrote the paper; JC, and LW performed experiments; JC, SL, and ZX performed the statistical analyses; HW, XQ, YW, XW, and JW were involved in field management. MK assisted in English correction. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Long-Term Irrigation Affects the Dynamics and Activity of the Wheat Rhizosphere Microbiome

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The Inland Pacific Northwest (IPNW) encompasses 1. 6 million cropland hectares and is a major wheat-producing area in the western United States. The climate throughout the region is semi-arid, making the availability of water a significant challenge for IPNW agriculture. Much attention has been given to uncovering the effects of water stress on the physiology of wheat and the dynamics of its soilborne diseases. In contrast, the impact of soil moisture on the establishment and activity of microbial communities in the rhizosphere of dryland wheat remains poorly understood. We addressed this gap by conducting a three-year field study involving wheat grown in adjacent irrigated and dryland (rainfed) plots established in Lind, Washington State. We used deep amplicon sequencing of the V4 region of the 16S rRNA to characterize the responses of the wheat rhizosphere microbiome to overhead irrigation. We also characterized the population dynamics and activity of indigenous Phz$^+$ rhizobacteria that produce the antibiotic phenazine-1-carboxylic acid (PCA) and contribute to the natural suppression of soilborne pathogens of wheat. Results of the study revealed that irrigation affected the Phz$^+$ rhizobacteria adversely, which was evident from the significantly reduced plant colonization frequency, population size and levels of PCA in the field. The observed differences between irrigated and dryland plots were reproducible and amplified over the course of the study, thus identifying soil moisture as a critical abiotic factor that influences the dynamics, and activity of indigenous Phz$^+$ communities. The three seasons of irrigation had a slight effect on the overall diversity within the rhizosphere microbiome but led to significant differences in the relative abundances of specific OTUs. In particular, irrigation differentially affected multiple groups of *Bacteroidetes* and *Proteobacteria,* including taxa with known plant growth-promoting activity. Analysis of environmental variables revealed that the separation between irrigated and dryland treatments was due to changes in the water potential (Ψ_m) and pH. In contrast, the temporal changes in the composition of the rhizosphere microbiome correlated with temperature and precipitation. In summary, our long-term study provides insights into how the availability of water in a semi-arid agroecosystem shapes the belowground wheat microbiome.

Keywords: microbiome, rhizosphere, wheat, soil moisture, *Pseudomonas*, phenazine

INTRODUCTION

Most wheat (*Triticum aestivum* L.) in the Inland Pacific Northwest (IPNW) of the U.S.A. is grown throughout the Columbia Plateau, an area encompassing more than 62,000 square kilometers that comprises the largest contiguous cropping system in the western United States. Cereal crops throughout the region support large populations (10^5–10^6 CFU/g of root) of indigenous rhizobacteria that produce the antibiotic phenazine-1-carboxylic acid (PCA) (Mavrodi et al., 2012a; Parejko et al., 2013). Phenazines are colorful, redox-active metabolites that act as electron shuttles (Hernandez et al., 2004; Pham et al., 2008; Wang et al., 2010) and contribute strongly to the morphology, physiology, and ecology of the strains that produce them (Mazzola et al., 1992; Maddula et al., 2006; Price-Whelan et al., 2006; Dietrich et al., 2008). Indigenous populations of PCA-producing (Phz^+) rhizobacteria from Columbia Plateau soils are diverse and are part of the *Pseudomonas fluorescens* species complex. They include at least 31 genotypes that are closely related to *Pseudomonas synxantha, P. orientalis,* and the provisional species *P. aridus,* and *P. cerealis* (Parejko et al., 2013). These microorganisms are exemplified by *P. synxantha* (formerly *P. fluorescens*) 2-79, a model biocontrol agent isolated from soil at the Washington State University's Lind Dryland Research Station that had been cropped to wheat for 14 consecutive years (Weller and Cook, 1983; Thomashow and Weller, 1988). The production of PCA in strain 2-79 is controlled by the seven-gene *phz* operon that has been cloned, sequenced, and shown to be regulated by quorum sensing (QS) via *N*-(3-hydroxy-hexanoyl)-L-homoserine lactone (Mavrodi et al., 1998; Khan et al., 2005). PCA produced by 2-79-like bacteria has been detected at nanomolar concentrations in the rhizosphere of field-grown wheat, and there is a direct relationship between the amount of phenazines extracted from the roots and the population density of Phz^+ *Pseudomonas* spp. (Mavrodi et al., 2012a). Phenazines produced by 2-79 and closely related species of Phz^+ pseudomonads exhibit broad-spectrum antibiotic activity and contribute to the capacity of these organisms to suppress several important plant pathogens (Thomashow and Weller, 1988; Chin-a-Woeng et al., 1998; Arseneault et al., 2016; Jaaffar et al., 2017). Our surveys of commercial wheat fields from throughout the Columbia Plateau revealed that the frequency of root systems colonized by Phz^+ bacteria is inversely correlated with annual precipitation and irrigation (Mavrodi et al., 2012a,b). This observation prompted us to hypothesize that soil moisture strongly affects the rhizosphere microbiome of dryland wheat and plays a key role in the establishment and proliferation of indigenous phenazine-producing pseudomonads.

The availability of water is a major challenge for agriculture in the IPNW. The region is divided into three annual precipitation zones, of which the low-precipitation zone (<300 mm) encompasses more than 1.6 million cropland hectares. Two-thirds of annual precipitation in the region occurs between October and March, one-fourth during April through June, and <10% in July through September. Across most of the IPNW, winter precipitation is efficiently stored in the soil, underpinning an alternating winter wheat-summer fallow rotation that has been the dominant cropping system for over 125 years (Schillinger and Papendick, 2008). Some parts of the IPNW were considered too dry to farm until the Yakima River Basin and Columbia River Basin projects resulted in the erection of a series of dams and deep wells that capture water for irrigation of over 0.6 million hectares of farmland throughout central Washington and northern Oregon (Schillinger et al., 2010). Much attention has been given to uncovering the effects of soil moisture through management practices on the physiology of wheat grown throughout the Pacific Northwest area. It is also known that the availability of water affects the complex of soilborne diseases of wheat. Take-all, caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici*, is one of the most important root diseases under irrigation and in higher precipitation areas. Under dryland conditions, take-all is less severe and crown rots caused by *Fusarium culmorum* and *F. pseudograminearum*, and root rots caused by *Rhizoctonia solani* AG-8 and *R. oryzae*, become more important soilborne diseases (Cook and Veseth, 1991). In contrast to the effects of water on cereal crops and soilborne pathogens in the IPNW, much less is known about the role of soil moisture in the establishment, maintenance, and activity of bacterial communities associated with roots of dryland wheat. The missing knowledge is crucial for the rational exploitation of beneficial microbial communities to improve crop performance under conditions of water shortage due to irrigation withdrawal from surface reservoirs and deep wells and shifting rainfall patterns, which are anticipated to become even less consistent as global climate changes (Stockle et al., 2010).

The present work aimed to investigate the effect of water on the population levels, diversity, and composition of microbial communities in the rhizosphere of wheat. The study was conducted by growing wheat for 3 consecutive years in adjacent irrigated and dryland (rainfed) field plots established at the WSU Dryland Research Station in Lind, WA. The experiment was designed to closely mimic conditions in the clusters of irrigated fields scattered across the vast semi-arid wheat-producing area of the Inland Pacific Northwest, US. Our results reveal the magnitude of changes in the wheat rhizosphere microbiome in response to soil moisture, temperature, and crop monoculture. Our findings also shed light on the impact of environmental factors on *in situ* production of a biologically active metabolite in the indigenous community of Phz^+ rhizobacteria.

MATERIALS AND METHODS

Field Trial and Sampling

Six plots, three irrigated, three non-irrigated, each 18.3 × 18.3 m separated by 3.1 m buffer zones, were established in a completely randomized design at a dryland (never previously irrigated) site at the WSU Lind Dryland Research Station (46.973°N, 118.616°W, 423.7 m above sea level). The soil at the site is a Shano silt loam (coarse-silty, mixed, superactive, mesic Xeric Haplocambids) with uniform texture throughout the profile (39% fine sand, 51% silt, 10% clay), pH 5.63–6.27, and organic matter content of 1.13–1.14%. There is a thin, weak layer of calcium carbonate accumulation at about the 50 cm depth, but otherwise

no restrictive layers or rocks within the 180 cm profile. Such soils are typical throughout the low-precipitation zone of east-central Washington (Wuest and Schillinger, 2011). Plots were sown annually from 2011 through 2013 in mid-March with the soft white spring wheat (*T. aestivum* L. cv. Louise). Irrigation (once weekly at night for 12–15 h) began in mid-May from sprinklers installed at the center of the irrigated plots, a regime simulating growers' water application in the same area. Soil matric potential and temperature were monitored with MPS-1 dielectric water potential and ECT temperature sensors and an Em50 data logger (Decagon Devices, Pullman, WA, USA). Sensors were calibrated to equate the relationship between output and soil water potential from −10 to −500 kPa and buried to a depth of 10 and 20 cm. Measurements were recorded hourly during the growing season and average values of water potential and soil temperature per day were calculated and plotted with SigmaPlot (version 10.0; SYSTAT Software, Richmond, CA, USA). Plots were harvested for yield estimation each year in August.

Wheat plants were sampled seven times each year from April through July. Clumps of plants were chosen at random every few meters along each of four perpendicular transects through each plot, dug with a shovel to a depth of about 18 cm, and placed in large plastic bags, four per plot. Plants were brought to the laboratory and stored at 4°C for no more than 24 h before processing. From each bag, four plants were assayed to determine population sizes of total culturable heterotrophic aerobic rhizobacteria, and the bacterial population size and frequency of individual root systems colonized by Phz^+ pseudomonads containing phenazine-1-carboxylic acid biosynthesis genes.

Phz+ and Total Culturable Heterotrophic Bacteria

Indigenous root-associated Phz^+ and total culturable heterotrophic bacteria were enumerated by the modified terminal dilution endpoint assay (Mavrodi et al., 2012b). The root system with adhering rhizosphere soil of a single plant was placed in a tube with sterile distilled water (10 or 20 mL), the tube was vortexed (1 min), and then treated in an ultrasonic cleaner (1 min). An aliquot (100 μL) of each root wash was serially diluted in water in a 96-well microtiter plate. The resulting dilutions were then used to inoculate two other microtiter plates pre-filled with: (i) a semi-selective growth medium for fluorescent *Pseudomonas* spp. comprised of one-third-strength King's medium B ($^1/_3$ KMB) (King et al., 1954) supplemented with cycloheximide, chloramphenicol, and ampicillin (100, 15, and 40 μg mL^{-1}, respectively); and (ii) medium for growth of total culturable heterotrophic aerobic bacteria consisting of one-tenth-strength Tryptic Soy Broth ($^1/_{10}$ TSB) (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with cycloheximide (100 μg mL^{-1}). Cycloheximide was used to inhibit soilborne fungi, while chloramphenicol and ampicillin were added to reduce the growth of competing soil bacteria because *Pseudomonas* spp. are naturally resistant to both antibiotics (Mavrodi et al., 2007). After incubation at room temperature in the dark for 72 h, the optical density at 600 nm was measured with a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). All wells with detectable bacterial growth ($OD_{600} \geq 0.1$) were screened for the presence of Phz^+ pseudomonads by PCR with the Ps_up1-Ps_low1 primer set targeting the key phenazine biosynthesis gene *phzF* (Mavrodi et al., 2010). Population densities of Phz^+ pseudomonads were calculated based on the final dilution with positive PCR amplification. All population data were converted to log CFU per gram (fresh weight) of rhizosphere and the detection limit of the bacteria with this assay was log 3.2 CFU g^{-1} root fresh weight. Since not every wheat plant carried Phz^+ bacteria, the mean population values were reported for colonized plants only. Frequencies of rhizospheres colonized by bacteria were calculated as a proportion of rhizospheres with populations above the limit of detection. The colonization frequency ratio was calculated as a ratio of colonization frequency in non-irrigated over irrigated treatments.

Extraction of Rhizosphere Soil DNA and Processing of 16S rRNA Gene Amplicons

Rhizosphere soil DNA was extracted from plants collected from each replicate irrigated and non-irrigated plot in the first (Sampling 1) and the third year of the experiment (Samplings 2, 4, and 6; **Figure 1**). The DNA was purified from root washes of 10 individual plants using an UltraClean Soil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) and the alternative protocol for wet soil samples as described by Mavrodi et al. (2007). The quality of the extracted DNA was verified by amplifying 16S rRNA with primers 8F and 1492R (Weisburg et al., 1991). The amplifications were carried out in 25 μL reactions containing 1 × GoTaq DNA polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, 1.5 mM $MgCl_2$, 20 pmol of each primer, and 0.06 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). The cycling program consisted of the initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 20 s, 55°C for 15 s, and 72°C for 1.5 min, with a final extension at 72°C for 3 min. For the microbiome analysis, barcoded 16S rRNA gene amplicons were generated from the extracted rhizosphere DNA by PCR with primers 515F and 806R following the protocol of Caporaso et al. (2010, 2012). The amplifications were performed in 25 μL reaction mixtures containing 5 pmol of each primer and the 5 Prime HotMasterMix (0.5 U HotMaster *Taq* DNA polymerase, 2.5 × HotMaster *Taq* buffer, 2.5 mM $MgCl_2$, and 200 μM each of dATP, dTTP, dGTP, and dCTP; Quanta Biosciences, Beverly, MA, USA). Samples were amplified with a T100 gradient thermal cycler (Bio-Rad) and the cycling program consisted of initial denaturation at 94°C for 3 min followed by 27 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min, a final extension at 72°C for 10 min. The amplicons were purified with a GeneJET PCR Purification kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantified on a Synergy 2 microplate reader (BioTek, Winooski, VT, USA) using a Fluorescent DNA Quantification kit (Bio-Rad). The purified 16S rRNA amplicons were normalized and shipped to AgriLife Genomics & Bioinformatics Services (Texas A&M University, College Station, TX, USA) for sequencing on a HiSeq 2500

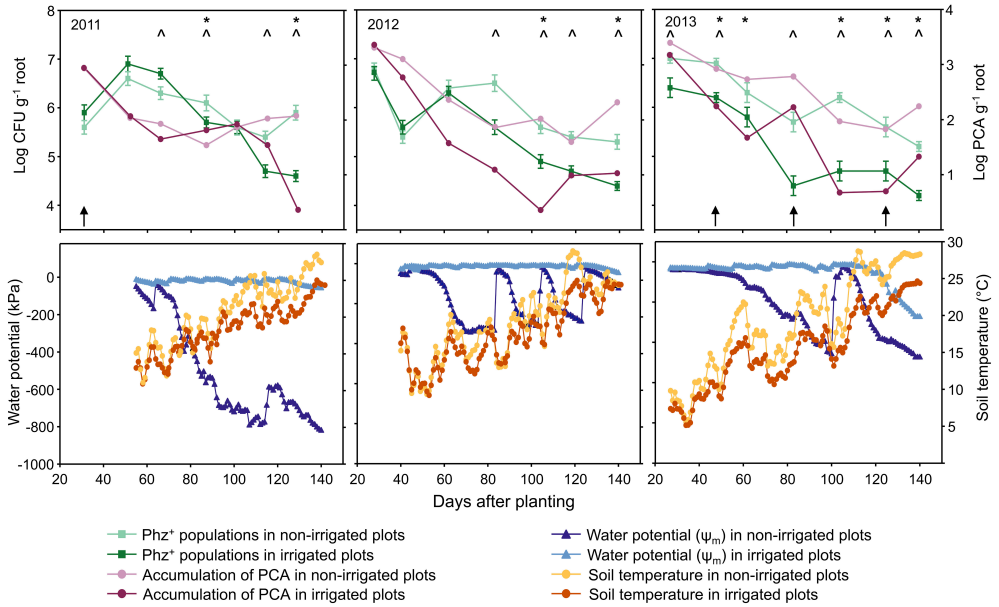


FIGURE 1 | Seasonal dynamics of Phz$^+$ *Pseudomonas* spp. and accumulation of PCA on roots of wheat grown in non-irrigated (dryland) and irrigated plots **(Top)**, and changes in the soil water potential (Ψ_m) and soil temperature at a depth of 10 cm **(Bottom)**. Asterisks and carat signs in the top panel indicate, respectively, sampling points with significant differences in the levels of PCA or Phz$^+$ pseudomonads between irrigated and non-irrigated treatments. Vertical black arrows indicate time points when rhizosphere soil DNA was extracted for microbial community analysis.

instrument (Illumina, San Diego, CA, USA) in 250 bp paired-end rapid mode. Replicates for the Illumina sequencing and subsequent bioinformatics analysis were generated by pooling equal amounts (300 ng) of purified amplicons originating from five randomly chosen wheat plants collected from a single irrigated or non-irrigated plot.

Microbial Community Analysis

Individual samples were processed with the FastQC toolkit (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to confirm sequence quality. Illumina HiSeq forward and reverse reads were merged into contiguous sequences with the mergepairs tool in USEARCH 1.2.22q (Edgar, 2010). The USEARCH fastx_uniques_command was used to dereplicate reads and the UCLUST algorithm was used to cluster reads at a maximum e-value of 0.1 (Edgar, 2010). Operational taxonomic units (OTUs) were characterized by matching to the RDP database (release 11.4; Cole et al., 2014) after clustering with a cutoff of 97% identity. Reads were subsequently mapped back to OTUs to determine OTU abundance for each sample. An OTU was defined as having a minimum of four reads in a cluster (-minsize = 4). The Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software suite (Caporaso et al., 2010) was used to calculate the taxonomic tree based on the RDP dataset for use in analyses with the R package phyloseq (Mcmurdie and Holmes, 2013). A hierarchically clustered heatmap was generated with an R (version 3.3.1) batch script ("run_R_heatmap.batch," provided in Supplementary Material). Any OTUs that accounted for less than 1% of the total OTUs were removed. Phyloseq was used to generate canonical correspondence analysis (CCA) to visualize the community relationships between and within each sample using Bray-Curtis similarity of Log (x+1) transformed values of the abundance of the OTUs. Phyloseq was also used to generate differential abundance plots between the conditions for each sample date using the DESeq2 R package (Anders and Huber, 2010) with *p*-value cutoff of 0.001 and fold change of 2 ($\log_2$ scale). Alpha diversity was calculated using the vegan package (Oksanen et al., 2013). A Tukey multiple comparisons of means test was performed to determine if there were significant differences in Shannon diversity indices. For specific pseudomonad OTUs, blastn searches were used to look at sequence identity with known pathogenic and biocontrol strains.

Detection of Phenazine-1-Carboxylic Acid in the Rhizosphere of Field-Grown Wheat

Phenazine-1-carboxylic acid was extracted from the rhizosphere of field-grown wheat and quantified as described by Mavrodi et al. (2012a). Briefly, root systems of wheat plants were excised from the shoots and stored in plastic bags at −80°C. Fifteen grams of frozen roots with adhering rhizosphere soil were cut into pieces and shaken for 2 h in 30 mL of 80% acetone acidified to pH 2.0 with 10% trifluoroacetic acid. The extraction efficiency of PCA was determined by spiking each sample with 2 μg of phenazine (Sigma, St. Louis, MO, USA) as an internal standard. The acetone root wash was extracted twice with 10 mL of ethyl acetate and the organic phase was collected, evaporated to dryness, and the dried samples were reconstituted in 1 mL of 98% acetonitrile−2% acetic acid and clarified by passage through a 0.22 μm filter

(Bonsall et al., 2007). PCA was detected and quantified with a Waters 2695 liquid chromatograph equipped with a 996 photodiode array and coupled to a quadrupole time-of-flight Q-Tof 2 mass spectrometer with an IonSABRE atmospheric chemical ionization probe (all from Waters Corp., Milford, MA, USA). Samples were separated on a Symmetry C_{18} column (Waters) and spectral scanning by photodiode array was from 180 to 470 nm with monitoring for PCA at 248 nm, its spectral maximum in this solvent system (Mavrodi et al., 2012a). Data were analyzed by using MassLynx, OpenLynx, and QuantLynx software (Waters). PCA was quantified by comparing values to a six-point calibration curve (0.2–10 μg). The determination coefficient (r^2) of the calibration equations ranged from 0.9942 to 0.9997 and the detection limit for PCA in rhizosphere samples was 35 ng per 15 g sample.

Statistical Analyses

Statistical analyses were performed by using appropriate parametric and nonparametric procedures with the Statistix 10 package (Analytical Software, Tallahassee, FL). All population data were converted to log CFU per rhizosphere or gram of root fresh weight. Differences among treatments were determined by the Two-sample *T*-test or Wilcoxon Rank Sum test ($p \leq 0.05$).

RESULTS

The Dynamics of Rhizobacteria and Accumulation of Phenazine-1-Carboxylic Acid in Irrigated and Dryland Wheat Plots

The Lind field site is located in the heart of the low-precipitation zone of central Washington State, which is characterized by cool, moist winters and warm, dry summers with an average of 244 mm annual precipitation (http://lindstation.wsu.edu/). In 2011 and 2013, the study site received 198 and 178 mm of precipitation, respectively (Supplementary Table 1). In contrast, the unusually high amount of rainfall in 2012 increased the annual precipitation to a total of 381 mm. For the part of the year when field samples were collected (April through July) the amounts of rainfall in 2011, 2012, and 2013 were 84.6, 137.2, and 63.5 mm, respectively. The mean monthly air temperature during the field sampling period rose from 6.8 to 9.9°C in April to 19.7–23.3°C in July (Supplementary Table 1). In the absence of irrigation, the soil water potential (Ψ_m) at the depth of 10 cm gradually decreased from −30 kPa in April to −430 kPa in July (**Figure 1**). With irrigation, the Ψ_m values recorded over the field season ranged between −10 and −250 kPa.

Seasonal changes in temperature and soil moisture were accompanied by fluctuations in populations of total culturable heterotrophic and phenazine-producing rhizobacteria (**Figure 1**, **Table 1**). Throughout the study, the counts of culturable rhizosphere bacteria were highest in late April and early May, when they ranged across all treatments between log 7.5 and log 9.2 g^{-1} of root fresh weight. Later in the season, the populations significantly declined, and during the last sampling in July were between log 6.9 and log 7.9 CFU g^{-1} of root fresh weight. There were some statistically significant differences in the levels of rhizobacteria in non-irrigated and irrigated plots, but these variations fluctuated throughout the field season without any apparent trend. The levels of Phz^+ bacteria exhibited similar seasonal dynamics and peaked (across all treatments) in April and May at between log 5.4 and 7.0 CFU g^{-1} of root fresh weight (corresponds to 0.8–6.3% of the total culturable community). The Phz^+ populations were lowest in July, when they declined to between log 4.2 and log 5.9 CFU g^{-1} of root fresh weight (0.2–1.3% of the culturable community). Notably, the dynamics of phenazine producers differed significantly in non-irrigated and irrigated plots. There was a significant decline in the number of plants colonized by Phz^+ rhizobacteria under irrigation, which was especially evident toward the end of the field season (**Figure 2**). The differences between treatments amplified over time and were highest in July of the third year when 60% of plants were colonized in non-irrigated plots vs. only 23% under irrigation (**Table 2**). The changes in the plant colonization frequency were accompanied by differences in Phz^+ population sizes, which declined faster in the irrigated plots (**Figure 1**, **Table 1**). On average, at the end of the field season the levels of phenazine producers in the rhizosphere of irrigated wheat were tenfold lower than in the rhizosphere of plants grown without irrigation.

The amounts of phenazine-1-carboxylic acid recovered from wheat roots closely mirrored the population trend of Phz^+ rhizobacteria. In both irrigated and non-irrigated plots, the highest amounts of PCA were detected in plants collected in April through mid-May (**Figure 1**, **Table 1**). Later in the season, the levels of phenazine-1-carboxylic acid declined and were at their lowest in July. The accumulation of PCA also negatively correlated with soil moisture, and roots of plants collected from irrigated plots during the second or third sampling contained significantly lower amounts of the metabolite (**Figure 1**).

Microbial Community Composition

The profiling of rhizosphere microbiomes was performed using pooled samples of soil DNA extracted from roots of plants collected from replicate irrigated and non-irrigated plots. The control set of DNA samples was collected at the start of the experiment, in April of 2011, and the rest of the samples were collected after three consecutive seasons, in April, June, and July of 2013 (**Figure 1**, **Table 2**). Following all quality filtering steps, a dataset of 15,282,474 sequences spanning the V4 region of the 16S rRNA gene from the 2011 controls and the subsequent 2013 samples ($n = 3$ per treatment condition per sampling point, respectively) were compiled (mean length in nucleotides 291, standard deviation 23), all merged reads were truncated to 275 nucleotides after filtering out reads under 275 to provide a uniform read length. After processing, 214 OTUs were detected and after mapping reads back to these OTUs, a total of 9,933,608 reads were utilized in further analyses. OTUs were observed from nine bacterial phyla: *Actinobacteria*, *Bacteroidetes*, *Fibrobacteres*, *Firmicutes*, *Gemmatimonadetes*, *Proteobacteria*, *Synergistetes*, *Tenericutes*, and *Verrucomicrobia*. The alpha diversity for each sample was

TABLE 1 | Levels of indigenous rhizobacteria and Phz$^+$ *Pseudomonas* and accumulation of phenazine-1-carboxylic acid (PCA) in the rhizosphere of non-irrigated and irrigated wheat during 2011–2013.

Sampling date (mm/dd/yy)	Treatment	Culturable heterotrophic rhizobacteria[a] (log CFU/g root ± S.D.)	Phz$^+$ rhizobacteria		PCA in the rhizosphere[a] (log per g root ± S.D.)
			Population[a] (log CFU/g root ± S.D.)	Plant colonization frequency (%)	
04/18/11	Non-irrigated	8.6 ± 0.4a*	5.6 ± 1.0a	96	2.9 ± 0.2a
	Irrigated	8.3 ± 0.6b*	5.9 ± 1.1a	98	3.0 ± 0.2a
05/09/11	Non-irrigated	8.6 ± 0.5a*	6.6 ± 1.0a	96	2.0 ± 0.3a
	Irrigated	8.7 ± 0.6a*	6.9 ± 1.1a	98	2.1 ± 0.7a
05/23/11	Non-irrigated	8.2 ± 0.6a*	6.3 ± 0.9b*	94	1.9 ± 0.1a*
	Irrigated	8.4 ± 0.4a*	6.7 ± 0.8a*	96	1.7 ± 0.5a*
06/13/11	Non-irrigated	7.8 ± 0.6a*	6.1 ± 1.0a*	85	1.5 ± 0.1b
	Irrigated	8.0 ± 0.4a*	5.7 ± 0.8b*	96	1.8 ± 0.0a
06/27/11	Non-irrigated	8.3 ± 0.9a	5.6 ± 0.9a	88	1.9 ± 0.2a*
	Irrigated	8.4 ± 0.6a	5.6 ± 0.9a	80	1.9 ± 0.0a*
07/11/11	Non-irrigated	8.0 ± 0.9a*	5.4 ± 0.8a	77	2.0 ± 0.3a
	Irrigated	7.0 ± 0.6b*	4.7 ± 0.7b	69	1.5 ± 0.1a
07/25/11	Non-irrigated	7.9 ± 0.4a	5.9 ± 0.9a*	75	2.1 ± 0.3a
	Irrigated	6.9 ± 0.4b	4.6 ± 0.6b*	60	0.4 ± 0.1b
04/10/12	Non-irrigated	7.5 ± 0.5b	6.8 ± 0.7a	90	3.3 ± 0.1a*
	Irrigated	7.9 ± 0.5a	6.7 ± 0.9a	94	3.4 ± 0.2a*
04/23/12	Non-irrigated	8.9 ± 0.5b	5.4 ± 0.8a	77	3.1 ± 0.4a
	Irrigated	9.2 ± 0.6a	5.6 ± 0.8a	69	2.8 ± 0.2a
05/14/12	Non-irrigated	8.1 ± 0.4b	6.4 ± 1.0a	79	2.4 ± 0.1a*
	Irrigated	8.6 ± 0.5a	6.3 ± 0.9a	77	1.6 ± 1.2a*
06/04/12	Non-irrigated	8.1 ± 0.5a	6.5 ± 1.1a	88	1.9 ± 0.3a
	Irrigated	8.2 ± 0.4a	5.6 ± 0.9b	75	1.1 ± 0.8a
06/25/12	Non-irrigated	7.7 ± 0.5a	5.6 ± 0.9a	88	2.0 ± 0.5a
	Irrigated	7.7 ± 0.6a	4.9 ± 0.8b	65	0.4 ± 0.1b
07/09/12	Non-irrigated	7.6 ± 0.4a*	5.4 ± 0.7a	81	1.6 ± 0.2a
	Irrigated	7.3 ± 0.5b*	4.7 ± 0.5b	52	1.0 ± 0.5a
07/30/12	Non-irrigated	7.0 ± 0.5a	5.3 ± 0.8a*	58	2.3 ± 0.1a*
	Irrigated	6.9 ± 0.5a	4.4 ± 0.4b*	35	1.0 ± 0.6a*
04/08/13	Non-irrigated	8.2 ± 0.5b	7.0 ± 0.8a*	98	3.4 ± 0.2a
	Irrigated	8.5 ± 0.4a	6.4 ± 1.1b*	100	3.2 ± 0.1a
04/29/13	Non-irrigated	8.8 ± 0.6a*	6.9 ± 0.9a*	96	2.9 ± 0.1a
	Irrigated	9.0 ± 0.4a*	6.2 ± 0.7b*	85	2.2 ± 0.3b
05/13/13	Non-irrigated	8.0 ± 0.5b	6.3 ± 1.0a	88	2.7 ± 0.1a
	Irrigated	8.3 ± 0.4a	5.8 ± 0.8b	52	1.6 ± 0.0b
06/03/13	Non-irrigated	8.3 ± 0.5a	5.7 ± 0.9a	77	2.8 ± 0.0a*
	Irrigated	8.0 ± 0.4b	4.4 ± 0.7b	38	2.2 ± 0.3a*
06/24/13	Non-irrigated	8.4 ± 0.6a	6.2 ± 0.8a	100	2.0 ± 0.3a
	Irrigated	8.0 ± 0.5b	4.7 ± 0.9b	46	0.7 ± 0.3b
07/08/13	Non-irrigated	7.3 ± 0.5a	5.6 ± 1.0a	75	1.8 ± 0.3a
	Irrigated	6.9 ± 0.4b	4.7 ± 0.9b	31	0.7 ± 0.3b
07/29/13	Non-Irrigated	7.1 ± 0.6a	5.2 ± 0.8a*	60	2.2 ± 0.2a
	Irrigated	6.9 ± 0.5b	4.2 ± 0.2b*	23	1.3 ± 0.3b

Numbers in the same column followed by different letters or different letters with asterisks are significantly different according to two-sample t-test (p = 0.05) or Wilcoxon Rank Sum test (p = 0.05), respectively.

calculated and the Shannon diversity measures ranged from 3.7 to 4.6 (Supplementary Figure 1). The Shannon diversity measure for each of the irrigated plots was larger than for equivalent non-irrigated plots, however this difference was not significant based on a Tukey's multiple comparisons of means test.

Community Structure and Drivers

To examine changes in relative abundance of the main OTUs across treatments and time, hierarchical clustering was performed on all OTUs contributing to more than 1% of the observed population (**Figure 3**). Looking at community composition across the sampling period, we observed increasingly divergent populations between irrigated and non-irrigated treatments. Delineation between the communities from different time points was clearly observed and all replicates were found to cluster with one another (**Figure 3**). Canonical correspondence analysis was used to determine what environmental variables contribute to the observed shifts in community composition (**Figure 4**). The total inertia of the plot was 1.0631; of this the constrained inertia was 0.4941. A total of 24% of the constrained inertia was expressed by the CCA1 axis, while CCA2 captured a further 14.2%. The CCA1 axis separates control samples from all treatment samples, with samples collected earlier in the growing season located closer to the controls. CCA1 was strongly correlated with average monthly temperature, soil temperature and, to a lesser degree, precipitation. The separation of samples along CCA1 also reflects changes in the plant age that ranged between the seedling development stage (Zadoks stage13) in late April, through late boot stage (Zadoks 47) in early June to heading stage (Zadoks 59) in mid-July (**Table 2**). The effect of the growth stage on root microbiome was statistically tied to changes in temperature and moisture (data not shown), and there was no difference in growth stage between plants sampled from non-irrigated and irrigated plots because irrigation commenced later in the field season. Microbial community composition for irrigated samples

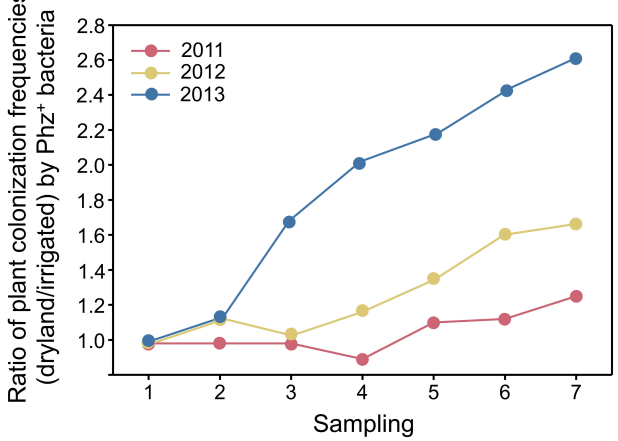


FIGURE 2 | Ratios (dryland/irrigated) of plant colonization frequencies by Phz$^+$ pseudomonads in the first, second, and third years of the experiment.

TABLE 2 | Metadata for sampling points at which soil DNA was extracted for microbial community analysis.

Metadata	Treatment	Year 1 (04/18/11)	Year 3 (04/29/13)	Year 3 (06/03/13)	Year 3 (07/08/13)
Water potential (Ψ_m) at 10 cm (kPa)[a]	Non-irrigated	−43	−57	−278	−13
	Irrigated	−36	−32	−16	−48
Water potential (Ψ_m) at 20 cm (kPa)[a]	Non-irrigated	−46	−50	−100	−324
	Irrigated	−45	−40	−26	−110
Soil temperature at 10 cm (°C)[a]	Non-irrigated	9.1	12.8	17.3	27.0
	Irrigated	7.1	10.5	13.7	21.9
Soil temperature at 20 cm (°C)[a]	Non-irrigated	8.1	12.3	15.9	25.4
	Irrigated	6.5	9.1	13.8	21.8
Phz$^+$ population (log CFU/g root ± *S.D.*)[b]	Non-irrigated	5.6 ± 1.0a[c]	6.9 ± 0.9a*	5.7 ± 0.9a	5.6 ± 1.0a
	Irrigated	5.9 ± 1.1a	6.2 ± 0.7a*	4.4 ± 0.7b	4.7 ± 0.9b
Plant colonization frequency by Phz$^+$ bacteria (%)	Non-irrigated	96	96	77	75
	Irrigated	98	85	38	31
Rhizosphere PCA (log per gram of root ± *S.D.*)[b]	Non-irrigated	3.0 ± 0.2a	2.9 ± 0.1a	2.8 ± 0.1a*	1.8 ± 0.3a
	Irrigated	2.9 ± 0.2a	2.2 ± 0.3b	2.2 ± 0.3a*	0.7 ± 0.3b
Wheat growth stage (Zadok's scale)[c]	Non-irrigated	10	13	47	59
	Irrigated	10	13	47	59
Monthly precipitation (mm)[d]		25.4	12.7	35.3	0
Air monthly temperature max (°C)[d]		13.6	16.7	25.4	33.9
Air monthly temperature min (°C)[d]		0.1	1.8	9.3	12.7
Mean monthly air temperature (°C)[d]		6.8	9.3	17.4	23.3

[a] *Water potential (Ψm) and soil temperature at 10 and 20 cm were measured with an Em50 data logger and MPS-1 dielectric water potential and ECT temperature sensors.*

[b] *Numbers in the same column followed by different letter or different letters with asterisks are significantly different according to two-sample t-test (p = 0.05) or Wilcoxon Rank Sum test (p = 0.05), respectively.*

[c] *Wheat growth development was assessed by the Zadok's system (10, tillering, emergence; 13, tillering, tillering begins; 47, stem extension, head in the "boot"; 59, heading, head completely emerged).*

[d] *Data were obtained from the Northwest Alliance for Computational Science & Engineering (NACSE) database maintained by the Oregon State University (http://www.prism.oregonstate.edu/index.phtml).*

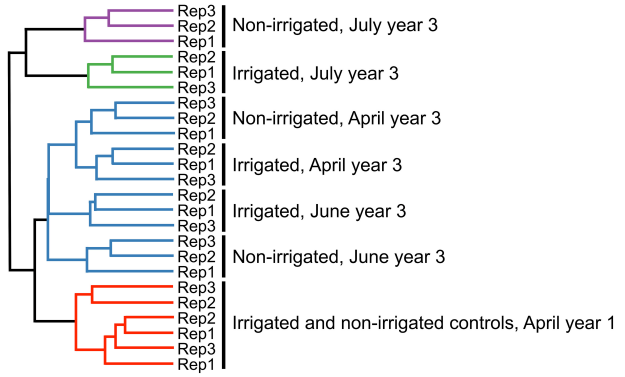


FIGURE 3 | Dendrogram based on Bray-Curtis distance metric hierarchical clustering of OTU relative abundance for each replicate sample.

was well separated from non-irrigated samples along the CCA2 axis and this correlated most strongly with soil water potential and pH.

Impact of Irrigation at the OTU Level

Differential abundance analyses were conducted in DESeq2 to determine which taxa were significantly different in non-irrigated compared to irrigated samples at the three 2013 time points used for this experiment. The taxonomic data from 2011 control samples (plots prior to irrigation commencing) were similarly examined using DESeq2. There were 195 OTUs observed in the 2011 control sample sets and there was no significant difference in the relative abundance of these OTUs across sampled control plots. After irrigation commenced, we observed large numbers of OTUs that differed significantly in abundance between irrigated and non-irrigated sites. In April of 2013, 29 OTUs were significantly more abundant in irrigated sites compared to the non-irrigated sites, while 15 were less abundant ($p < 0.001$). In June, this had increased to 47 OTUs significantly more abundant in irrigated sites and 36 less abundant, while the July sample had 44 OTUs significantly more abundant and 29 less abundant in irrigated sites. The degree of abundance differentiation was also higher in the later time points. For the April sample, log2 fold change ranged from 7.8 to −3.9, while in the June and July samples the range has increased, spanning from 11.2 to −6.6, and 12.3 to −5.3, respectively (**Figure 5**).

For each time point, the five OTUs that showed the highest differential abundance shifts (both up and down) were examined. The OTUs most increased in abundance under irrigation belonged to a relatively small number of phyla (*Bacteroidetes, Proteobacteria, Actinobacteria*; **Figure 5**, **Tables 3**, **4**). In contrast, representatives of six phyla were observed in the set strongly decreasing in relative abundance under irrigation (*Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Tenericutes*, *Gemmatimonadetes*; **Figure 5**, **Table 4**). Interestingly, at the first sample time point OTU_101, classified to the genus *Mucilaginibacter,* was amongst the most increased under irrigation whilst a second OTU (OTU_58) classified to the same genus was observed to show a strong decrease in abundance under irrigation (**Figure 5**, **Table 3**). For OTUs representing pseudomonads, the closest sequence match was determined to ascertain if they were likely soil plant commensals. OTU_4 showed 100% identity to the grass phyllosphere isolate *Pseudomonas cedrina* subsp. *fulgida*, while OTU_10 showed 100% identity to *Pseudomonas syringae* pv. *tomato,* a known plant pathogen. OTU_4 contributed to 4.4% relative abundance in the control sample communities, while OTU_10 contributed to 3.5% relative abundance in the control sample communities. The relative abundance of these OTUs was similar in subsequent sampled communities until the third time point, when the relative abundance of both OTUs declined (Supplementary Figure 2), but was still not significantly different between irrigated and non-irrigated samples.

DISCUSSION

A field survey we conducted in 2008–2009 revealed the presence of large indigenous populations of beneficial phenazine-producing (Phz$^+$) *Pseudomonas* spp. in the rhizosphere of wheat grown across 22,000 km^2 of arid parts of central Washington and northeastern Oregon (Mavrodi et al., 2012a; Parejko et al., 2013). Although these Phz$^+$ pseudomonads were ubiquitous and colonized almost 100% of wheat in regions of low precipitation, they were less abundant or non-detectable in irrigated fields or neighboring higher rainfall areas (Mavrodi et al., 2012b). These findings prompted us to hypothesize that precipitation plays an important role in the establishment of indigenous communities and the activity of beneficial Phz$^+$ rhizobacteria. In this study, we tested our hypothesis by examining the effect of irrigation on the seasonal dynamics of Phz$^+$ pseudomonads and levels of phenazine-1-carboxylic acid in the rhizosphere of field-grown spring wheat. Results of this work strongly supported our hypothesis and revealed that just three successive seasons of overhead irrigation were sufficient to significantly reduce the incidence of Phz$^+$ pseudomonads and amounts of PCA in the field (**Figures 1**, **2**). The observed differences between irrigated and non-irrigated plots were reproducible and amplified over the course of the 3-year study, thus identifying precipitation as a key abiotic factor that affects the dynamics and activity of indigenous Phz$^+$ *Pseudomonas* communities. Although the mechanism behind this phenomenon is currently unknown, we speculate that increased soil moisture in the irrigated plots perturbs interactions within the rhizosphere microbiome and alters rhizodeposition and soil properties. We attempted to gain additional insights into the effect of irrigation on fluorescent pseudomonads via the sequence-based profiling of the rhizosphere microbiome (see below), but identified only two *Pseudomonas* OTUs that matched the plant pathogen *P. syringae* and *P. cedrina*, which belongs to the *P. fluorescens* group. The levels of these OTUs in the rhizosphere of wheat fluctuated over the duration of the study and did not significantly differ between irrigated and non-irrigated treatments (Supplementary Figure 2). It is likely that differential responses of different *Pseudomonas* to irrigation were obscured by the lack of resolution at finer taxonomic levels due

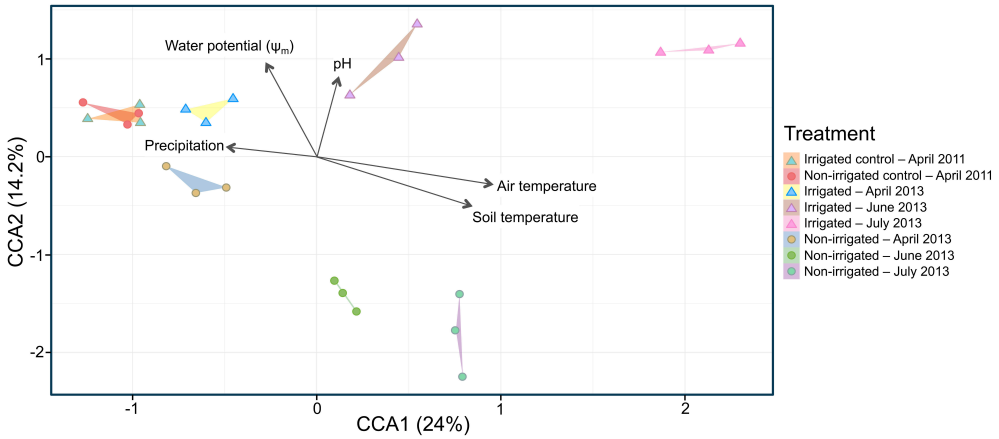


FIGURE 4 | Canonical correspondence analysis (CCA) plot of microbial community composition showing explanatory environmental variables. Each point represents a sampled rhizosphere community. Arrows show quantitative explanatory environmental variables with arrowheads indicating their direction of increase. Samples from irrigated and non-irrigated plots are indicated with triangles and circles, respectively. Colors correspond to control and treatment time points in 2011 and 2013 as shown in the figure legend.

to the relatively short length (300–350 bp) of V4 amplicons used in the microbiome analysis.

Our results also provide the first comprehensive picture of the seasonal dynamics of indigenous Phz$^+$ pseudomonads and accumulation of PCA in dryland wheat fields. Over the course of the study, the levels of PCA on wheat roots remained high and peaked in April when the soil was wet and cool and then gradually declined toward the end of the field season. However, the short half-life of PCA in the rhizosphere (Mavrodi et al., 2013) suggests that the production of microbial phenazines on wheat roots is a sustained and highly dynamic process, and continues even as the soil dries to lower matric potential. These findings also indicate that the observed populations of phenazine producers in the field (between 10^5 and 10^7 CFU g^{-1} of root fresh weight) were sufficiently high to support HSL-mediated quorum sensing that is necessary for the induction of PCA biosynthesis genes. The sustained production of PCA may play a crucial role in the establishment and maintenance of indigenous communities of Phz$^+$ pseudomonads associated with the rhizosphere of dryland wheat. Pseudomonads have respiratory metabolism but can tolerate low oxygen environments such as waterlogged soils. The opportunistic human pathogen *P. aeruginosa* uses redox-active phenazines as alternative terminal electron acceptors, and the PCA-mediated electron shuttling promotes survival of this organism under anaerobic conditions (Wang et al., 2010; Glasser et al., 2014). It is plausible that PCA performs a similar function in rhizosphere Phz$^+$ pseudomonads and supports their growth during parts of the year when the soil is wet and hypoxic or even anoxic. Phenazine biosynthesis also modulates the surface adhesion and biofilm architecture in *P. chlororaphis* and *P. aeruginosa* (Maddula et al., 2008; Ramos et al., 2010). In its spatial and temporal characteristics, root colonization by rhizobacteria resembles biofilm growth (Angus and Hirsch, 2013), and PCA may help the Phz$^+$ populations to form stress-resistant biofilms and proliferate on roots of dryland wheat growing in soils with intermittent availability of water. Phenazine-1-carboxylic acid has broad-spectrum antimicrobial and antihelmintic properties (Smirnov and Kiprianova, 1990; Cezairliyan et al., 2013) and may aid Phz$^+$ pseudomonads in competition with indigenous microflora and resistance to predation by bacteriovorus nematodes. Finally, phenazines play a crucial role in the ability of Phz$^+$ rhizobacteria to control soilborne fungal pathogens (Thomashow and Weller, 1988; Chin-a-Woeng et al., 1998; Arseneault et al., 2016; Jaaffar et al., 2017). The combination of higher soil moisture and lower temperatures exacerbates the severity of damage by Rhizoctonia root rot, which is among the most important soilborne diseases of dryland wheat (Smiley and Uddin, 1993; Gill et al., 2001; Smiley et al., 2012). Therefore, the interplay between the precipitation, Phz$^+$ populations and the accumulation of rhizosphere PCA early in the spring may have significant implications for managing root and crown diseases of cereal crops in the IPNW soils.

In addition to the effect of irrigation on Phz$^+$ rhizobacteria, we examined the impact of altered precipitation on the structure and dynamics of the entire wheat rhizosphere microbiome. Our analysis revealed that roots of dryland wheat harbor diverse communities dominated by *Proteobacteria, Bacteroidetes, Actinobacteria*, and *Acidobacteria*, which is in agreement with earlier assessments of microbial diversity in the rhizosphere of this crop (Yin et al., 2013; Mahoney et al., 2017). The three seasons of irrigation had a slight effect on the overall diversity within the rhizosphere microbiome (Supplementary Figure 1), which was in contrast with the apparent separation of irrigated and dryland treatments by the Bray–Curtis similarity measures that indicated significant differences in the relative abundances of specific OTUs (**Figure 3**). The observed delineation between rhizosphere populations from irrigated and dryland wheat was particularly pronounced later in the season after the onset of irrigation. The analysis of measured environmental

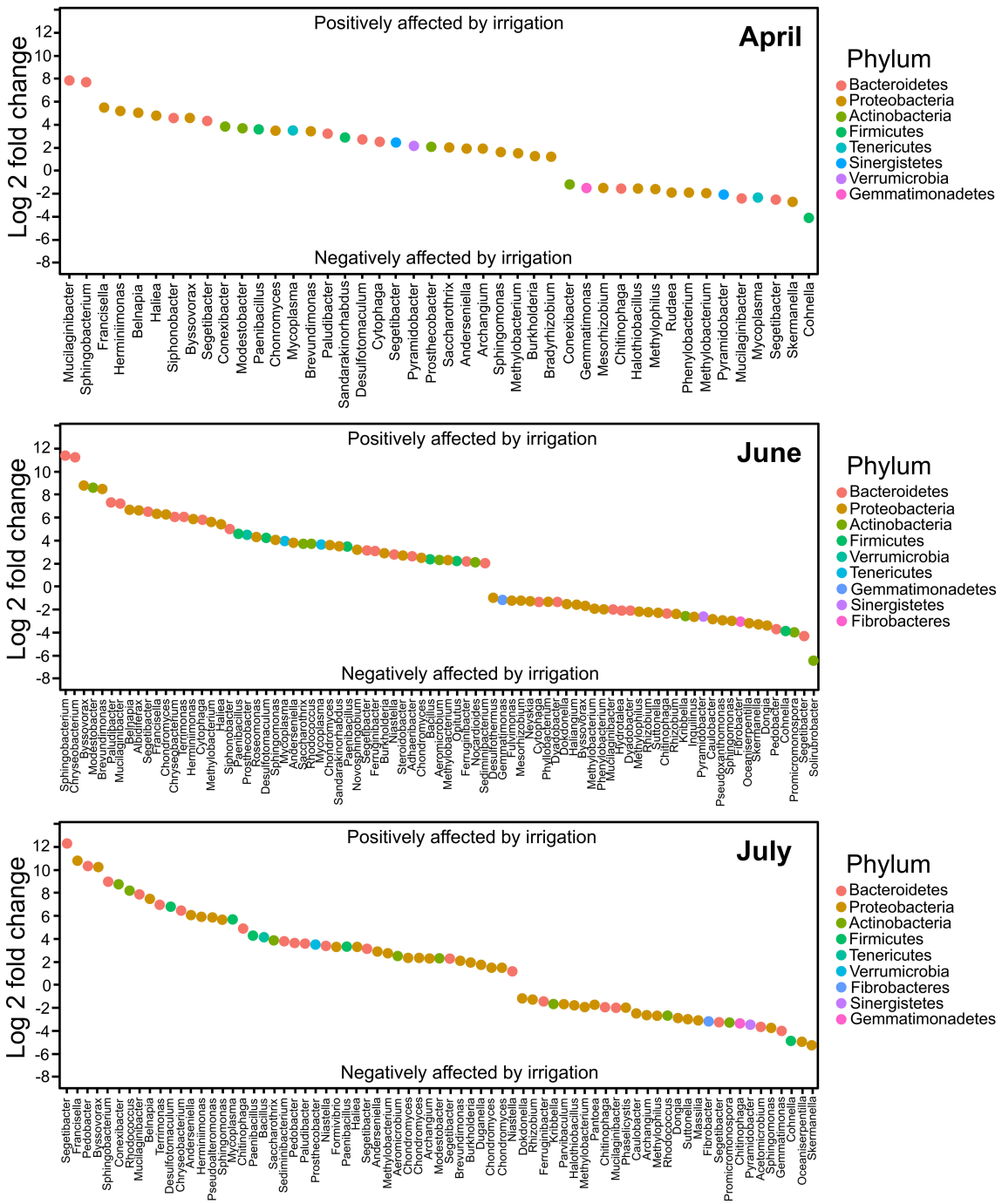


FIGURE 5 | DESeq analysis of taxa differentially distributed in irrigated samples compared to non-irrigated at the three 2013 sampling time points. Analyses were performed with a *p*-value maximum of 0.001. Comparisons were performed with respect to irrigated samples.

variables contributing to the observed shifts in the community composition revealed that the separation between irrigated and dryland treatments was due to changes in the water potential (Ψ_m) and pH. In contrast, the temporal changes in the composition of the wheat rhizosphere microbiome were driven by temperature and precipitation (**Figure 4**). These findings are in agreement with the emerging consensus regarding abiotic factors with marked effects on the variety and abundance of soil microbial taxa (Lauber et al., 2009; Fierer, 2017). The combination of the measured environmental factors explained only part (38.2%) of the total change in the structure of rhizosphere bacterial communities, and the remaining portion

TABLE 3 | OTUs with highest relative increase in abundance under irrigation in April (S1), June (S2), or July (S3) of 2013.

Sample	OTU	log2 FC	Phylum	Class	Order	Family	Genus	Species
S3	OTU_106	12.3	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Segetibacter*	OTU_106__Segetibacter__*Bacteroidetes*
S2	OTU_179	11.0	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*	OTU_179__Chryseobacterium__*Bacteroidetes*
S3	OTU_155	10.3	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Pedobacter*	OTU_155__Pedobacter__*Bacteroidetes*
S2,3	OTU_198	8.6	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	*Polyangiaceae*	*Byssovorax*	OTU_198__Byssovorax__*Proteobacteria*
S2	OTU_116	8.5	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Geodermatophilaceae*	*Modestobacter*	OTU_116__Modestobacter__Actinobacteria
S2	OTU_86	8.3	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	OTU_86__Brevundimonas__*Proteobacteria*
S1	OTU_101	7.8	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Mucilaginibacter*	OTU_101__Mucilaginibacter__*Bacteroidetes*
S1,2,3	OTU_97	7.6	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingobacterium*	OTU_97__Sphingobacterium__*Bacteroidetes*
S1,3	OTU_176	5.5	*Proteobacteria*	*Gammaproteobacteria*	*Thiotrichales*	*Francisellaceae*	*Francisella*	OTU_176__Francisella__*Proteobacteria*
S1	OTU_216	5.2	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herminiimonas*	OTU_216__Herminiimonas__*Proteobacteria*
S1	OTU_109	5.0	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*	*Belnapia*	OTU_109__Belnapia__*Proteobacteria*

TABLE 4 | OTUs with highest relative decrease in abundance under irrigation in April (S1), June (S2), or July (S3) of 2013.

Sample	OTU	log2FC	Phylum	Class	Order	Family	Genus	Species
S2	OTU_64	−6.6	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Solirubrobacter*	OTU_64__Solirubrobacter__Actinobacteria
S3	OTU_57	−4.8	*Proteobacteria*	*Gammaproteobacteria*	*Oceanospirillales*	*Oceanospirillaceae*	*Oceaniserpentilla*	OTU_57__Oceaniserpentilla__Proteobacteria
S2	OTU_126	−4.5	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Segetibacter*	OTU_126__Segetibacter__*Bacteroidetes*
S2	OTU_37	−4.2	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Promicromonosporaceae*	*Promicromonospora*	OTU_37__Promicromonospora__Actinobacteria
S1,2,3	OTU_194	−3.9	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Cohnella*	OTU_194__Cohnella__Firmicutes
S2	OTU_8	−3.8	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Pedobacter*	OTU_8__Pedobacter__*Bacteroidetes*
S3	OTU_189	−3.8	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*	OTU_189__Gemmatimonas__Gemmatimonadetes
S3	OTU_92	−3.6	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	OTU_92__Sphingomonas__Proteobacteria
S1,3	OTU_28	−2.6	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Skermanella*	OTU_28__Skermanella__Proteobacteria
S1	OTU_126	−2.5	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Segetibacter*	OTU_126__Segetibacter__*Bacteroidetes*
S1	OTU_159	−2.4	*Tenericutes*	*Mollicutes*	*Mycoplasmatales*	*Mycoplasmataceae*	*Mycoplasma*	OTU_159__Mycoplasma__Tenericutes
S1	OTU_58	−2.3	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Mucilaginibacter*	OTU_58__Mucilaginibacter__*Bacteroidetes*

of the variation could be due to the complex interaction of other abiotic and biotic factors (e.g., spatial heterogeneity, soil disturbances, dispersal ability, competition, and niche differentiation; Fierer, 2017). The temporal changes in the rhizosphere microbiome are also likely to be driven by plant age and shifts in the composition and amounts of rhizodeposits at distinct stages of plant development. A recent study by Donn et al. (2015) reported a strong effect of the vegetative plant stage on motile, aerobic, or facultatively anaerobic taxa that are tightly associated with wheat roots.

Although water strongly influences soil microorganisms, surprisingly few studies have examined the effect of irrigation on the structure of soil microbial communities across space or time. A recent study by Hartmann et al. (2017) characterized the impact of long-term irrigation on the soil microbiome of a semi-arid pine forest in the Rhone Valley of Switzerland. Results of that study revealed that a decade of irrigation in an ecosystem with a history of water limitation caused a pronounced shift in the microbiome from oligotrophic to more copiotrophic lifestyles. The increased soil moisture and stimulated plant-derived inputs promoted the occurrence of copiotrophic *Proteobacteria* and displaced oligotrophic groups that are more tolerant of water stress such as *Actinobacteria, Gemmatimonadetes, Acidobacteria,* and *Armatimonadetes* (Hartmann et al., 2017). In contrast, our results identified most taxa with strong positive and negative responses to irrigation as *Bacteroidetes* and *Proteobacteria*, and at the class level the largest group of rhizobacteria influenced by irrigation was represented by *Sphingobacteria* (**Figure 5**; **Tables 3**, **4**). These discrepancies are not surprising and can be attributed to some significant differences between the two experimental systems and sampling procedures. Our study was conducted in an intensively managed agroecosystem dominated by the monoculture of wheat, whereas Hartmann and colleagues studied a natural forest ecosystem located in the European Alps and dominated by Scots pine (*Pinus sylvestris*). Also, it is known that the proximity of plant host strongly influences the composition of soil microbiome (Lareen et al., 2016), and Hartmann and colleagues conducted their analysis by extracting DNA from bulk soil, while we specifically focused on microbial communities that are tightly associated with the surface of wheat roots. Several non-pseudomonad taxa that we identified as differentially responding to irrigation have been previously shown to affect plant health positively. Yin et al. (2013) demonstrated that *Chryseobacterium* and *Pedobacter* from the IPNW wheat field soils produced antifungal compounds and antagonized the mycelial growth of *R. solani* AG-8 under *in vitro* conditions. Furthermore, several isolates of *C. soldanellicola* significantly reduced the severity of Rhizoctonia root rot of wheat in greenhouse assays. A synergistic interaction within a mixture of strains of *Brevundimonas*, *Pseudomonas,* and *Pedobacter* resulted in a significant growth reduction of phytopathogenic fungi *F. culmorum* and *R. solani* (de Boer et al., 2007). Strains of *Promicromonospora* and *Sphingobacterium* have been found to promote plant growth by secreting gibberellins and modulating the levels of stress-induced ethylene via the production of 1-aminocyclopropane-1-carboxylic acid deaminase (Kang et al., 2012; Feng et al., 2017). Interestingly, although prominently associated with wheat roots, the aforementioned taxa are dynamic and, in addition to irrigation, differentially respond to other agronomical practices such as organic farming, tillage regimens, and the use of different wheat cultivars (Li et al., 2012; Mahoney et al., 2017; Yin et al., 2017).

During the rest of the twenty-first century, all general circulation models predict warmer temperatures and extreme weather events around the globe (Stockle et al., 2010; Zhao and Running, 2010; Seneviratne et al., 2012). As a consequence, crops will increasingly be exposed to abiotic stresses caused by changes in average temperatures, temperature extremes, and moisture availability. Plant roots host distinct bacterial communities that positively influence plant development, vigor, disease resistance, productivity, and response to stressors associated with global climate change (Adriaensen et al., 2005; Rodriguez et al., 2008; Kawasaki et al., 2012; Lau and Lennon, 2012). Much attention has been given to uncovering the mechanisms of water stress in plants. In contrast, the complex effects of altered precipitation on rhizobacteria remain poorly understood. Here, we characterized the responses of biocontrol pseudomonads and the entire wheat rhizosphere microbiome to overhead irrigation. To the best of our knowledge, this is the first study that examined this topic by conducting a long-term field experiment in an agroecosystem with a strong history of water limitation. Our results provide direct experimental evidence that soil water status drives the development of populations of beneficial antibiotic-producing rhizobacteria that contribute to the natural suppression of soilborne diseases of cereal crops. We further demonstrated that these rhizobacteria produce copious amounts of phenazine-1-carboxylic acid that persists in the rhizosphere of wheat over the course of the field season. Phenazines are broad-host-range antimicrobials that inhibit the growth of certain groups of bacteria and fungi (Mavrodi et al., 2006), serve as a source of carbon and energy for others (Costa et al., 2015) and affect the amount and composition of plant rhizodeposition (Phillips et al., 2004), thus raising questions about the broader role of these metabolites in the shaping of the belowground wheat microbiome. More broadly, results of this long-term study provide new insights into how the availability of water in a semi-arid agroecosystem shapes the belowground wheat microbiome. Our findings also suggest that soilborne pathogens in wheat fields across the Inland Pacific Northwest, U.S.A. are kept in check by the concerted action of multiple groups of rhizosphere bacteria, and suggest that the dynamics of these beneficial rhizobacteria may be strongly influenced by the interplay between soil moisture and agricultural management practices. These findings will aid in understanding the impact of agricultural management practices and climate change on the dynamics of the rhizosphere microbiome and soilborne pathogens in cereal crops.

AUTHOR CONTRIBUTIONS

DM, LT, DW, and IP: conceived the research project; OM, DM, JP, and MY: collected field samples; OM: generated amplicons for

Illumina sequencing; RB: extracted and quantified phenazine-1-carboxylic acid; LE and ST: performed the microbiome analysis; DM, OM, LE, ST, DW, and LT: wrote the manuscript and all authors contributed to the manuscript revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00345/full#supplementary-material

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Impacts of Atmospheric CO_2 and Soil Nutritional Value on Plant Responses to Rhizosphere Colonization by Soil Bacteria

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Concerns over rising atmospheric CO_2 concentrations have led to growing interest in the effects of global change on plant-microbe interactions. As a primary substrate of plant metabolism, atmospheric CO_2 influences below-ground carbon allocation and root exudation chemistry, potentially affecting rhizosphere interactions with beneficial soil microbes. In this study, we have examined the effects of different atmospheric CO_2 concentrations on Arabidopsis rhizosphere colonization by the rhizobacterial strain *Pseudomonas simiae* WCS417 and the saprophytic strain *Pseudomonas putida* KT2440. Rhizosphere colonization by saprophytic KT2440 was not influenced by sub-ambient (200 ppm) and elevated (1,200 ppm) concentrations of CO_2, irrespective of the carbon (C) and nitrogen (N) content of the soil. Conversely, rhizosphere colonization by WCS417 in soil with relatively low C and N content increased from sub-ambient to elevated CO_2. Examination of plant responses to WCS417 revealed that plant growth and systemic resistance varied according to atmospheric CO_2 concentration and soil-type, ranging from growth promotion with induced susceptibility at sub-ambient CO_2, to growth repression with induced resistance at elevated CO_2. Collectively, our results demonstrate that the interaction between atmospheric CO_2 and soil nutritional status has a profound impact on plant responses to rhizobacteria. We conclude that predictions about plant performance under past and future climate scenarios depend on interactive plant responses to soil nutritional status and rhizobacteria.

Keywords: CO_2, PGPR, global change, rhizosphere, ISR

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INTRODUCTION

Atmospheric CO_2 influences microbial biomass and diversity in the rhizosphere (Paterson et al., 1997). The plant-mediated effects of atmospheric CO_2 on soil microbial communities are well documented (Wiemken et al., 2001; Montealegre et al., 2002), indicating a dominant, plant-mediated mechanism. It is likely that the variation in root- microbe interactions under different

atmospheric conditions are due to changes in root exudates which are estimated to contain between 5 and 40% of plant photosynthetically fixed carbon (Lynch and Whipps, 1990; Hinsinger et al., 2006; Marschner, 2012). Since rhizodeposition of carbon (C) increases under elevated CO_2 (eCO_2; Phillips et al., 2009; Eisenhauer et al., 2012), it can be expected that rhizosphere colonization by microbes relying on C from plant exudates will also be enhanced (Lipson et al., 2005; Kassem et al., 2008; Eisenhauer et al., 2012). While it is clear is that CO_2 alters overall microbial community composition across a range of different soil-types (Montealegre et al., 2002; Janus et al., 2005), the extent to which eCO_2 affects microbial interactions in the rhizosphere remains controversial. Using chloroform fumigation extraction to estimate microbial biomass, previous studies have reported both positive and negative relationships with eCO_2 (Rice et al., 1994; Ross et al., 1995; Kassem et al., 2008; Eisenhauer et al., 2012). It also remains contentious in how far eCO_2 induces shifts between fungal or bacterial communities, and the resultant effects on the functioning on rhizosphere microbes (Ross et al., 1995; Lipson et al., 2005; Drigo et al., 2008).

Early research on plant growth responses and the presence of specific rhizosphere microbes to eCO_2 have suggested a possible relationship between eCO_2, plant growth and increases in colonization by plant growth-promoting rhizobacteria (PGPR; O'Neill et al., 1987). PGPRs are often closely associated with plant roots and should, therefore, be more reliant on plant-derived C (Denef et al., 2007). Although many studies have addressed the effects of eCO_2 on plant-rhizobia and plant-mycorrhiza interactions (e.g., Rogers et al., 2009; Mohan et al., 2014), little is known about the specific impacts of eCO_2 on PGPR (Drigo et al., 2008). Considering that PGPR modulate a range of agronomically important plant traits, including plant growth, abiotic stress tolerance and resistance to pests, and diseases (Lugtenberg and Kamilova, 2009), this knowledge gap limits our ability to predict how anthropogenic global change will impact crop production and food security. Furthermore, the impacts of CO_2 across a range of CO_2 conditions, including sub-ambient CO_2 ($saCO_2$), remain poorly documented (Field et al., 2012). In a CO_2 gradient study (200–600 ppm), microbial biomass and soil respiration from a grassland ecosystem were not clearly related to CO_2 concentration (Gill et al., 2006). By contrast, analysis of fungal communities, using pyrosequencing of internal transcribed spacer sequences, revealed a positive relationship between operational taxonomic unit richness and CO_2 concentration that was soil-type dependent (Procter et al., 2014). While these studies suggest that atmospheric CO_2 impacts on plant-beneficial microbes in the rhizosphere, it remains difficult to ascertain the underpinning mechanisms and predict the corresponding plant responses to altered colonization by these microbes. Most studies on the effects of CO_2 gradients on rhizosphere microbes involved field experiments, which are prone to environmental variability, such as nutrient availability, soil moisture, temperature, soil pH, and plant species present (Freeman et al., 2004; Castro et al., 2010; Classen et al., 2015; Dam et al., 2017) and do not allow the manipulation of bacteria in the rhizosphere, hence preventing examination of their function.

In this study, we have investigated the impacts of a pre-industrial concentration of $saCO_2$ and a worst-case scenario projected concentration of eCO_2 on rhizosphere colonization of Arabidopsis roots by two well- characterized soil bacteria: the rhizosphere colonizer *Pseudomonas simiae* WCS417 (previously named *Pseudomonas fluorescens* WCS417; Berendsen et al., 2015) and the saprophytic soil colonizer *Pseudomonas putida* KT2440. We demonstrate that increasing CO_2 levels boost root colonization by WCS417 in soil with relatively low C and nitrogen (N) content. Interestingly, these effects were associated with contrasting growth and resistance responses by the host plant, demonstrating that high atmospheric CO_2 concentration can have profound and counterintuitive effects on plant growth and resistance due to altered rhizosphere interactions.

RESULTS

Impacts of Atmospheric CO_2 on Rhizosphere Colonization by Soil Bacteria Depends on Soil Quality and Bacterial Species

C and N content are markers for soil quality (Gil-Sotres et al., 2005), which has a direct impact on the performance of PGPR (e.g., Egamberdiyeva, 2007; Agbodjato et al., 2015). To examine the importance of soil quality on rhizosphere colonization by two well-studied soil bacteria, Arabidopsis was cultivated either in artificial nutrient-poor soil (1:9 sand:compost; v/v) with low C- and N-contents, or in nutrient-rich soil (2:3 sand:compost; v/v) with relatively high C and N content (**Table 1**). Soils were inoculated with 5×10^7 colony forming units (CFU).g^{-1} soil of *P. simiae* WCS417, a rhizosphere colonizer (Rainey, 1999; Zamioudis et al., 2014), or *P. putida* KT2440, a more generalist saprophytic soil colonizer (Weinel et al., 2002). Soil with and without Arabidopsis plants (accession Col-0) were left for 4 weeks before sampling for quantification of bacterial colonization through enumeration of CFU on selective agar medium. Two-way ANOVA of CFU values revealed a statistically significant interaction between soil and bacterial strain ($P = 0.023$; **Figure 1** and **Supplementary Table S1**), indicating that the two strains colonize the soil-types and soil compartments to different extents. Indeed, statistical analysis by Tukey *post hoc* tests revealed that titres of the rhizobacterial strain WCS417 were significantly higher in the rhizosphere of Arabidopsis compared to those in plant-free bulk soil, where the level of colonization by this strain remained below the CFU detection limit (**Figure 1**). This rhizosphere-specific colonization by WCS417 was apparent in both soil-types (**Figure 1**). By contrast, the generalist saprophyte KT2440 colonized rhizosphere and bulk

TABLE 1 | C and N concentrations in nutrient-rich and poor-soil.

	Carbon (C)		Nitrogen (N)		C:N
Nutrient-poor	2.58%	−0.15	0.21%	−0.01	12.29
Nutrient-rich	18.78%	−0.48	0.37%	−0.03	51.02

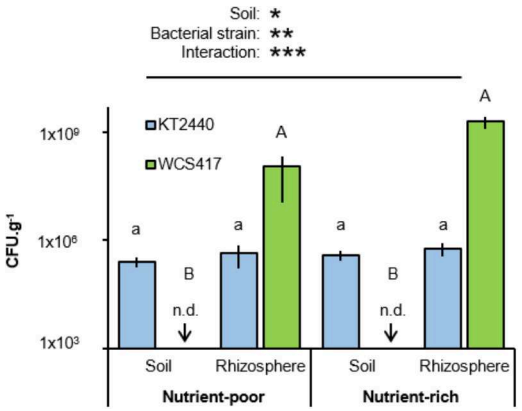


FIGURE 1 | Effects of soil nutritional status on colonization by *P. simiae* WCS417 and *P. putida* KT2440. Bacteria were introduced into nutrient-poor soil or nutrient-rich soil at 5×10^7 Colony forming units (CFU).g^{-1}. CFU.g^{-1} of KT2400 (blue) and WCS417 (green) were determined after 4 weeks. Samples were taken from root-associated rhizosphere soil (Rhizosphere), or bulk soil without plants (Soil). Data represent mean CFU.g^{-1} values (± SE, $n = 8$). Asterisks on top of the graph indicate statistical significance of 2-way ANOVA (*: $0.05 < P < 0.01$, **: $0.01 < P < 0.001$, and ***: $P < 0.001$). Different letters of same font indicate statistically significant differences between soil-types for each strain (1-way ANOVA + Tukey multiple comparisons test; $P < 0.05$). n.d: not detected; bacterial titres were below the limit of detection.

soil from both soil-types with equal efficiencies, although its levels of rhizosphere colonization remained orders of magnitude lower than that of WCS417 (**Figure 1**).

To examine whether atmospheric CO_2 alters rhizosphere colonization by WCS417 and KT2440, Arabidopsis was cultivated for 4 weeks in both soil-types at *sa*CO_2 (200 ppm), ambient CO_2 (*a*CO_2; 400 ppm) or *e*CO_2 (1200 ppm) before quantification of rhizosphere colonization. Interestingly, in nutrient-poor soil, rhizosphere titres of WCS417 bacteria increased statistically from *sa*CO_2 to *e*CO_2, whereas this effect of CO_2 was absent in nutrient-rich soil (**Figure 2A**). Furthermore, the statistically significant interaction between CO_2 and soil-type indicates that the stimulating effect of CO_2 on rhizosphere colonization by WCS417 depends on soil nutritional status (two-way ANOVA; $P = 0.006$; **Figure 2A** and **Supplementary Table S2A**). By contrast, rhizosphere titres of KT2440 were not statistically altered by CO_2, soil-type, or the interaction thereof (two-way ANOVA; $P = 0.541$; **Figure 2B** and **Supplementary Table S2B**), indicating that the colonization by this saprophytic strain is unaffected by soil nutritional status and atmospheric CO_2. Hence, the stimulatory impacts of atmospheric CO_2 on rhizosphere colonization by soil bacteria depend on soil quality and bacterial species.

Atmospheric CO_2 Influences Plant Growth Responses to *P. simiae* WCS417 on Nutrient-Poor Soil

To assess the influence of CO_2 on plant growth responses to rhizobacteria, control- (i.e., mock inoculated) and WCS417-inoculated plants were examined for rosette areas after 5 weeks of growth. In the absence of WCS417, rosette sizes increased statistically from *sa*CO_2 to *e*CO_2, which was apparent in both nutrient-poor and nutrient-rich soil (**Figure 3A** and **Supplementary Table S3A**). Furthermore, application of WCS417 did not influence the growth of plants on nutrient-rich soil (**Figure 3A**). This was confirmed by two-way ANOVA, which did not indicate a statistically significant interaction between bacterial treatment and CO_2 ($P = 0.432$; **Supplementary Table S3B**). Conversely, in nutrient-poor soil, WCS417 had a statistically significant effect on rosette size and also showed a statistically significant interaction with CO_2 by 2-way ANOVA ($P < 0.001$; **Supplementary Table S3B**). This indicates that the effects of WCS417 on shoot growth are dependent on atmospheric CO_2 concentration. Subsequent *t*-tests revealed that WCS417 statistically increased rosette size at *a*CO_2 and repressed at *e*CO_2 (**Figure 3A**). Since PGPR have been reported to affect root and shoot growth differentially through impacts on auxin and cytokinin levels (Vacheron et al., 2013), we also determined root biomass. As is shown in **Figure 3B**, root dry weights in nutrient-poor soil mirrored the effects of WCS417 on rosette area on this soil-type: the bacteria increased root biomass at *a*CO_2, while they increased root biomass at *e*CO_2. As for the average rosette area, the effects of WCS417r on root biomass were statistically significant and showed a statistically significant interaction with CO_2 (**Supplementary Table S3C**). Together, these results suggest that WCS417 has a plant growth-promoting effect at *sa*CO_2 and *a*CO_2, but that it reduces plant growth at *e*CO_2.

Atmospheric CO_2 Influences Systemic Resistance Responses to *P. simiae* WCS417 on Both Nutrient-Poor and Nutrient-Rich Soil

Arabidopsis develops induced systemic resistance (ISR) upon root colonization by WCS417 (Pieterse et al., 1996). Since WCS417 colonization of the Arabidopsis rhizosphere is CO_2-dependent (**Figure 2A**), we examined impacts of CO_2 on ISR. To this end, leaves of control- and WCS417-inoculated plants were challenge-inoculated with the necrotrophic leaf fungus *Plectosphaerella cucumerina*. Disease progression was quantified at 8 and 13 days post-inoculation (dpi) by lesion diameter in both nutrient-poor and nutrient-rich soil. For each time-point/soil-type combination (apart from 8dpi in nutrient-rich soil), two-way ANOVA revealed a statistically significant effect of CO_2 on disease resistance (in each case $P < 0.001$; **Supplementary Tables S4A–D**), which manifested itself as increased resistance at *e*CO_2 compared to *sa*CO_2 and *a*CO_2 (**Figure 4**). There was also a statistically significant interaction between bacterial treatment and CO_2 in nutrient-poor soil which was apparent at 8 and 13 dpi in nutrient-poor soil, but was not significant at 13 dpi in nutrient-rich soil (two-way ANOVA; $P < 0.001$, $P = 0.004$, and $P = 0.087$, respectively; **Supplementary Tables S4A–D**). This indicates that the effects of WCS417 on systemic resistance depend on atmospheric CO_2 concentration. Subsequent *t*-tests revealed that WCS417 reduced lesion diameters at both *a*CO_2 and *e*CO_2 in nutrient-poor and nutrient-rich soils, which

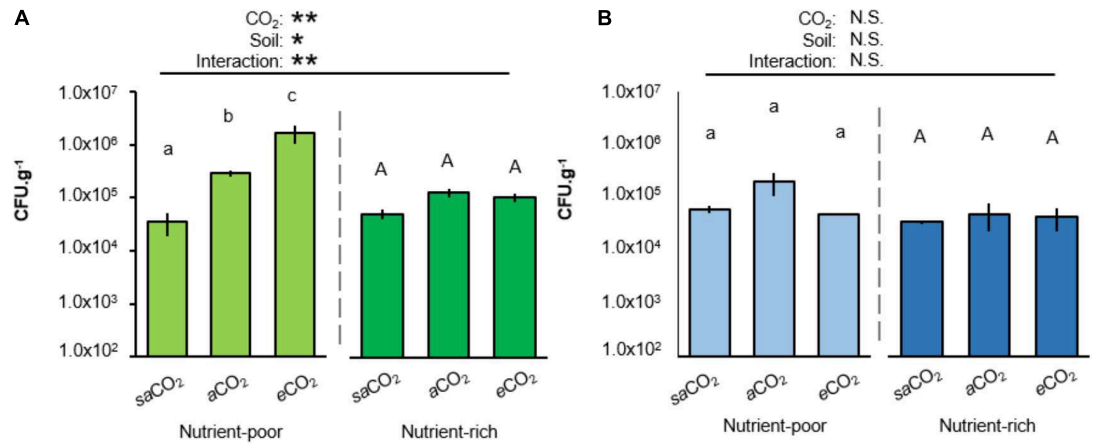


FIGURE 2 | Impacts of atmospheric CO_2 and soil-type on Arabidopsis rhizosphere colonization by *P. simiae* WCS417 **(A)** and *P. putida* KT2440 **(B)**. Bacteria were introduced at 5×10^7 CFU.g^{-1} into nutrient-poor (left panels) or nutrient-rich (right panels) soil prior to planting Arabidopsis seeds. Rhizosphere colonization was determined after 4 weeks of growth at sub-ambient CO_2 (200 ppm), ambient CO_2 (400 ppm), or elevated CO_2 (1200 ppm). Data shown represent mean CFU.g^{-1} (±SE, $n = 10$). Asterisks on top of the graph indicate statistical significance of 2-way ANOVA (*: $0.05 < P < 0.01$, **: $0.01 < P < 0.001$, and ***: $P < 0.001$). Different letters of the same font indicate statistically significant differences between CO_2 conditions for each soil-type (Tukey multiple comparisons *post hoc* test, $P < 0.05$). Patterns of colonization with WCS417 were consistent over two independent experiments.

was statistically significant at either 8 or 13 dpi (**Figure 4**). Surprisingly, at *sa*CO_2, treatment of nutrient-poor soil with WCS417 statistically increased lesion diameters at both 8 and 13 dpi, suggesting induced systemic susceptibility (ISS). This response was absent when plants were grown on nutrient-rich soil at *sa*CO_2, where WCS417 did not have a statistically significant effect on lesion diameter by *P. cucumerina* (**Figure 4**). Hence, the effect of WCS417 on systemic plant immunity varies from induced susceptibility to induced resistance, depending on the atmospheric CO_2 concentration and soil nutritional status.

DISCUSSION

To date, only few studies have investigated effects of atmospheric CO_2 on rhizosphere colonization by PGPRs. While previous work has shown that *e*CO_2 increases bacterial and fungal biomass in the rhizosphere (Kassem et al., 2008), our study is the first to report effects of *sa*CO_2 and *e*CO_2 on rhizosphere colonization by selected soil bacteria. Procter et al. (2014) reported an increase in fungal species richness and enhanced relative abundance of selected fungi with *e*CO_2, which varied according to soil-type (Procter et al., 2014). Furthermore, a grassland free air CO_2 enrichment (FACE) experiment revealed that initial C accumulation occurred predominantly in arbuscular mycorrhizal fungi (AMF; Denef et al., 2007), which are symbiotic and rely on host-derived carbon (e.g., Lindahl et al., 2010). Although mycorrhizal root colonization is influenced by different factors than rhizobacterial root colonization, it is plausible that increased C deposition at *e*CO_2 has more pronounced effects in C-poor soil-types, where root-associated microbes will be more reliant on plant-derived C. Indeed, the rhizobacterial WCS417 strain showed increasing rhizosphere colonization at rising CO_2 concentrations, which was most pronounced in nutrient-poor soil (**Figure 2**). Moreover, the differential effects of CO_2 on KT2440 and WCS417 help to explain why CO_2 has been reported to have effects on some bacterial soil communities, while others remain unaffected (Rice et al., 1994; Ross et al., 1995; Kassem et al., 2008; Eisenhauer et al., 2012). Exactly what changes in rhizosphere chemistry drive these community effects, requires further research.

KT2440 was originally isolated from benzene-contaminated soils in Japan (Nakazawa and Yokota, 1973). Accordingly, it survives well in root-free bulk soils. However, this strain has also been reported to colonize the rhizosphere of plants, in particular of grasses (Molina et al., 2000). The rhizosphere of many grass species, such as maize, contain relatively high concentrations of aromatic benzoxazinoids (Neal et al., 2012). KT2440 is highly tolerant to the antimicrobial activity of benzoxazinoids and responds to these chemicals by positive chemotaxis (Neal et al., 2012), explaining why this strain is a strong colonizer of the maize rhizosphere. By contrast, KT2440 did not show increased colonization of the Arabidopsis rhizosphere in comparison to plant-free control soil (**Figure 1**), suggesting that KT2440 is not majorly influenced by the rhizosphere chemistry of Arabidopsis. WCS417, on the other hand, showed relatively high levels of colonization in the rhizosphere, but failed to sustain colonies in plant-free control soil (**Figure 1**), which is typical for a rhizobacterial species. WCS417 was originally isolated from the rhizosphere of wheat (Lamers et al., 1988) and has since been shown to colonize the rhizosphere of a wide range of plant species (Berendsen et al., 2015). Interestingly, a recent report has shown the iron-regulated secondary metabolite scopoletin in Arabidopsis root exudates selectively inhibits soil-borne pathogens, while ISR-inducing rhizobacteria, including WCS417, are highly tolerant to the antimicrobial effect of scopoletin (Stringlis et al., 2018). Hence, the recruitment

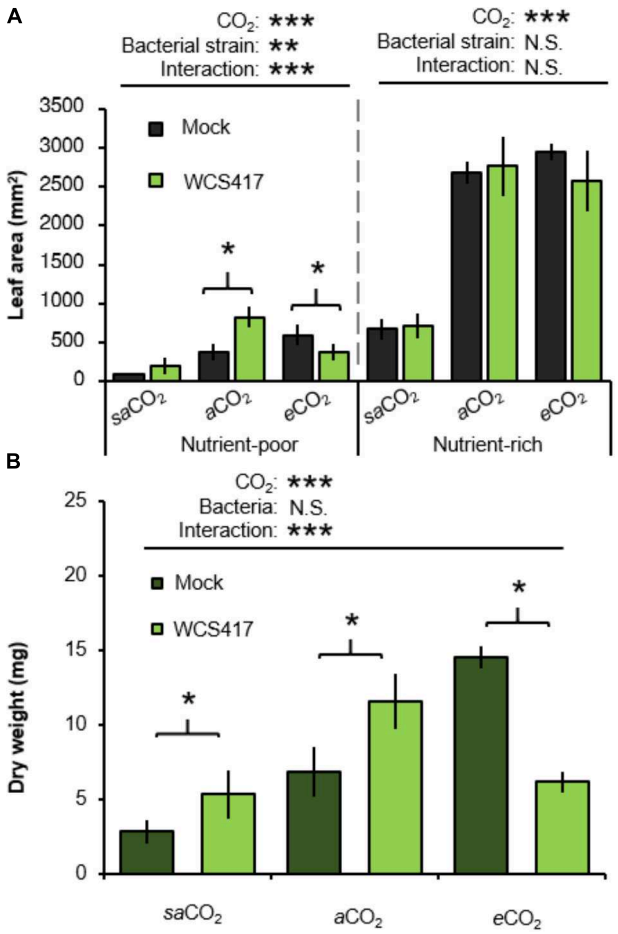


FIGURE 3 | Effects of atmospheric CO_2 and soil nutritional status on plant growth responses to *P. simiae* WCS417. **(A)** Effects of WCS417 on total leaf area of Arabidopsis at increased CO_2 concentrations in nutrient-poor (left) and nutrient-rich (right) soils. Soil were inoculated with WCS417 (5×10^7 CFU.g^{-1} soil), or or mock treated with $MgSO_4$ prior to planting. Leaf area was quantified by image analysis after 4 weeks of growth. Shown are mean leaf areas (±SE, $n = 10$). **(B)** Effects of WCS417 root biomass at increased CO_2 concentrations and in nutrient-poor soil. Data represent mean dry root weight values (± SE, $n = 10$). Asterisks on top of the graph indicate statistical significance of 2-way ANOVA (*: $0.05 < P < 0.01$, **: $0.01 < P < 0.001$, and ***: $P < 0.001$). Asterisks and parentheses indicate statistically significant differences between mock- and WCS417-treated soils (Student's *t*-test; $P < 0.05$).

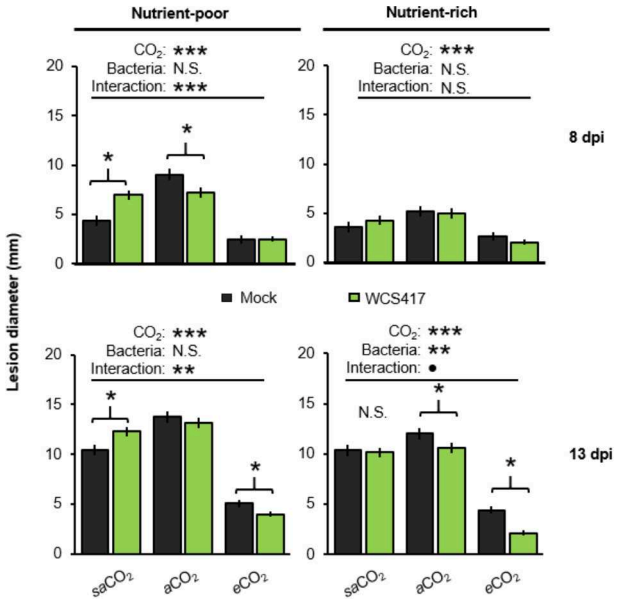


FIGURE 4 | Effects of atmospheric CO_2 and soil nutritional status on systemic resistance responses of Arabidopsis to *P. simiae* WCS417. Soil were inoculated with WCS417 (5×10^7 CFU.g^{-1} soil), or mock treated with $MgSO_4$ prior to planting. To quantify systemic resistance effects, 4-week-old plants were challenge-inoculated with *P. cucumerina* by applying 6-µL droplets of 5×10^6 spores.mL^{-1} onto 4 fully expanded leaves per plant. Data shown are mean lesion diameters (± SE, $n = 10$) at 8 and 13 days post inoculation (dpi). Asterisks on top of the graph indicate statistical significance of 2-way ANOVA (: $0.1 < P < 0.05$, *: $0.05 < P < 0.01$, **: $0.01 < P < 0.001$, and ***: $P < 0.001$). Asterisks and parentheses indicate statistical differences (Student's *t*-test; $P < 0.05$).

and establishment of rhizosphere-colonizing bacteria not only depends on primary metabolites, but also on their sensitivity to secondary metabolites. The extent to which the exudation of scopoletin, and other possible rhizosphere chemicals, are influenced by atmospheric CO_2 in Arabidopsis requires further investigation.

Rhizosphere colonization by PGPR promotes shoot and root development through different mechanisms (Lugtenberg and Kamilova, 2009). For instance, *Pseudomonas fluorescens* WCS365 has been shown to convert exuded tryptophan into the plant growth hormone auxin (Kamilova et al., 2006). In nutrient-poor soil, growth promotion by WCS417 was apparent under both *sa*CO_2 and *a*CO_2 (**Figure 3**). However, WCS417 repressed plant growth at *e*CO_2 (**Figure 3**), indicating potentially pathogenic activity. This hypothesis is supported by the colonization data (**Figure 2**), which revealed >10 fold higher colonization of WCS417 at *e*CO_2 compared to that at *a*CO_2. It is tempting to speculate that such high densities at the root surface are perceived as hostile by the host immune system, triggering a growth-repressing immune response. The continuum between mutualism and pathogenic lifestyles is a recognized phenomenon for fungal endophytes (Schulz and Boyle, 2005) and other root colonizers (Bever et al., 2012). Interestingly, this plasticity is partially driven by environmental factors, including CO_2 (Anderson et al., 2004; Schulz and Boyle, 2005). Although the relationship between plant-microbial mutualism and environmental factors remains poorly understood (Garrett et al., 2006, 2011; Johnson and Gehring, 2007), the growth repression by WCS417 at *e*CO_2 was marked by relatively high levels of resistance against *P. cucumerina* (**Figure 3**). While this resistance appears to be an additive result of ISR and *e*CO_2-induced resistance (Williams et al., 2018), it is plausible that these high levels of resistance are associated with costs to plant growth, which become apparent under nutrient-limiting conditions. ISR has been associated with priming of jasmonic acid and ethylene-controlled defenses (Pieterse

et al., 2002). Even though priming is generally considered to be a low-cost defense strategy (van Hulten et al., 2006), the additive effect of eCO_2 and ISR may result in constitutive up-regulation of inducible defenses that incur a detectable cost on plant growth under nutrient-limiting conditions. This hypothesis gains support from the observation that WCS417 only represses growth at eCO_2 in nutrient-poor soil (**Figure 3**).

Our study has shown that two well-characterized soil bacteria display different rhizosphere behavior in response to changes in atmospheric CO_2. Moreover, the plant responses to colonization by the rhizobacterial colonizing strain revealed a range of outcomes, including growth repression and induced systemic susceptibly. These findings demonstrate that predictions about impacts of global change and soil quality on crop performance need to take into account the complex interactions taking place in the rhizosphere. This outcome highlights the need for further research on the impacts of future global change on rhizosphere chemistry and the associated root microbiome.

MATERIALS AND METHODS

Plant Cultivation and Growth Conditions

Arabidopsis thaliana (Arabidopsis), accession Columbia (Col-0) was cultivated in mx flow 6000 cabinets (Sanyo, United Kingdom) under ambient conditions (aCO_2; 400 ppm, i.e., $\mu L\ L^{-1}$), sub-ambient CO_2 ($saCO_2$; 200 ppm), or elevated CO_2 (eCO_2; 1200 ppm). CO_2 concentrations were chosen specifically to reflect two aspects of global change; 200 ppm was used as a post-glacial and pre-industrial atmospheric concentration, to imitate Arabidopsis' ancestral habit (Beilstein et al., 2010; Beerling and Royer, 2011), and 1200 ppm was selected as worse case representative concentration scenario, as highlighted in the most recent intergovernmental panel on climate change report (IPCC, 2013). Growth chambers were supplemented with compressed CO_2 (BOC, United Kingdom) or scrubbed with Sofnolime 797 (AP diving, United Kingdom) to maintain constant CO_2 levels at indicated concentrations. Plants were cultivated under short-day conditions (8.5: 15.5 h light: dark; 20°C light, 18°C dark; 65% relative humidity). Seeds were stratified for 2 days (d) in the dark at 4°C and planted in 60-mL pots, containing a sand (silica CH52): dry compost (Levington M3) mixture, in a ratio of 2: 3 for nutrient-rich soil, or 1: 9 for nutrient-poor soil (v:v in both instances). Pots with plant-free control soil were set up and maintained under the same growth conditions. All pots were placed in trays to allow for bi-weekly watering. At 7 days after germination, seedlings were thinned to prevent crowding. To limit variation between different CO_2 conditions, and compensate for pseudoreplication generated via chamber effects, experiments were conducted in identical climate chamber models, the exact same batches of seed and soil were used throughout each experiment. Furthermore, plant trays within each chamber were rotated weekly in a randomized fashion to counter positional effects.

Soil Carbon (C) and Nitrogen (N) Concentrations

C and N concentrations in soil-types were determined by the complete combustion method followed by gas chromatography, using an ANCA GSL 20-20 Mass Spectrometer (Sercon PDZ Europa; Cheshire).

Soil Treatment With Pseudomonas Simiae Wcs417 and Pseudomonas Putida KT2440 and Quantification of Bacterial Colonization

To determine impacts of CO_2 on colonization of rhizosphere bacteria, yellow fluorescent protein (YFP)-expressing *P. simiae* WCS417 (Berendsen et al., 2012) was cultivated on selective Lysogeny broth (LB) agar (5 $\mu g\ mL^{-1}$ tetracycline and 25 $\mu g.mL^{-1}$ rifampicin). One YFP-fluorescent colony was selected for propagation in an overnight culture of liquid LB, containing the same selective concentrations of tetracycline and rifampicin. The medium was incubated in an orbital shaking incubator for 16 h at 28°C at 200 revolutions per minute (rpm). A similar method was employed for the cultivation of a green fluorescent protein (GFP)-expressing *P. putida* KT2440, which carries a stable chromosome-inserted $PA_{1/04/03}$-RBSII-*gfp*mut3*-T0-T1 transposon at a negligible metabolic cost (Dechesne and Bertolla, 2005). However, in this case, the bacteria were grown on minimal solid media (M9), after which one GFP-fluorescent colony was selected for propagation in LB liquid medium without selective antibiotics. Soils were inoculated with WCS417 or KT2240 bacteria by adding a bacterial suspension in 10 mM $MgSO_4$ at a final density of 5×10^7 $CFU.g^{-1}$, or a mock treatment of 10 mM $MgSO_4$ alone. Seeds were planted directly on the soil. Four weeks after germination, samples of root adhering rhizosphere soil and control soil (∼2 g) were collected, serially diluted and stamp-plated, using a 96-well Replica plater (Sigma-Aldrich, R2383) onto selective LB agar with tetracycline and rifampicin for WSC417, and M9 without antibiotics for GFP-expressing KT2240. Fluorescent colonies were enumerated using a Dark Reader DR195M Transilluminator (Clare Chemical) and normalized to sample weight. The colonization experiments (**Figure 2**) were repeated once with comparable results.

Plant Growth Analysis

To determine the size of the plants, rosette area was estimated non-destructively from digital photographs (Canon EOS 500D) of rosettes, taken with a size standard. Image analysis involved converting pixels per rosette into area (mm^2), using imaging software (Corel Paintshop Pro, ver. X7). To determine root growth, root material plus soil was collected and oven dried using an economy incubator 2 (Weiss Technik, United Kingdom; 60°C). Subsequently, roots were carefully extracted from the surrounding soil and weighed, using an analytical balance (Mettler Toledo AJ100).

Induced Systemic Resistance Assays

To quantify WCS417-mediated ISR, plants were grown in soil with and without WCS417 bacteria as described above.

After 5 weeks of growth, plants were challenge-inoculated with *P. cucumerina* (strain BMM). Lesion diameters were enumerated at 8 and 13 dpi and analyzed using Student's test ($P < 0.05$). To ensure necrotrophic infection, *P. cucumerina* was applied by droplet inoculation (6 μL, 5×10^6 spores mL^{-1}) on 4 to 6 fully expanded leaves of plants ($n = 8$), as described previously (Pétriacq et al., 2016). Disease progression was determined by quantification of lesion diameters at 8 and 13 dpi, which correlates with fungal colonization disease progression (Pétriacq et al., 2016; Williams et al., 2018). Four lesion diameters per plant were averaged and treated as one biological replicate ($n = 8$). Differences in average lesion diameter between treatments were analyzed for statistical significance by ANOVA (using R, v. 3.1.2).

AUTHOR CONTRIBUTIONS

JT and DB conceived the project. AW, PP, TC, and JT planned the experiments. AW, TC, and PP performed the experiments. JT and DB provided reagents, equipment, and facilities. AW, PP, and JT analyzed the data. AW and JT wrote the paper with feedback from all co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01493/full#supplementary-material

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Stereoisomers of the Bacterial Volatile Compound 2,3-Butanediol Differently Elicit Systemic Defense Responses of Pepper against Multiple Viruses in the Field

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The volatile compound 2,3-butanediol, which is produced by certain strains of root-associated bacteria, consists of three stereoisomers, namely, two enantiomers (2R,3R- and 2S,3S-butanediol) and one meso compound (2R,3S-butanediol). The ability of 2,3-butanediol to induce plant resistance against pathogenic fungi and bacteria has been investigated; however, little is known about its effects on induced resistance against viruses in plants. To investigate the effects of 2,3-butanediol on plant systemic defense against viruses, we evaluated the disease control capacity of each of its three stereoisomers in pepper. Specifically, we investigated the optimal concentration of 2,3-butanediol to use for disease control against *Cucumber mosaic virus* and *Tobacco mosaic virus* in the greenhouse and examined the effects of drench application of these compounds in the field. In the field trial, treatment with 2R,3R-butanediol and 2R,3S-butanediol significantly reduced the incidence of naturally occurring viruses compared with 2S,3S-butanediol and control treatments. In addition, 2R,3R-butanediol treatment induced the expression of plant defense marker genes in the salicylic acid, jasmonic acid, and ethylene signaling pathways to levels similar to those of the benzothiadiazole-treated positive control. This study reports the first field trial showing that specific stereoisomers of 2,3-butanediol trigger plant immunity against multiple viruses.

Keywords: bacterial volatile compound, 2,3-butanediol, plant viral disease, ISR, pepper

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INTRODUCTION

A wide range of plant pathogens cause diseases in crops, leading to serious losses in yields, increased labor and costs, and human health problems caused by the use of harmful chemicals (Agrios, 2005; Strange and Scott, 2005; Lamberth et al., 2013). Among plant diseases, the incidence of viral diseases has been increasing yearly due to global warming, which is causing serious problems because there are currently no practical control methods for these diseases (Mandadi and Scholthof, 2013; Guerret et al., 2016; Jones, 2017). To date, the control of viral disease has primarily involved monitoring and chemical management of vectors that mediate disease progression, as well

as virus resistance-based breeding and transgenic-based gene technology (Perring et al., 1999; Kang et al., 2005; Hashimoto et al., 2016; Khalid et al., 2017). However, the use of these technologies is limited due to environmental safety issues and the large amount of time it takes to apply them to the field (Agrios, 2005; Valkonen, 2015).

Many studies have focused on the use of induced resistance, i.e., systemic acquired resistance (SAR) and induced systemic resistance (ISR), against a variety of viruses as an alternate method for plant virus control. In particular, SAR against viral diseases in plants can be induced by weakened viruses, and plants can be protected from various viral diseases through systemic or localized viral infection (Ross, 1961; Ryals et al., 1994). However, the use of viruses to induce SAR is limited by treatment difficulties and the low success rate of inducible resistance in plants. As an alternative, microbes (pathogenic and beneficial bacteria) and chemicals have been used to induce plant resistance. However, it is difficult to apply pathogens in order to elicit plant resistance in the field, and some chemicals have significant negative effects on vegetative and generative growth (Ross, 1961; Tally et al., 1999; Kloepper et al., 2004a). Therefore, an increasing number of studies have focused on the induction of resistance in crops without perturbing their growth using plant growth-promoting rhizobacteria (PGPR) (Raupach et al., 1996; Zehnder et al., 2000; Zehnder et al., 2001; Murphy et al., 2003; Ryu et al., 2004). The application of *Pseudomonas fluorescens* CHAO, *Pseudomonas putida* 89B61, and *Serratia marcescens* 90-166 reduced the development of symptoms caused by *Tobacco necrosis virus* or *Cucumber mosaic virus* (CMV) in cucumber and tomato via eliciting ISR (Maurhofer et al., 1994; Raupach et al., 1996; Zehnder et al., 2000). Spraying of *Bacillus amyloliquefaciens* strain 5B6 (isolated from a cherry tree leaf) was recently reported to delay the development of symptoms caused by CMV in pepper (Lee and Ryu, 2016). To obtain PGPR-mediated ISR in the field, the activity and population instability of the treated bacteria are important issues. To overcome the disadvantages of microbial instability in the field, scientists have attempted to identify bacterial determinants, such as lipopolysaccharides, siderophores, and bacterial metabolites, with similar effects *in vitro* and in greenhouse experiments (Leeman et al., 1995; Duijff et al., 1997; Ongena et al., 2005; Ryu et al., 2013).

B. amyloliquefaciens strain IN937a was recently shown to stimulate systemic resistance due to the release of bacterial volatile compounds (BVCs) (Kloepper et al., 2004b; Ryu et al., 2004; Choi et al., 2014). Treatment with BVCs from PGPR reduced symptom development and CMV accumulation in pepper seedlings (Song et al., 2013; Choi et al., 2014). BVCs are important chemicals in plant virus control because they can affect plant resistance and can be applied on a wide range of scales (Kesselmeier and Staudt, 1999; Ryu et al., 2004; Kai et al., 2009).

One such BVC, 2,3-butanediol, is produced by various bacteria such as *Bacillus* spp., *Aerobacter* spp., and *Klebsiella* spp. (Barrett et al., 1983; Voloch et al., 1985; Ryu et al., 2004; Kopke et al., 2011). This compound is used in the manufacture of synthetic industrial products, aviation fuel, explosives, plasticizers, and pharmaceuticals (Gupta et al., 2005). Treatment with 2,3-butanediol has beneficial effects on plants, such as growth promotion and the induction of systemic resistance in *Arabidopsis thaliana* and tobacco (Ryu et al., 2003, 2004; Han et al., 2006). Both 2,3-butanediol and 2-butanol affect plant physiology, promote growth, and induce defense against the insect pest *Spodoptera littoralis* in maize (*Zea mays*) (D'Alessandro et al., 2014). However, little is known about the efficacy and applicability of other 2,3-butanediol isomers to plants to control plant viruses.

In this study, we evaluated the efficacy of bacterial volatiles for managing common, economically important viral diseases of pepper in South Korea under greenhouse and field conditions. Since the effect of 2,3-butanediol on induced resistance against viruses has not yet been studied, we investigated its effect on multiple viral diseases. First, we determined the optimal concentration of 2,3-butanediol to use to control CMV and *Tobacco mosaic virus* (TMV) under greenhouse conditions. In addition, we examined the effect of 2,3-butanediol isomers on induced resistance against TMV. We then examined the use of two enantiomers (2R,3R and 2S,3S-butanediol) and one meso compound (2R,3S-butanediol) in the field to control viral diseases, which were alleviated by these treatments compared with the control. We measured virus levels in plants using five representative pepper viruses in South Korea, including CMV, TMV, *Pepper mottle virus* (PepMoV), *Tomato yellow leaf curl virus* (TYLCV), and *Tomato spotted wilt virus* (TSWV), by qRT-PCR using specific primers. We confirmed the induction of plant defense systems by examining the expression of molecular markers of induced resistance, including genes related to/encoding *Capsicum annuum pathogenesis-related 4* (*CaPR4*), *Ca chitinase 2* (*CaChi2*), *Ca phenylalanine-I ammonia-lyase* (*CaPAL*), Ca*SAR8.2*, *Ca 1-aminocyclopropane-1-carboxylic acid oxidase* (*CaACC*), and *Ca proteinase inhibitor 2* (*CaPIN2*). Treatment with 2R,3R- and 2R,3S-butanediol reduced the severity of diseases caused by naturally occurring plant viruses and increased the yield of mature pepper fruits. Collectively, 2,3-butanediol treatments had different effects on inducing systemic resistance and viral suppression, depending on the isomer. This is the first report on the use of different isomers of BVCs to induce resistance to viral pathogens in pepper.

MATERIALS AND METHODS

Greenhouse Trials

The induction of resistance against CMV and TMV by 2,3-butanediol isomers (Sigma–Aldrich Corp., Korea; Lot number 237639 (2R,3R-), 300349 (2S,3S-), 361461 (2R,3S-) was investigated in the greenhouse. Pepper (*Capsicum annum* L. cv. Bukwang) seeds were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water, and then maintained on Murashige and Skoog agar medium (Duchefa, Haarlem, Netherlands) at 25°C for 3 days until germination. Germinated seeds were then transplanted into soil-less medium (Punong Horticulture Nursery Media LOW, Punong Co. Ltd., Gyeongju, Korea). Seedlings were grown in

controlled conditions at 25 ± 2°C under fluorescent light with an intensity of approximately 7,000 lux and a 12 h/12 h day/night cycle, and were then transferred to the KRIBB greenhouse facility in Daejeon, South Korea.

2,3-Butanediol was prepared at concentrations of 1, 5, and 10 mM. Four-week-old pepper seedlings were drenched with 20 mL of each diluted solution of 2,3-butanediol. Alternatively, seedlings were treated with 1 mM benzothiadiazole (BTH; Syngenta, Durham, NC, United States) and water as positive and negative controls, respectively.

CMV and TMV were maintained in *Nicotiana tabacum* by mechanical passage in a temperature-controlled greenhouse. The virus inoculum used throughout the experiments consisted of systemically infected *N. tabacum* tissue ground in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM sodium sulfite (1 g of tissue: 10 ml of buffer). All tissues were chilled prior to use and maintained on ice during inoculation.

The primary leaves were rub-inoculated with CMV or TMV 7 days after treatment with 2,3-butanediol, BTH, or water (control). 'Mock' inoculated plants were rub-inoculated with 50 mM potassium phosphate buffer. The experiment was repeated three times with 10 replications per experiment. Virus quantification was performed via qRT-PCR as described below at 2 weeks after virus inoculation.

Field Trial

The field trial was conducted in Geumsan-gun, Chungcheongnam-do, South Korea (36°35′ 32.27″North, 127°30′ 34.75″East), where plants are affected by multiple viral diseases each year, in mid-April of 2016 and 2017. For the field study, all necessary permits were obtained from the owners of private lands. Pepper seedlings (*Capsicum annum* L. cv. Bukwang) were transplanted at a distance of 30 cm. Before transplanting, the furrows were covered with black polyethylene plastic film to prevent weed problems. To test induced resistance under field conditions, 1-month-old seedlings were drenched with 100 ml per plant of 1 mM 2,3-butanediol isomers (2R,3R form, 2S,3S form, and 2R,3S form) (Sigma–Aldrich Corp., Korea) and 1 mM BTH solution three times per month. The same volume of sterilized water was used as a negative control. Each treatment was replicated five times in a completely randomized block design and consisted of 20 plants per treatment.

Measurement of Pepper Fruit Yields

To investigate whether 2,3-butanediol isomers and BTH influence pepper fruit yields under field conditions compared with the water control, yields were measured 100 days post-treatment (dpt). Red-colored fruits (only) were harvested twice from mid-August to the end of August, depending on the growing season. Total yield (g/plant) per treatment was estimated, and the total fruit weight per plant was calculated for each harvest time. In addition, the number of fruits per plant was recorded for each harvest, and the total harvest was then calculated as the number of fruits/plant.

Diagnosis of Viral Diseases

To evaluate plant virus levels in the field, 30 leaves per replication were randomly sampled 90 days after the application of 2,3-butanediol isomers and BTH. The leaves were immediately frozen in liquid nitrogen for total RNA extraction. Total RNA was isolated from the leaves using Tri reagent (Molecular Research Inc., Cincinnati, OH, United States) according to the manufacturer's instructions and as described in our previous study (Lee et al., 2017). First-strand cDNA synthesis was performed with 2 μg of DNase-treated total RNA, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase (Enzynomics, Daejeon, Korea). The qRT-PCR assays consisted of cDNA, iQ™ SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, United States), and 10 pM of each primer. The cycling parameters were as follows: initial polymerase activation for 10 min at 95°C; followed by 40 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s at 72°C. The identification of plant viruses as CMV, TMV, PepMoV, TYLCV, or TSWV was performed using the specific primer pairs shown in **Table 1**. *CaUBQ* (ubiquitin) was used as a loading control to ensure

TABLE 1 | Primer sets used for the amplification of plant viruses and expression analysis of defense-related genes in this study.

	Primer	Sequence (5′-3′)
Plant pathogenic virus	CMV CP Forward	5′-CGTTGCCGCTATCTCTGCTAT-3′
	CMV CP Reverse	5′-GGATGCTGCATACTGACAAACC-3′
	TMV Forward	5′-ATGTCTTACAGTATCACTAC-3′
	TMV Reverse	5′-TCAAGTTGCAGGACCAGAGG-3′
	PepMoV Forward	5′-CAAGCAAGGGTATGCATGT-3′
	PepMoV Reverse	5′-AAGATCAGACACATGGA-3′
	TYLCV Forward	5′-CGCCCGCCTCGAAGGTTC-3′
	TYLCV Reverse	5′-TCGTCGCTTGTTTGTGCCTTG-3′
	TSWV Forward	5′-ATGTCTAAGGTTAAGCTCAC-3′
	TSWV Reverse	5′-TCAAGCAAGTTCTGCGAGTT-3′
Plant defense-related gene	*CaUBQ Forward*	5′-GCACAAGCACAAGAAGGTTAAG-3′
	CaUBQ Reverse	5′-GCACCACACTCAGCATTAGGA-3′
	CaPR1 Forward	5′-ACTTGCAATTATGATCCACC-3′
	CaPR1 Reverse	5′-ACTCCAGTTACTGCACCATT-3′
	CaPR2 Forward	5′-TTTTAGCTATGCTGGTAATCCGCG-3′
	CaPR2 Reverse	5′-AAACCATGAGGACCAACAAAAGCG-3′
	CaPR4 Forward	5′-AACTGGGATTGAGAACTGCCAGC-3′
	CaPR4 Reverse	5′-ATCCAAGGTACATATAGAGCTTCC-3′
	CaPIN2 Forward	5′-TGGGACTTTCATTTGTGAAGGAGAG-3′
	CaPIN2 Reverse	5′-GACACAGTGAATAGGCAATATTTGG-3′
	CaPAL Forward	5′-ATTCGCGCTGCAACTAAGAT-3′
	CaPAL Reverse	5′-CACCGTGTAAGGCCTTGTTT-3′
	CaACC Forward	5′-AGTGGCCTTCAACTCCTCAA-3′
	CaACC Reverse	5′-TTCCGTTTGTGATCACCTCA-3′
	CaSAR8.2 Forward	5′-TAGTGAGACTAAGAAAGTTGGACG-3′
	CaSAR8.2 Reverse	5′-AAGAGTGCATGCAGTATCACAAAG-3′
	CaChi2 Forward	5′-ATTGGACGATGGAAGCCATCACCAG-3′
	CaChi2 Reverse	5′-ATATTCCGAATGTCTAAAGTGGTAC-3′
	CaActin Forward	5′-CACTGAAGCACCCTTGAACCC-3′
	CaActin Reverse	5′-GAGACAACACCGCCTGAATAGC-3′

that equal amounts of RNA were used in each assay. Relative transcript levels were normalized with respect to *CaUBQ* mRNA levels and calculated using the $2^{-\Delta\Delta C_T}$ method. Standard error of mean values among replicates were calculated using Bio-Rad manager (version 2.1) (Bio-Rad CFX Connect).

Assessment of Defense-Priming Gene Expression

To analyze the expression of defense-priming genes, the expression of candidate priming genes was analyzed using the primers shown in **Table 1** (Yang et al., 2009, 2011; Song et al., 2013). The expression of defense-priming genes in pepper leaves was analyzed using the same leaves sample as the assay of viral disease. As a control to ensure that equal amounts of RNA were analyzed in each experiment, relative RNA levels were calibrated and normalized to the level of *CaActin* mRNA (GenBank accession no. AY572427).

Statistical Analysis

The experimental data sets were subjected to analysis of variance using JMP software ver. 4.0 (SAS Institute Inc., Cary, NC, United States[1]). The significance of differences among treatments was determined based on the magnitude of the *F* value at $P = 0.05$. When a significant *F* value was obtained for the treatments, separation of means was accomplished using Fisher's protected least-significant difference (LSD) at $P = 0.05$.

RESULTS

Optimization of 2,3-Butanediol Levels to Elicit Induced Resistance under Greenhouse Conditions

In our previous study (Choi et al., 2014), we found that the use of 1 mM 2,3-butanediol reduced the severity of bacterial spot caused by *Xanthomoas axonopodis* pv. *vesicatora* in pepper. However, it was not clear whether this compound induces resistance activity against plant viral pathogens. To evaluate whether 2,3-butanediol induces plant immunity against CMV and TMV under greenhouse conditions, we quantified the viruses 2 weeks after infection in the greenhouse. After TMV inoculation, we observed yellowing caused by the virus in the leaves of the control plants, whereas no symptoms were observed in the leaves of plants under 2,3-butanediol treatment (**Figure 1A**). In addition, the quantitative value of the virus (i.e., the relative gene expression level compared with *CaUBQ*) in the 2,3-butanediol treatment group was 0.1, which was dramatically less than that of the control, at 16.25 (**Figure 1B**). Furthermore, to determine the minimum concentration of 2,3-butanediol needed, we examined the effect of 10, 5, and 1 mM 2,3-butanediol on CMV and TMV. Based on the quantification of CMV, there was no statistically significant difference among the 10, 5, and 1 mM 2,3-butanediol treatments, with relative gene expression levels of 1.07, 1.67, and 1.69, respectively; these values were three times less than the control (3.45) under all treatments. CMV levels were lowest after BTH treatment, at 0.63 (**Figure 1B**). The relative gene expression levels of TMV were 0.52, 0.54, and 1.06 under 10, 5, and 1 mM 2,3-butanediol treatment, respectively. Therefore, the concentration of virus was highest under 1 mM 2,3-butanediol treatment, but there was no statistically significant difference among treatments. Under all treatments, CMV levels were significantly lower than those in the control. Under BTH treatment, the relative gene expression level of CMV was 0.68, which was not significantly different from that detected under 1 mM 2,3-butanediol treatment, the lowest concentration of this compound tested (**Figure 1B**). Therefore, the minimum effective concentration of 2,3-butanediol was 1 mM ($P < 0.05$, $n = 10$), which was used in subsequent experiments.

Assessment of the Protective Effects of 2,3-Butanediol against Viral Diseases under Field Conditions

To evaluate virus resistance under field conditions in 2016, we treated field-grown pepper plants with 2,3-butanediol three times at 1 month intervals. We sampled leaves at 90 days after planting and performed quantitative analysis of five viruses: CMV, PepMoV, TMV, TSWV, and TYLCV. In the water-treated control, the five viruses (CMV, PepMoV, TMV, TSWV, and TYLCV) were detected at levels of 1.00, 5.38, 4.87, 5.38, and 6.53, respectively. Plants treated with 2,3-butanediol had viral levels of 0.41, 0.06, and 0.00 for CMV, TMV, and TYLCV, respectively, which did not significantly differ from the values obtained under BTH treatment (0.33, 0.36, and 0.06, respectively). Under 2,3-butanediol treatment, PepMoV and TSWV were detected at levels of 0.53 and 0.00, respectively, which were lower than the levels detected under BTH treatment (3.08 and 1.36, respectively). Therefore, 2,3-butanediol can be successfully used to control various viruses under field conditions (**Figures 2A,B**).

To investigate the effect of 2,3-butanediol on red pepper fruit yields as well as the ability of the treatment to control viruses, we examined fruit yield in 2017. For the yield measurements, only red pepper was harvested in the second round, and the weight and number of fruits were measured. In the field experiment, the number of fruits did not significantly differ between the treatments; however, fruit weight per plant was 113.7 g upon 2,3-butanediol treatment, an increase of 1.7-fold compared with the control value of 67.2 g (**Figure 2C**).

Previous studies showed that viral suppression in plants is caused by the immune response (Mandadi and Scholthof, 2013). Therefore, in the current study, we investigated the mechanism underlying viral suppression in response to 2,3-butanediol treatment by examining the expression of genes related to jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) biosynthesis and signaling, as these plant hormones play a central role in the regulation of plant immune responses. The specific genes in the JA, SA, and ET pathways that we examined were *CaPAL* (SA), *CaPIN2* (JA), *CaChi2* (JA), *CaSAR8.2* (SA), *CaACC* (ET), and *CaPR4* (SA and JA). The relative expression levels of these genes in response to 2,3-butanediol treatment

[1]www.sas.com

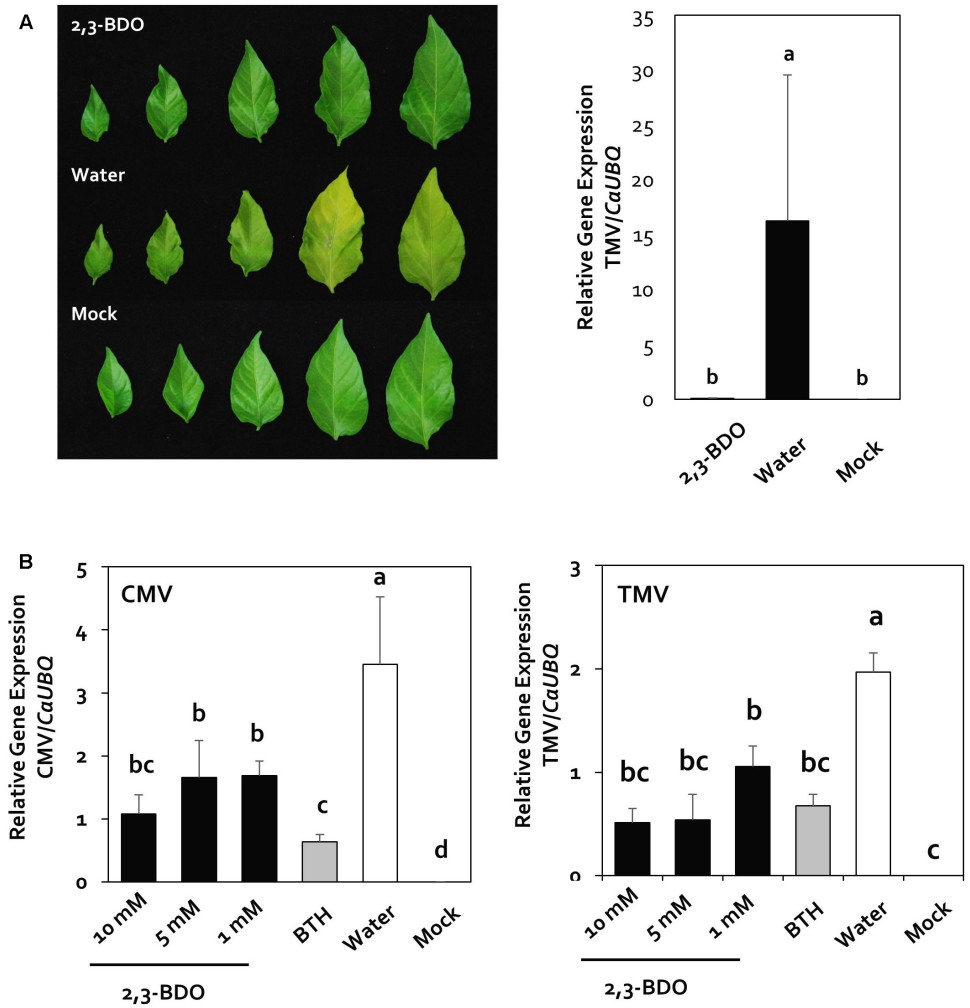


FIGURE 1 | Disease suppression of rub-inoculated viruses by drench application of 2,3-butanediol (2,3-BDO). **(A)** Disease symptoms caused by rub-inoculated *Cucumber mosaic virus* (CMV) were evaluated 2 weeks post-inoculation, and the induction of resistance against *Tobacco mosaic virus* (TMV) by 2,3-BDO was validated in the greenhouse. **(B)** Validation of the induction of resistance against CMV and TMV by treatment with three concentrations of 2,3-BDO in the greenhouse. Expression of viral-specific genes was measured 2 weeks after treatment of pepper plants with 1, 5, and 10 mM 2,3-BDO, 1 mM benzothiadiazole (BTH), and water. Bars represent mean ± SEM (n = 10). The housekeeping gene *CaUBQ* was used as a reference in qPCR.

(compared with *CaActin*) were 1.46, 6.98, 1.00, 0.69, 0.66, and 0.41, with increases of 46.4-, 3.3-, 10-, 5.2-, 1.7-, and 2.8-fold, respectively, compared with the control (**Figure 3**). In particular, *CaPIN2* and *CaPR4* were more highly upregulated by 2,3-butanediol than by BTH treatment (**Figure 3**). Taken together, these results indicate that 2,3-butanediol induces the expression of plant defense marker genes in pepper, which could be associated with the induction of three major defense hormone signaling pathways, the SA, ET, and JA pathways, in these plants.

Effect of 2,3-Butanediol Isomers on Induced Resistance against Naturally Occurring Viral Diseases

In the natural ecosystem, microorganisms can produce three 2,3-butanediol isomers, i.e., the R form (2R,3R-butanediol), S form (2S,3S-butanediol), and meso form (2R,3S-butanediol) depending on the species (Herold et al., 1995; Celińska and Grajek, 2009; Ji et al., 2011; Kandasamy et al., 2016). After the first year of greenhouse and field trials, we investigated whether the different isomers of 2,3-butanediol affect ISR. Treatment with 2,3-butanediol has an effect on virus control through increasing plant immunity. Therefore, we investigated the effects of the other isoforms of 2,3-butanediol in a second field experiment in 2016. We performed a quantitative assay of pepper viruses using the same five viruses as in the first field experiment but failed to detect TYLCV in the second field experiment. Treatment with 2R,3R-butanediol (R form) led to a 2.8-, 689.7-, 122.9-, and 25.9-fold decrease in CMV, TMV, PepMoV, and TSWV levels, respectively. Compared with 2R,3R-butanediol, the effect of 2R,3S-butanediol (meso form) treatment on these four viruses did not significantly differ from that of treatment

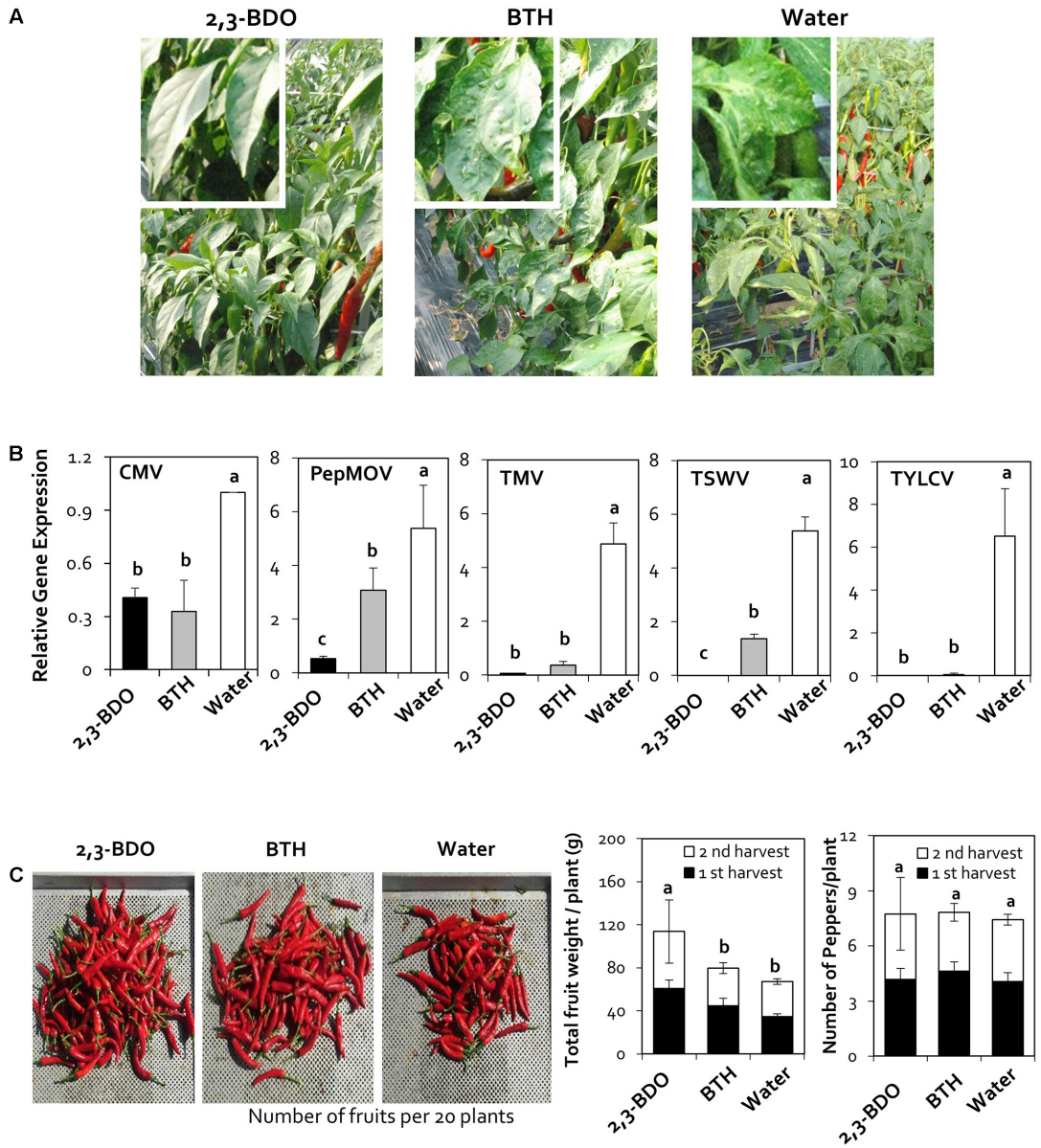


FIGURE 2 | Disease suppression of naturally occurring viruses by treatment with 2,3-butanediol (2,3-BDO). **(A)** Disease symptoms caused by naturally occurring viruses were evaluated at 90 days post-transplantation (dpt). **(B)** The induction of resistance against *Cucumber mosaic virus* (CMV), *Pepper mottle virus* (PepMoV), *Tobacco mosaic virus* (TMV), *Tomato spotted wilt virus* (TSWV), and *Tomato yellow leaf curl virus* (TYLCV) by application of 2,3-BDO was evaluated at 90 dpt in the field. Bars represent mean ± SEM (n = 100). The housekeeping gene *CaUBQ* was used as a reference in qPCR. **(C)** Fruit fresh weight per plant and the fruit yield of 20 plants treated with 2,3-BDO, benzothiadiazole (BTH), and water were assessed at 100 dpt. Different letters indicate statistically significant differences (P = 0.05). Error bars represent mean ± SEM.

with the other forms of 2,3-butanediol and BTH (**Figure 4A**). Plants treated with 2S,3S-butanediol (S form) showed a 9.2-fold decrease in TSWV levels compared with the control, which was not significantly different from the other treatments. However, under this treatment, the levels of CMV, TMV, and PepMoV were reduced by only 1.3-, 1.4-, and 1.7-fold, making 2S,3S-butanediol less effective than the other forms of 2,3-butanediol and BTH (**Figure 4A**). Therefore, the effects of the R form and meso form of 2,3-butanediol were similar to that of BTH treatment, whereas the S form of 2,3-butanediol had less of an effect than the other isomers.

To confirm the effects of these compounds, we investigated the expression of the plant immunity genes by qRT-PCR. Under 2R,3R-butanediol treatment, *CaPIN2*, *CaPR1*, *CaChi2*, *CaPAL*, *CaSAR8.2*, and *CaPR4* were upregulated 2.9-, 15.7-, 2.1-, 2.4-, 54.8-, and 1.7-fold, respectively, compared with water treatment (**Figure 5**). Under 2R,3R-butanediol treatment, the expression levels of these six genes were the highest

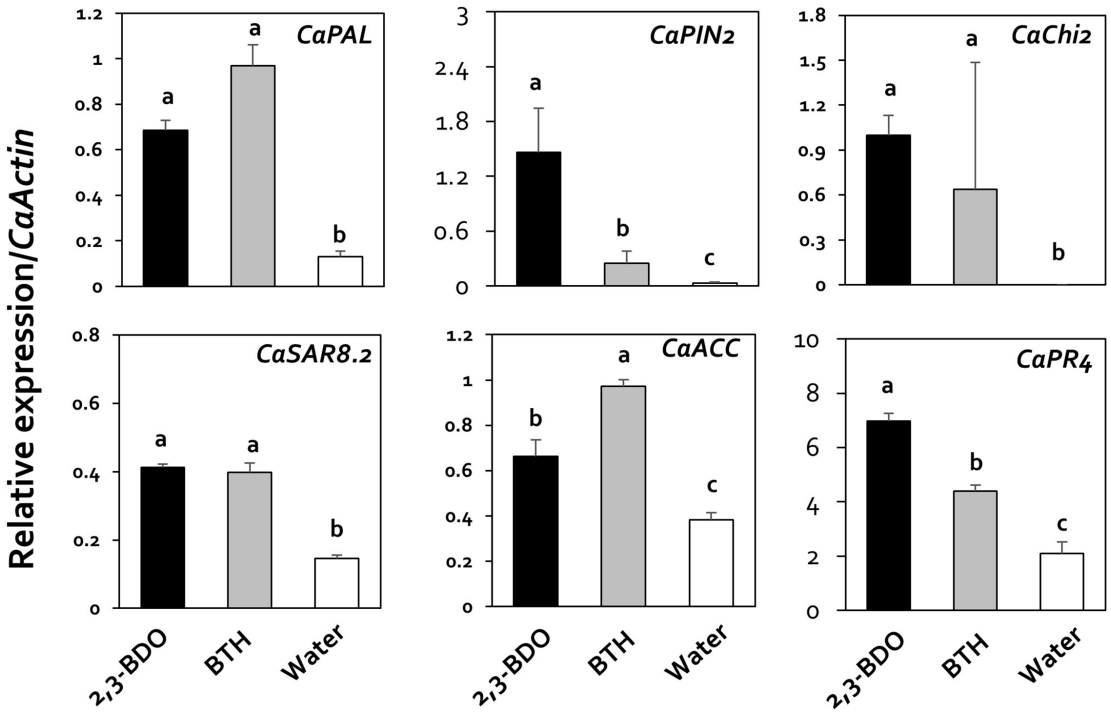


FIGURE 3 | Induction of pepper defense-related gene expression by 2,3-butanediol under field conditions. Quantification of the expression of marker genes of the salicylic acid (*CaPAL*, *CaPR4,* and *CaSAR8.2*), ethylene (*CaACC*), and jasmonic acid (*CaPIN2*, *CaPR4*, and *CaChi2*) signaling pathways in plants treated with 1 mM 2,3-butanediol, 1 mM benzothiadiazole (BTH), and water. The housekeeping gene *CaActin* was used for normalization. Different letters within panels indicate significant differences between treatments according to LSD at a significance level of $P < 0.05$. Error bars represent mean ± SEM. Sample size: $n = 100$ plants per treatment in 2016.

among 2,3-butanediol isomer treatments, with no significant difference from BTH treatment (**Figure 5**). The expression levels of *CaPAL* and *CaSAR8.2* under 2R,3S-butanediol treatment were 0.7 and 0.2, respectively, i.e., 1.7- and 12.5-times that of the water-treated control. However, these levels were lower than those measured under 2R,3R-butanediol and BTH treatments (**Figure 5**). By contrast, in plants treated with 2S,3S-butanediol, the expression levels of these genes did not significantly differ from those detected in the water-treated control (**Figure 5**).

Therefore, the decrease in virus levels detected under 2R,3R-butanediol and BTH treatment was confirmed by the expression patterns of the plant defense genes.

Measurement of Pepper Fruit Yields

To investigate the effects of 2,3-butanediol isomers on red pepper fruit yields as well as the ability of the treatment to control viruses, we examined fruit yield in 2017. For the yield measurements, only red pepper was harvested in the second round, and the weight and number of fruits were measured. When we measured the yield of red pepper fruit in response to isomer treatment in the field in 2017, the number of fruits per plant was 7, 6, and 7 per plant for plants treated with the R, S, and meso form of 2,3-butanediol, respectively, which was significantly higher than that of the BTH-treated group (3) and the control (4). The fruit weight was 116.8, 94.7, and 117 g in plants treated with the R, S, and meso forms of 2,3-butanediol, respectively, which was significantly higher than in plants treated with the BTH (40 g) and control (66 g) treatments (**Figure 4B**).

Based on the results of the 2 year field experiments, the R form of 2,3-butanediol is effective in controlling plant viral diseases and increasing the yields of red pepper fruit. These results are important for the practical application of volatile substances produced by microorganisms under field conditions. In addition, this is the first report that different 2,3-butanediol isomers have different effects on the control of plant viruses under field conditions.

DISCUSSION

Our study demonstrates that drench application of the BVC 2,3-butanediol to roots induces plant systemic defense responses in pepper resulting in the reduced accumulation of viruses in leaves. In addition, we found that treating plants with different isomeric compounds of 2,3-butanediol had different effects on inducing plant immunity, depending on the structure of the bacterial-derived volatile substance. This is the first study showing that 2,3-butanediol controls plant viral diseases and the plant's immune response is dependent on the presence of specific 2,3-butanediol isomers. The results are based on the quantification of plant

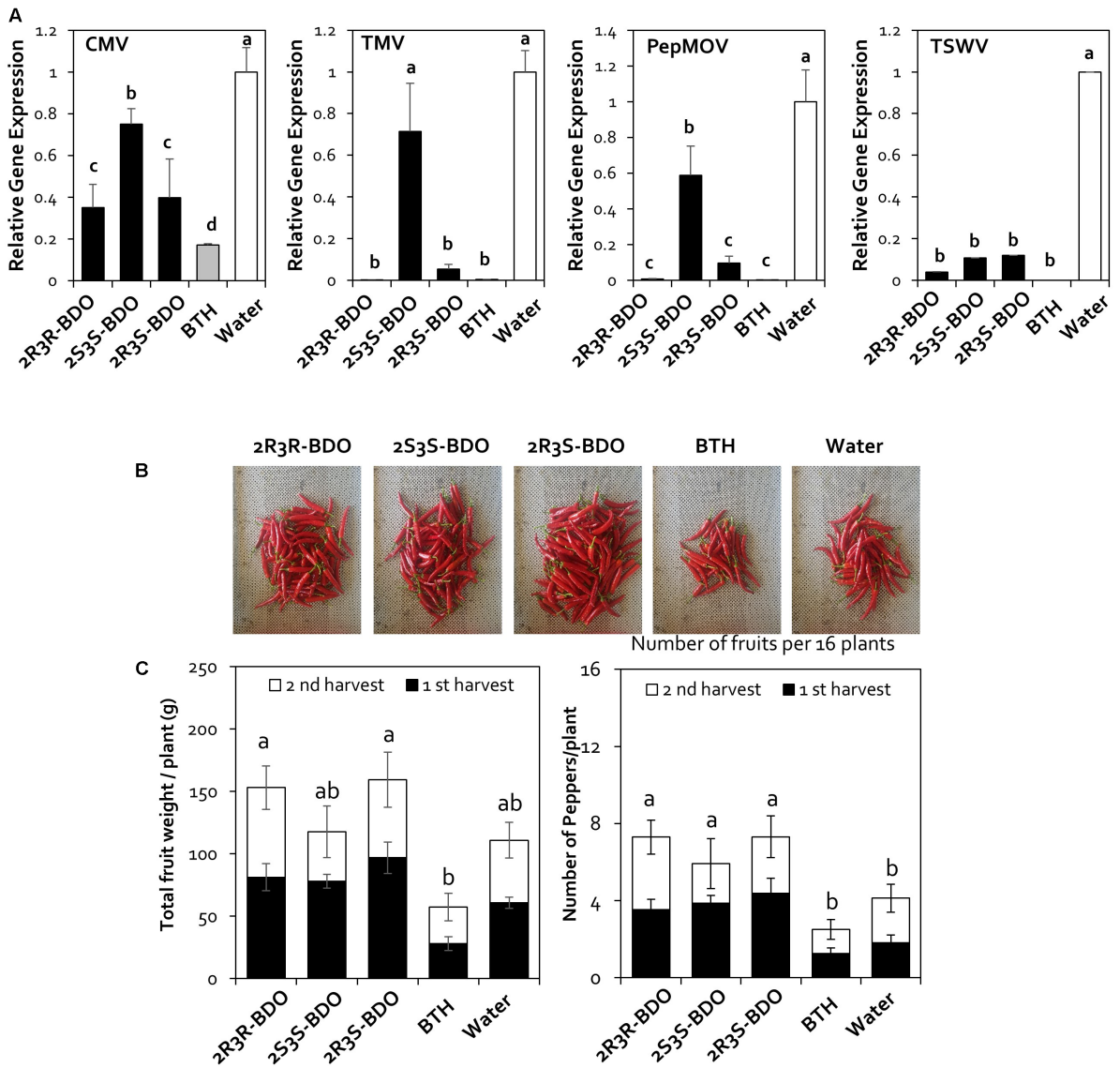


FIGURE 4 | Disease suppression of naturally occurring viruses by treatment with 2,3-butanediol (2,3-BDO) isomers. **(A)** Disease symptoms caused by naturally occurring viruses were evaluated at 90 days post-transplantation (dpt). **(B)** The induction of resistance against *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Pepper mottle virus* (PepMoV), and *Tomato spotted wilt virus* (TSWV) by application of each 2,3-BDO isomer (2R,3R-, 2R,3S-, and 2S,3S-butanediol) was evaluated at 90 dpt in the field. Bars represent mean ± SEM (n = 100). The housekeeping gene *CaUBQ* was used as a reference in qPCR. **(C)** Fruit fresh weight per plant and the fruit yield of 16 plants treated with 2,3-BDO, benzothiadiazole (BTH), and water were assessed at 100 dpt. Different letters indicate statistically significant differences ($P < 0.05$). Error bars represent mean ± SEM.

defense gene expression through qRT-PCR. Therefore, 2,3-butanediol isomers can potentially be used as a bioprotectant of plants against viruses under field conditions.

Pepper fruit is an important commercial vegetable product. Viral diseases have recently been reducing the yield and quality of red peppers yearly in South Korea. Under natural field conditions, various plant viruses infect pepper, such as CMV, TMV, TSWV, PepMoV, and TYLCV. TSWV, PepMoV, and TYLCV occur sporadically, whereas CMV and TMV are the most important red pepper viruses. CMV, PepMoV, and TMV belong to group IV (viruses with positive sense single-stranded RNA); the majority of plant viruses are included in this group. TSWV belongs to group V (viruses with negative sense single-stranded RNA), and TYLCV belongs to group II (viruses with single-stranded DNA). In field experiments, different viral diseases may occur depending on the abiotic conditions (topography, climate, and soil type). Plant viruses depend on insect vectors for their survival, transmission, and spread. Consequently, changes in insect vectors affect the occurrence of viral diseases.

The effectiveness of many bacteria in controlling viral diseases has been demonstrated (Ryu et al., 2004; Dashti et al., 2012). We recently evaluated the effects of leaf-colonizing

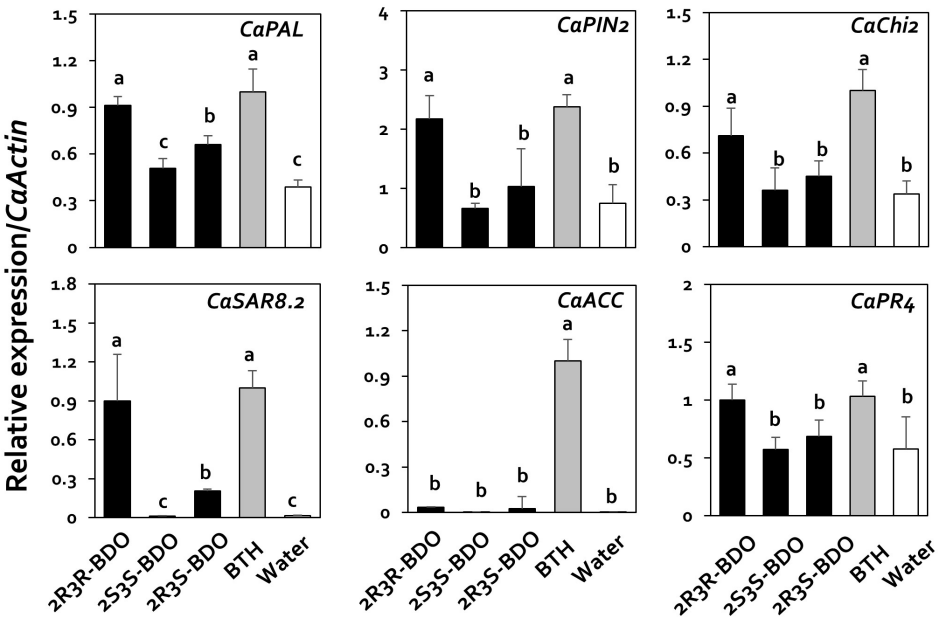


FIGURE 5 | Induction of pepper defense-related gene expression by 2,3-butanediol isomers under field conditions. Quantification of the expression of marker genes of the salicylic acid (*CaPAL*, *CaPR4*, and *CaSAR8.2*), ethylene (*CaACC*), and jasmonic acid (*CaPIN2*, *CaPR4*, and *CaChi2*) signaling pathways in plants treated with 1 mM of each 2,3-butanediol isomer (2R,3R-, 2R,3S-, and 2S,3S-butanediol), 1 mM benzothiadiazole (BTH), and water. The housekeeping gene *CaActin* was used for normalization. Different letters within panels indicate significant differences between treatments according to LSD at a significance level of $P < 0.05$. Error bars represent mean ± SEM. Sample size: $n = 100$ plants per treatment in 2017.

B. amyloliquefaciens strain 5B6 in protecting crop plants against CMV in pepper (Lee and Ryu, 2016). Although bacterial isolates are effective in controlling viral diseases, their application in the field is limited due to the decreased survival and activity of introduced bacteria in the natural environment (Vejan et al., 2016). Therefore, the inhibition of plant viral diseases using active substances produced by bacteria has been proposed, and BVCs released from bacteria have been shown to promote growth and induce defense responses in the host plant (Bailly and Weisskopf, 2012; Yi et al., 2016). However, the effect of 2,3-butanediol on plant viral disease has not previously been elucidated. In this study, we investigated the effects of soil application of 2,3-butanediol on protecting plants from various viruses. We determined the optimal concentration of 2,3-butanediol for field application through analysis in the greenhouse and monitored the reduction in virus levels in the field. The qRT-PCR assay showed that viral accumulation continuously decreased in 2,3-butanediol-treated plants in both the greenhouse and field (**Figures 1**, **2**). The levels of TYLCV, TSWV, and PepMoV also decreased under 2,3-butanediol treatment, suggesting that 2,3-butanediol could be used as a commercial formulation for the biological control of viral diseases.

The direct inhibition of virus accumulation in plants by 2,3-butanediol treatment might be due to its effect on the induction of ISR in plants. Indeed, the inhibition of viral disease through induced resistance mediated by bacteria and bacterial metabolites in plants has been reported (Murphy et al., 2003). The effect of 2,3-butanediol treatment appears to be dependent on SA, JA, and ET for ISR induction under field conditions (**Figure 3**). These results suggest that the reduction in viral infection by 2,3-butanediol treatment is not due to its antiviral effect against viruses but is instead due to its direct effect on increasing plant resistance to overall viral diseases through the induction of plant immunity. Similarly, the direct effect of 2,3-butanediol on pepper roots was reflected by increases in plant defense gene expression. Indeed, *CaPAL*, *CaSAR8.2*, *CaACC*, and *CaPR2* were previously shown to be upregulated in plants whose roots were drenched with 2,3-butanediol (Yi et al., 2016). However, 2,3-butanediol has not previously been shown to protect plants from viral diseases, although treatment with the bacterial volatile derivative 3-pentanol was found to mitigate the severity of disease in pepper caused by *Xanthomonas axonopodis* and naturally occurring CMV. RT-PCR analysis showed that this treatment increased the expression of defense-related genes involved in the SA, JA, and ET signaling pathways (Choi et al., 2014). The suppression of plant viral diseases by both 2,3-butanediol and 3-pentanol treatment suggests that additional BVCs with this effect might also be discovered.

All three isomeric forms of 2,3-butanediol are reportedly produced by specific types of bacteria. Therefore, using field experiments, we confirmed that the plant defense response against plant viruses likely occurs in an isomer-dependent manner. Specifically, in plants treated with the R form and meso form of 2,3-butanediol, the accumulation of viruses continuously decreased, as was observed in the other field experiment using mixed isomers (**Figure 4**). However, in plants treated with the S form of this compound, there was no significant difference in viral levels compared with the control.

The mechanism underlying the induction of plant resistance by specific 2,3-butanediol isomers is unclear. Furthermore, we examined the expression levels of *CaPR4*, *CaPIN2*, *CaPAL*, *CaACC*, *CaSAR8.2*, and *CaChi2* to directly determine the effects of the 2,3-butanediol isomers on inducing plant immunity. We found that plant defense-related genes were induced only by 2R,3R-butanediol (**Figure 5**). Indeed, the stereochemistry of 2R,3R-butanediol is important for ISR in tobacco to *Erwinia carotovora* subsp. *carotovora* SCC1, as 2S,3S-butanediol did not activate plant resistance to this pathogen (Han et al., 2006). These findings suggest that the induction levels of plant defense responses differ depending on the isomers of the BVCs utilized.

The isomer-dependent activity of 2,3-butanediol in plants could be due to three underlying mechanisms. First, the 2,3-butanediol isomers investigated in this study are enriched in bacteria that produce similar isomers in the rhizosphere. Indeed, 2,3-butanediol is produced by various bacteria including *B. amyloliquefaciens* (Celińska and Grajek, 2009), *Bacillus subtilis* (Xiao and Xu, 2007), *Enterobacter aerogenes* (Byun et al., 1994), *Klebsiella pneumoniae* (Biebl et al., 1998), *Klebsiella oxytoca* (Syu, 2001), *Lactococcus lactis* (Hugenholtz and Starrenburg, 1992), *Paenibacillus polymyxa* (De Mas et al., 1988), and *S. marcescens* (Zhang et al., 2010). Soil bacteria and fungi synthesize the three stereoisomers (2R,3R-, 2S,3S-, and 2R,3S-) of 2,3-butanediol from pyruvate via a dehydrogenation and oxidation step (Wang et al., 2014; Kandasamy et al., 2016; Yi et al., 2016). While the exact role played by 2,3-butanediol between the bacteria and plant host is largely unknown, the production of various 2,3-butanediol isomers, depending on the bacteria, suggests that they might be used for bacterial interactions. For example, 2,3-butanediol from *B. subtilis* negatively regulates *RsmA*, encoding a post-transcriptional regulator of virulence factors, in *P. carotovorum* subsp. *carotovorum* (Kõiv et al., 2013; Broberg et al., 2014). Therefore, 2R,3R- and 2R,3S-butanediol, which are mainly produced by *Bacillus* spp., are likely enriched in *Bacillus* spp. producing the same isomer in the rhizosphere. Specific strains of *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* elicit significant reductions in disease levels in various hosts (Kloepper et al., 2004b). The number of whitefly nymphs that transmit *Tomato mottle virus* (ToMoV) was significantly reduced by treatment with *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b, and significant reductions in the severity of ToMoV and in disease incidence resulted from *B. subtilis* IN937b and *B. pumilus* SE34 treatment (Murphy et al., 2000).

Second, 2,3-butanediol has direct effects on the host plant, depending on the isomer used. Treatment with 2,3-butanediol directly affects plant immunity. Treatment with the 2,3-butanediol production mutant *B. subtilis* failed to stimulate plant defense responses, whereas treatment with wild-type *B. subtilis* successfully induced plant defense responses against bacterial pathogens, indicating that 2,3-butanediol plays a direct role in plant immunity (Ryu et al., 2004; Rudrappa et al., 2010). The different effects of the three 2,3-butanediol isomers on pepper indicate that plants have distinct receptors for each of these isomers. Consistent with our data, only 2R,3R-butanediol effectively improves plant growth and resistance to *P. carotovorum* subsp. *carotovorum* in *Nicotiana*, suggesting that plants have specific receptors for this isomer (Han et al., 2006). BVCs from *B. subtillis* GB03 do not promote the growth of *Arabidopsis thaliana* with mutated *cre1*, which encodes a cytokinin receptor (Ryu et al., 2003). This suggests that plant receptors play a role in the recognition of bacterial BVCs. BVCs may be recognized by ET receptors in plants. Indeed, it has been proposed that BVCs are recognized by ETR1-like ET receptors (Bailly and Weisskopf, 2012). However, we do not have any evidence that CRE1 can physically bind to bacterial volatiles such as 2,3-butanediol. Plants recognize bacteria by detecting microbe-associated molecular patterns via membrane-localized receptor kinases. Currently, receptor signaling is best understood at the genetic level. Indeed, genetic analyses demonstrated that ET receptor signaling negatively regulates ET responses (Hua et al., 1998). Specifically, this is achieved via receptor activation of CONSTITUTIVE RESPONSE1 (CTR1) protein kinase, which represses ET signaling mediated by ETHYLENE INSENSITIVE2 (EIN2). This mechanism was demonstrated by the findings that mutants with null mutations in multiple ET receptor genes and *ctr1* loss-of-function mutations display similar ET responses, whereas those with gain-of-function receptor mutations are insensitive to ET (Hua et al., 1998). Isomer-specific receptors in plants should be investigated based on the results of this and previous plant receptor studies.

Third, 2,3-butanediol might reduce the incidence of viral diseases in plants by providing an alkaline environment that protects bacterial cells from unfavorable acidic conditions (Yoon and Mekalanos, 2006; Pradhan et al., 2010; Bari et al., 2011). In addition, this treatment increases bacterial robustness against harmful compounds, such as root exudates. Furthermore, root exudates from 2,3-butanediol-treated pepper exhibited selective antagonism against *Ralstonia solanacearum*. By contrast, application of 2,3-butanediol-elicited root exudate increases robustness of the PGPR *Bacillus subtilis* GB03 and the saprophyte *Pseudomonas protegens* Pf-5 (Yi et al., 2016). These findings suggest that 2,3-butanediol increases the robustness of the acidic rhizosphere environment (Hinsinger et al., 2003; Huang and Chen, 2003). The increased robustness of the bacteria may be dependent on the activities of particular 2,3-butanediol isomers, which have different effects on the induction of ISR in plants. Based on these three possible mechanisms, the effects of 2,3-butanediol on plant immune responses can be explained not only by its direct effect on rhizosphere bacteria, but also by the direct induction of plant resistance and the changes in rhizosphere conditions such as plant root exudate.

CONCLUSION

This is the first report demonstrating that 2,3-butanediol protects pepper plants from viral diseases under field conditions. In particular, through field experiments, we confirmed that different isomers of 2,3-butanediol have different effects on controlling plant viruses via the induction of plant immunity. Active compounds derived from bacteria (such as 2,3-butanediol) could be highly valuable for industrial applications. Despite the

efficacy of 2,3-butanediol in protecting plants from viral diseases, the limitation of formulation technology to control volatility currently poses a challenge for the commercialization of 2,3-butanediol. However, further experiments are needed to assess the mechanism underlying the induction of ISR and the effects of the rhizosphere environment on a particular 2,3-butanediol isomer.

AUTHOR CONTRIBUTIONS

C-MR designed the study. HK, TS, and TK performed all the experiments. TS and TK helped with the *in vitro* experiments and *in vivo* treatments. C-MR contributed to scientific discussions that guided the project. C-MR and HK wrote the paper.

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REVIEW
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Challenges and Approaches in Microbiome Research: From Fundamental to Applied

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We face major agricultural challenges that remain a threat for global food security. Soil microbes harbor enormous potentials to provide sustainable and economically favorable solutions that could introduce novel approaches to improve agricultural practices and, hence, crop productivity. In this review we give an overview regarding the current state-of-the-art of microbiome research by discussing new technologies and approaches. We also provide insights into fundamental microbiome research that aim to provide a deeper understanding of the dynamics within microbial communities, as well as their interactions with different plant hosts and the environment. We aim to connect all these approaches with potential applications and reflect how we can use microbial communities in modern agricultural systems to realize a more customized and sustainable use of valuable resources (e.g., soil).

Keywords: microbial community, root interactions, cropping systems, gnotobiotic, omics, microbial function

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INTRODUCTION

Soil is considered as one of the most diverse habitats on Earth containing billions of bacteria and millions of fungi (comprising thousands of taxa), as well as larger organisms such as nematodes, ants, or moles (Bardgett and van der Putten, 2014). Recent advances in high throughput sequencing techniques and the increasing number of microbial culture libraries are contributing to show an expanded version of the tree of life dominated by bacterial diversification (Hug et al., 2016). This enormous diversity is driven by the ability of microbes to perform lateral gene transfer across disparate phylogenetic groups (McDonald and Currie, 2017). Moreover, microbial communities are built on high numbers of individuals for each species (Robbins et al., 2016), that can quickly proliferate and have high mutation rates (in the range of 10^{-4} in *E. coli*) (Kibota and Lynch, 1996; Boe et al., 2000; Denamur and Matic, 2006) as compared to higher organisms like humans [10^{-8}] (Kuroki et al., 2006; Xue et al., 2015). These characteristics increase the diversification of microbes and microbial communities, where individual microbes of the same species could potentially bear different genetic endowments and thus functional characteristics.

Soil microbes play key roles in the cycling of nutrients such as nitrogen or phosphorus as well as providing plant protection against biotic and abiotic stress (Bender et al., 2016; Lladó et al., 2017). Intensive agriculture has contributed to increases in crop yields but at the same time it has had detrimental effects on the physical and biological properties of soils (Pimentel et al., 1995; Bouwman et al., 2009). In intensively managed agricultural systems, the application of fertilizers can compensate for a loss of soil fertility, while tillage disrupts microbial communities (Johnson et al., 1997). This is particularly relevant in the light of current crop production systems with the degradation of more than one half of the global agricultural land while we face massive

challenges associated with the disturbance of nitrogen and phosphorous cycles. This situation is very likely to worsen under the prospect of the climate change (Rockström et al., 2009; Yuan et al., 2018). As a consequence the United Nations has suggested the re-introduction of sustainable land management practices to minimize land degradation (Sanz, 2017). These practices include crop diversification, use of local adapted species or intercropping in order to maintain soil fertility, carbon sequestration, and nutrient cycling as well as to control soil erosion (Sanz, 2017). Interestingly, these procedures also enhance general soil disease suppression (Weller et al., 2002; Bonilla et al., 2015). In addition, sustaining microbial community diversity, structure and composition can help to support ecosystem functions, e.g., by regulating nutrient cycles.

During the last decade, microbiome research has modified our perception on the complexity and structure of microbial communities. However, we are only just starting to understand the organization of such complex communities, the interdependencies among themselves and with the biotic (e.g., plant) and abiotic (e.g., edaphic) environment. The increasing need for alternative experimental approaches, as well as the development of new tools has provided new insights into our understanding of the dynamics that occur within the microbiomes and their interaction with host organisms (Goodrich et al., 2017). In studying the human microbiome, the complexity of microbial interactions and the importance of analyzing them separately for each individual has already resulted in novel therapies. Considering the unique microbiome signature of each host, we could move toward a personalized application of microbiome, where we would be able to handle each case independently and better tailor the microbiome to the host's needs, thus increasing the efficiency of the treatment and the potential of the host (Human Microbiome Project Consortium, 2012). Such "personalized" microbiome approaches would be particularly facilitated by the genetic uniformity of host genotypes of a given crop plant species in the field. Similar to human microbiome studies, there have been efforts to understand the complexity of soil and plant microbiomes (Bulgarelli et al., 2012; Lundberg et al., 2012) and to fuel new innovations in sustainable crop production as part of the next green revolution (Jez et al., 2016). However, to exploit the full potential of microbiomes, we require the development of new analytical strategies to comprehend the array of functional capabilities of microbial communities (Bashiardes et al., 2018). The importance of maintaining a diverse and well-balanced microbiome at the plant–soil interface is vital in crop production. Any microbiome applications, however, have to focus on improving key determinants of crop production such as nutrient availability, soil fertility and soil health (Syed Ab Rahman et al., 2018). In this respect, the key challenge is to transfer lab-generated knowledge to the field. In addition to unraveling the structure of the plant/soil microbiome (Schlaeppi and Bulgarelli, 2015), it especially requires us to connect microbial community dynamics with microbiome functioning (Sánchez-Cañizares et al., 2017). In this review we present the challenges and latest efforts that have been made in order to advance our understanding of the different dimensions of microbiomes (e.g., structure, dynamics) and how it affects plants. We further introduce future approaches to access the full potential of the soil microbiome, including beneficial microbes, in improving crop production.

THE EXPANSION OF MICROBIOME RESEARCH IN THE "OMICS" ERA

The reduction in sequencing costs in addition to advances in sequencing technologies and increased computational power has facilitated an overwhelming number of soil and rhizosphere-related microbiome studies (Prosser, 2015; Fierer, 2017). Researchers commonly employ three main types of sequencing: (1) metataxonomic primer-based amplicon sequencing, which focuses on the amplification of specific regions of ubiquitous genetic markers, usually 16S rRNA (Bacteria and Archaea) or the intergenic spacer (ITS) region (Eukaryotes), (2) shotgun sequencing of the entire genomic or transcriptomic information within a given sample (metagenomics and metatranscriptomics) (3) detection of separated and fragmented proteins (metaproteomics), usually by combining liquid chromatography mass spectrometry (LC MS-MS), and (4) detection of metabolites, normally through MS or nuclear magnetic resonance (NMR) (metabolomics). The technical limitations of such metaomics and amplicon sequencing, such as sampling errors, primer or processing biases, computational power and adequate analytical algorithms have been extensively discussed in several comprehensive reviews and will not be discussed here (Hirsch et al., 2010; Carvalhais et al., 2012; Pinto and Raskin, 2012; Scholz et al., 2012; Temperton and Giovannoni, 2012; Tkacz and Poole, 2015). Encouragingly, major progress has been made in alleviating these limitations, but even with "perfect" metaomics techniques, many conceptual limitations, such as extrapolating accurate information from metaomics datasets to draw meaningful conclusions, still exist and require careful experimental design (Prosser, 2015). For example, mis-annotation of genes in datasets is a major limitation on extrapolating data from omics datasets (Lidbury et al., 2014; Fierer, 2017). In addition, particularly with regards to metagenomics, we tend to focus on genes with known functions and ignore a whole suite of genetic information that harbors the potential to perform novel functions, particularly with regards to genes that encode ecologically important extracellular proteins (Christie-Oleza et al., 2015).

New Efforts Toward the Characterization of Complex Microbial Communities

One clear benefit of amplicon sequencing is that multiple samples can be processed simultaneously in one sequencing experiment allowing for increased spatiotemporal resolution and the ability to test multivariate factors. To this end, amplicon sequencing has been invaluable in determining general patterns of microbial diversity within the plant microbiome (Bulgarelli et al., 2012; Lundberg et al., 2012; Peiffer et al., 2013; Tkacz et al., 2015). Amplicon sequencing is now frequently employed with more sophisticated techniques, for example exometabolic profiling of plant exudates (Badri et al., 2013; Zhalnina et al., 2018),

multi-generation plant trait selection experiments (Haney et al., 2015; Panke-Buisse et al., 2015), crop mutant line experiments (Senga et al., 2017), and microscopy (Rybakova et al., 2017) to help answer specific questions about plant-mediated bacterial recruitment and functioning in the rhizosphere. Indeed, using 16S rRNA gene data has also revealed that members of the "rare" biosphere are actively recruited in the rhizosphere suggesting that they may play an important role despite their low abundance (Dawson et al., 2017). Furthermore, Bulgarelli et al. (2015) suggested the need to combine alternative markers to 16S rRNA, such as 18S rRNA or internal transcribed spacers (ITSs), in order to access and characterize a broader range of microbes and to get a more representative picture of the microbial diversity and structure. Indeed, metagenomics or metatranscriptomics can partially alleviate this problem by sequencing all the genomic content in a sample simultaneously (Chaparro et al., 2014; Bulgarelli et al., 2015).

Challenges and New Efforts in Assigning Function to Microbes

Beyond determining microbiome structures under different environments, it is particularly challenging to assign a function to individual microbiome members or groups and we tend to consider each microbial group as a functional group. However, even species within a particular genus can have completely different lifestyles - from pathogen to mutualist depending on the environment (Hacquard et al., 2016; Hiruma et al., 2016), or due to horizontal transfer of specific functional genes (Qiu et al., 2009). This variability can lead to dramatic changes in microbial phenotypes of desired traits, such as phosphorus mobilization (Lidbury et al., 2016). More sensitive methods to characterize the microbiome beyond the genus level are needed, along with a better functional characterization of each species, which would require large scale/high throughput techniques (Schlaeppi and Bulgarelli, 2015). With the rate at which technology advances, combining, e.g., computational/modeling methods are very promising. For instance, there is a transition from metagenomics to metaphenomics, which combine the product of the metagenome (or "expressed functions encoded in microbial genomes") with the environment, taking all the parameters into account that may influence the dynamics of the interactions within the community and the environment (Jansson and Hofmockel, 2018). In this respect, metagenomics can be potentially powerful and provide information about the broad functional capabilities (e.g., secondary metabolite production or carbohydrate utilization) (Bulgarelli et al., 2015) or about specific gene sets (e.g., metabolic pathways) of the microbiome (Wang et al., 2015; Ofaim et al., 2017). With new analytical methods, we can also gain deeper insights into the specific taxa responsible for harboring key functional traits (Prosser, 2015). With the development of easy-to-use commercial kits, extracting DNA from a given sample is straightforward. However, DNA only gives the functional "potential" of a microbial community. Furthermore, particularly in soils, the vast majority (>90%) of microbial biomass (or genetic information) is inactive or dormant (for a comprehensive review, see Fierer, 2017). Nevertheless, this number is likely to drop significantly in the rhizosphere and root, due to the step-wise selection of microbes by plant-mediated factors meaning more microbes are metabolically active in these niches (Bulgarelli et al., 2013). Efforts have been made to extract RNA from rhizosphere samples to look at those microbes that are metabolically active and reveal which genetic pathways they are inducing in response to plant and microbial stimuli (Turner et al., 2013b; Chaparro et al., 2014; Yergeau et al., 2014). Therefore, many studies combine the enrichment of 13C-labeled CO_2 with metatranscriptomics to identify microbes responding to plant exudates to improve our understanding on the interactions between microbiome and plant host (Haichar et al., 2016; Lueders et al., 2016).

Unlike, the shorter turnover times of RNA, which reduces the simplicity and robustness of sampling efficiency (Prosser, 2015), proteins, especially exoproteins, are more stable in the environment (Armengaud et al., 2012). Thus, sampling is methodologically easier prone to sampling errors/artifacts. Metaproteomics also provides an exciting opportunity in omics research as it gives a profile of expressed proteins, and hence, (metabolic) activities in a given sample (Heyer et al., 2015). In turn, exoproteomics or exometaproteomics, enriches for the more ecologically important proteins that are involved in nutrient acquisition and microbial–microbial and microbial–host interactions (e.g., extracellular hydrolytic enzymes and transporter systems) (Armengaud et al., 2012; Lidbury et al., 2016). However, there are several drawbacks to metaproteomics, particularly a requirement for sufficient starting material (sometimes up to 100 g of soil is needed) (Johnson-Rollings et al., 2014), as well as the accurately assignment of peptides detected to the correct proteins, which relies on a comprehensive databases (metagenome) and sufficient computational power (Muth et al., 2013, 2016; Timmins-Schiffman et al., 2017). Perhaps this is why meta(exo)proteomics has not been extensively utilized in rhizosphere research, in comparison to studies of other less complex microbial niches, e.g., seawater and anaerobic digesters (Sowell et al., 2009; Williams et al., 2012; Heyer et al., 2015), and enriched chitin-degrading sandy soil samples (Johnson-Rollings et al., 2014). Similarly, this approach can be very powerful for identifying the major extracellular enzymes involved in phosphate mobilization within the rhizosphere (Lidbury et al., 2016; Lidbury et al., unpublished results).

Efforts to Isolate, Characterize, and Use Microbial Strains in Synthetic Communities

As the major rhizobacterial phyla (Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes) are amenable to cultivation, a number of new studies have reverted to extensive isolation efforts followed by genome sequencing and phenotypic characterisation (Bai et al., 2015; Mauchline et al., 2015; Levy et al., 2018). These are often combined with the reconstruction of synthetic communities to determine keystone species and patterns of recruitment in the rhizosphere (Bai et al., 2015; Niu et al., 2017). Reconstruction of microbial communities can help to identify microbe–microbe interactions that have an

effect on plant growth (Hartman et al., 2017). The advantage of the isolation approach is that sequencing and assembling of individual genomes is much simpler and usually provides a higher resolution of data than assembling metagenomes collected *in situ*. More, the isolation approach is a sophisticated tool to functionally validate isolates within a community and/or their interaction with host plants (Levy et al., 2018). Any isolates exhibiting either novel or improved functionality can easily be deployed for further investigation to identify the precise molecular mechanisms and associated rate kinetics of key enzymes. For example, studying the transcriptomic or proteomics response of individual bacterial or fungal isolates to the plant microbiome or associated nutrient stresses has provided useful information on the genes involved in potentially important plant growth promotion (PGP) processes and recruitment of beneficial microbes (Mauchline et al., 2006; Fernández et al., 2013; Lidbury et al., 2016; Martino et al., 2018). This approach can also be particularly useful for the discovery of novel traits associated with PGP activities mediated by microbes (Bruto et al., 2014). Since the *in vitro* screening methods for PGP traits do not necessarily reveal phenotypes associated with plants, the use of genomic screening tools could provide a fast, large scale screening while encouraging the discovery of novel PGP traits/genes (Finkel et al., 2017). Combining these methods with complementary molecular approaches, such as mutagenic and bio-reporter expression systems (Wetmore et al., 2015; Cheng et al., 2016; Pini et al., 2017) will uncover the role of these PGP traits/genes and improve our predictions about the mechanisms driving interactions within plant microbiomes.

EXPERIMENTAL SET UPS TO STUDY MICROBIOMES

The Need to Understand the Interactions

Plants can affect the structure of their root microbiome in favor of beneficial microbes and against pathogens or other deleterious microbes (Berendsen et al., 2012; Bulgarelli et al., 2015). In turn, microbes can also manipulate the host for their own benefit, e.g., altering host metabolism (Jones and Dangl, 2006; Engelstädter and Hurst, 2009; Hacquard et al., 2015; Levy et al., 2018). In addition, the abiotic environment (e.g., edaphic factors) influences both the plant and microbial communities, further enhancing the complexity of ecosystems (Bakker et al., 2014). Network analyses have shown the importance of "microbial hubs," which are strongly interconnected microbial taxa that severely influence communities and that are thought to be a key to understand microbiome dynamics, and the effect of single microbes on the structure of microbial communities (Agler et al., 2016). For instance, Niu et al. (2017) created synthetic communities using seven representative bacterial strains from the three most abundant phyla obtained from maize roots. Employing this simplified community, they aimed to uncover mechanisms that determine the dynamics of this system. Interestingly, the removal of one strain led to the complete collapse of the community, highlighting the importance of individual members of the microbiome (Niu et al., 2017). It further suggests that small or even subtle changes can lead to significant effects on microbiome structures. Therefore, deciphering underlying inter-microbial dynamics driving community structures can be key in validating stable synthetic communities. There are new efforts to analyse those complex interactions and establish reliable systems that can (i) overcome the soil ecosystem complexity and (ii) build our fundamental knowledge of microbial and plant–microbe interactions.

Advances to Overcome Soil Ecosystem Complexity

Since the revelation of the *Arabidopsis thaliana* core root microbiome, which gave more detailed insights into plant microbiome structures (Bulgarelli et al., 2012), numerous studies have collectively highlighted the importance of microbiomes in ecosystem functioning (Agler et al., 2016). Furthermore, the isolation and characterisation of microbial species together with the development of defined gnotobiotic systems (Lebeis et al., 2015; Finkel et al., 2017) allows the targeted functional characterisation of individual members of plant microbiomes (Bai et al., 2015). Gnotobiotic systems, in particular, have been recognized as being essential for microbiome research as they allow to distinguish between the effect of microbes or microbial combinations and the environment (by applying defined conditions) on plant phenotypes. By revealing individual processes in multicomponent plant–microbe–environment interactions, it gives the possibility to associate genotypes with phenotypes. However, the reduced complexity of such systems as well as the "artificial" or *de novo* assembly of microbiomes can prevent the recapitulation and, hence, full functionality of natural systems (Vorholt et al., 2017).

Hartman et al. (2017) presented a different way of microbe application as an effort to understand complex interactions within microbiomes. They isolated microbes from the roots of *Trifolium pratense* and chose one representative strain from each of the four most abundant microbial groups (OTUs) to inoculate sterile microcosms. They reported a negative effect of *Flavobacterium* on the growth of *Trifolium*, which was alleviated in the presence of either of the three other bacterial representatives from *Pseudomonas*, *Janthinobacterium,* and *Microbacterium*. Interestingly, none of the three bacteria affected the abundance of the *Flavobacterium* in the synthetic community. Therefore, the negative activity of *Flavobacterium* was somehow "buffered" by *Pseudomonas*, *Janthinobacterium*, and *Microbacterium* (Hartman et al., 2017). Such a reductionist approach can reveal new "keystone" players in regulating microbiome function and its interaction with the plant.

All these analytical approaches have greatly benefitted from the advancements in computational analyses and machine learning and their significance for the development of microbiome research (Knight et al., 2018). COREMIC, for example, is a bioinformatics tool that allows the generation and confirmation of hypothetical models, by associating microbes with certain plants or habitants using existing databases (Rodrigues et al., 2018). As for other omics-based analyses,

the need for reproducibility as well as the development of "golden standards" to improve consistency and comparability of experiments have been particularly highlighted (Knight et al., 2018). Furthermore, network analyses have equipped microbiome research with sophisticated tools that can analyse and explain the complexity of microbial communities (Adair and Douglas, 2017; Wang H. et al., 2017). While network analyses often build the basis in revealing the function of microbial taxa and the nature of microbial interactions (Poudel et al., 2016), it has certain limitations in identifying synergistic, additive and antagonistic effects. As a result, key functions of certain low abundant microbes might be underestimated or not even recognized (Shade et al., 2014; Shi et al., 2016). The ultimate aim of these efforts, the identification of interactions within microbial communities on plants as a result of inter–microbial communication, therefore requires sensitive tools uncovering correlative interactions that can be verified in biological assays.

Advances in Fundamental Research on Microbe–Microbe and Plant–Microbe Interactions

Besides comprehending the complexity of microbial communities and interactions, there is the need to uncover basic regulatory (communicative) principles of interactions that can inform experimental design. Recently, cytology-based systems have been developed to study microbial interactions. Hennessy et al. (2017) established a microplate reader-based system, to quantify the activities and interactions between living microbes. This method represents a potential high-throughput screen by using live imaging of fluorescing metabolites and microbial growth to identify and trace the expression of certain genes in defined microbial communities. In their study, they measured the rate of the production of fluorescent metabolites of *Pseudomonas fluorescens* in response to the presence of *Fusarium graminearum*, as an indicator for their interaction (Hennessy et al., 2017). Alternatively, Massalha et al. (2017) published recently a microfluidics-based system for *in vivo* imaging of plant root–microbe interactions. Using a transparent chamber, they could record root zone preferably colonized by a fluorophore-tagged microbe. By adding a second microbe to the system they were able to study microbe–microbe interactions in real time. Despite their minimalistic set-up, such studies reveal fundamental insights into basic principles that shape microbe–microbe and root–microbe interactions. In this respect, the application of transparent soil represents an innovative approach to study and live image microbes on plant roots in an environment which mimics different soil textures (Downie et al., 2012). It allows to detect processes driving the distribution of microbes in bulk substrate along the root (Downie et al., 2014) and study the effects of major root pests such as nematodes on microbe/community behavior (O'Callaghan et al., 2018).

In addition to bacterial and fungal microbiomes, soil and plant processes are directly influenced by other organisms including viruses, archaea, nematodes, and insects. Viruses play a very important role in soil biochemical processes and act as gene reservoirs for horizontal gene transfer, although their function is not completely understood (Pratama and van Elsas, 2018). Similarly, Archaea and nematodes significantly contribute to microbiome diversity and in interaction with other microbes to soil-plant processes and ecosystem functioning (Adam et al., 2017; Castillo et al., 2017; Elhady et al., 2017). In this respect, Benítez et al. (2017) has given a very interesting insight into plant–microbe–insect interactions. They reported that soil microorganisms can affect aboveground interactions between plants and insects, by modulating the release of plant volatiles (Pineda et al., 2015; Beck and Vannette, 2017; Benítez et al., 2017). These studies indicate that we need more comprehensive, holistic studies on multitrophic interactions in order to understand which edaphic and biotic factors determine the structure and, hence, function of soil and plant microbiomes structures.

Exploiting the full potential of microbes and microbial communities will depend on expertise from different fields. In addition to improving our understanding of complex plant–microbe and multitrophic interactions using plant biology and microbiology-based approaches, we need to develop new ecological systems with growing complexity. Most critically, in order for this knowledge to be successfully transferred to agriculture it is essential to understand the impact of various farming practices on the microbiome and how this is translated to plant health and, thus, crop productivity. In addition, it is necessary to test microbial community function in a highly complex and diverse system (e.g., field), bridging the gap between the lab and the farm.

BRIDGING THE LAB-FIELD GAP

Limitations on the Experiments Performed in Controlled Conditions (The Lack of Context)

Increasing evidence is showing that plant–microbe interactions can be beneficial or detrimental for either the host or microbial symbiont depending on the balance of associated biotic and abiotic factors. Whilst, experiments involving pairwise interactions under controlled conditions have increased our knowledge about gene and metabolite expression profiles involved in plant–microbe interactions, these experiments give us little information about microbial function in a natural ecosystem (de Boer, 2017). Although this was stated by de Boer (2017) for fungal–bacterial interactions it is applicable to many other interactions (even to those such as plant–rhizobia or plant–mycorrhizal interactions). For example, even some species of plant-growth promoting arbuscular mycorrhizae fungi (AMF) have been shown to inhibit plant growth under certain conditions, e.g., low light, low temperature or phosphorous (P) availability (Smith and Smith, 1996; Johnson et al., 1997). In addition, AMF activity can also be suppressed by the soil microbiota (Svenningsen et al., 2018) highlighting the practical need for field experiments to fully understand microbe behavior.

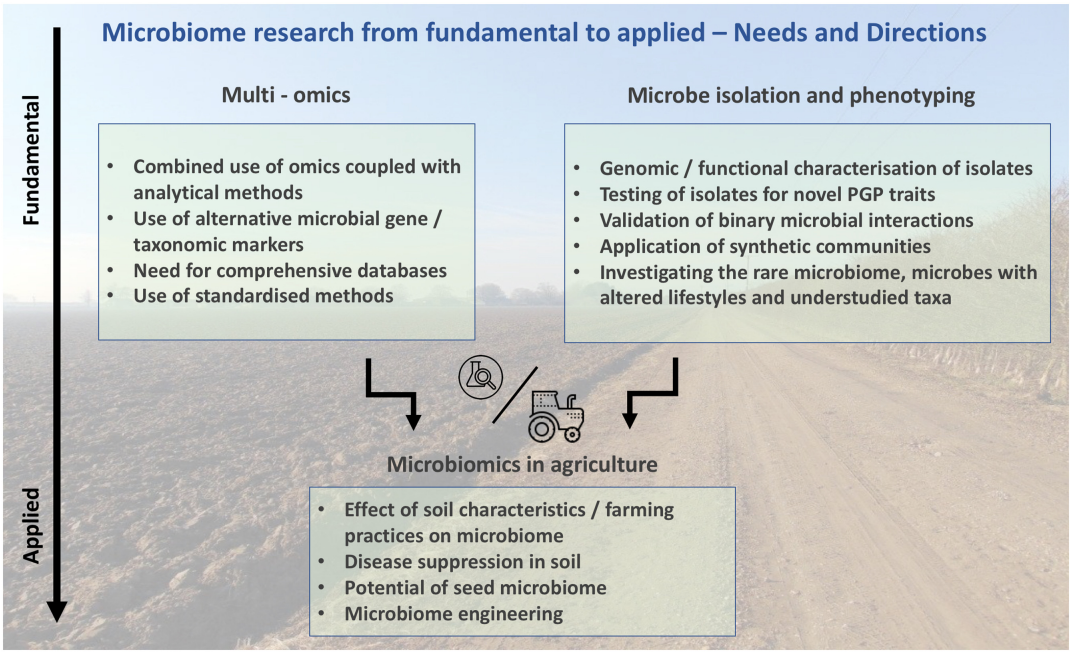


FIGURE 1 | Potential of microbiome research; connecting fundamental microbiomics with applied microbiomics in agriculture.

The main reason for the existence of this lab-farm gap is that lab studies generally do not capture the complexity of microbe–microbe interactions that occur in a natural setting. However, it is widely known now that microbial communities and plant–microbe interactions are highly dependent on the entire ecosystem (Bulgarelli et al., 2013; de Boer, 2017; Lewis et al., 2018). For instance, host genotypes have been shown to shape plant microbial communities (Bulgarelli et al., 2013; Horton et al., 2014) and a genome wide association study (GWAS) analysis revealed that both bacterial and fungal communities are structured by the same host biological processes (such as defense response or signal transduction). However, different genes seem to be involved in the interaction (Horton et al., 2014) and microbial communities are further fine-tuned during plant development according to host requirements (Chaparro et al., 2014). Host-dependent control of the microbial community is likely controlled by the flow of organic compounds from the root to the rhizosphere (rhizodeposition) (Chaparro et al., 2014; Baran et al., 2015) which has been shown to attract beneficial microbes and refrain pathogen attack. The legume-rhizobia symbiosis is an elegant example of rhizodeposits selecting for beneficial microbes. In response to low nitrogen, the host releases flavonoid compounds that initiate the molecular dialog with nitrogen-fixing rhizobia, resulting in root nodulation and nitrogen fixation (Oldroyd, 2013). In addition, rhizodeposition also functions as a chemical signal for the establishment of inter–root or root–microbe interactions (Jones et al., 2009). Therefore, different hosts, holding different gene sets, will trigger different responses to the same inoculant. Moreover, the same host will release different root exudates depending on the soil nutrient and microbial environment. All these examples represent the cyclic feedback between all the components of this ecosystem (plant–soil–microbes). This likely explains why field microbial inoculants fail to persist for long periods (Finkel et al., 2017). Moreover, the soil ecosystem plays a key role on the establishment of root microbiome (Edwards et al., 2015; Zarraonaindia et al., 2015), which means, that even if the inoculant survives within the soil community, it is not guaranteed that it would colonize the plant host.

Any benefits mediated by microbes observed under controlled conditions will ultimately need to be operative in the field. This implies their persistence in the field over time and successful plant colonization over a wide range of varying biotic, abiotic, and climate conditions. Therefore, finding single inoculants that can perform in such a variety of scenarios will be highly unlikely, which increases the need for the development of microbial precision agriculture mirroring the concept of human personalized medicine (Hamburg and Collins, 2010). In fact, the abundance of similarities between human gut and plant root microbiomes is striking and reveals the importance of the root microbiome in controlling plant fitness (Berendsen et al., 2012). Specifically, it has been shown that complex microbial inoculums can improve plant disease resistance and promote growth better than individual inoculums (reviewed in Finkel et al., 2017), highlighting the synergistic effects of a community. However, these findings still need to consider the soil context to address their potential use as soil amendments. In addition, a deeper understanding of a microbe's function within a community and within a host would require functional studies where the ecosystem is challenged with different conditions (e.g., temperature, light, humidity). Those studies would validate

their community interactions and their beneficial or detrimental outcome for plants as a prerequisite to justify for further field experiments (**Figure 1**).

Addressing Field-Based Microbiome Studies

The immense microbial biodiversity in soil is regulated at very different scales, for example changes in soil texture, biotic interactions or plant root exudates have enormous effects down to the smallest (microbial) scales. Variations in the physical and chemical properties of soil, such as pH, nutrient distribution or water retention, have effects on soil biodiversity on the medium (field) scales with soil pH as a suggested major driver of microbial diversity (Fierer and Jackson, 2006) linking microbial community structure with soil nutrient availability and cycling (Li et al., 2017). Finally, at larger scales, geo-localization and climate might play more relevant roles on controlling soil biodiversity (Bardgett and van der Putten, 2014).

The existence of disease suppressive soils is living proof that microbial communities cannot just promote plant growth (Chaparro et al., 2012; Van Der Heijden et al., 2008) but also provide protection against plant pathogens (Alabouvette, 1986; Andrivon, 1994; Shiomi et al., 1999). Disease suppression can be due to competition with native soil fauna (general suppression) or to the presence of specific subsets of microbes (specific suppression). These soil protection strategies are comparable to immunity strategies of animals (Raaijmakers and Mazzola, 2016). As common to all complex ecosystems, general suppression is also common to all soils. Specific suppression, in turn, is removed by soil pasteurization and can be transferred to other soils via soil transplants. Soils can lose their suppressiveness if non-host plants are grown and can be recovered if the susceptible host and pathogen are grown back in them (Wiseman et al., 1996; Berendsen et al., 2012; Raaijmakers and Mazzola, 2016).

The ability of plants to attract beneficial root microbes might represent a crucial strategy to survive under unfavorable environmental conditions. Several studies point out to the possibility of engineering microbiomes to control plant traits that can be used to increase and sustain plant production (Mueller and Sachs, 2015; Panke-Buisse et al., 2015; Herrera Paredes et al., 2018; Orozco-Mosqueda et al., 2018). Plants challenged with pathogens can recruit protective microbes in the rhizosphere and endosphere that can modulate the host immune responses (Berendsen et al., 2018). This strategy has been exploited to formulate bioorganic fertilizers that manipulate banana rhizosphere microbial structure and subsequently decrease the incidence of Panama disease (Xue et al., 2015). All these studies have demonstrated the feasibility to engineer plant microbiomes as a sustainable solution to increase yields as well as biotic and abiotic stress resistance.

Another recent breakthrough discovery highly relevant for microbiome research is the analysis of seed microbiomes. It is supposed to have been co-selected and evolved with the plant providing valuable traits that have driven and still drive plant evolution (Puente et al., 2009; Johnston-Monje and Raizada, 2011; Turner et al., 2013a; Bouffaud et al., 2014; Delaux et al., 2014; Hardoim et al., 2015). Seed microbiomes seem to consist of a limited range of microbial species and this restricted number is probably due to the requirement of these species to survive all seed developmental processes, even the most extreme such as desiccation (Truyens et al., 2015). These studies are in accordance with recent experiments that have shown the possibility to transfer the plant microbiota to the next generation (Mitter et al., 2017). All these findings have put plant microbial engineering and breeding at the forefront of sustainable agriculture (Wei and Jousset, 2017).

To ultimately bridge the lab-field gap, we need to take into account that field experiments (in contrast to glasshouse experiments) are subject to agricultural practices, and these have a significant influence on microbiomes and microbial diversity. According to the UN, sustainable land management practices such as (i) crop rotation, intercropping and use of local plant varieties, (ii) tillage and organic farming should be re-introduced to minimize land degradation (Sanz, 2017). However, it is not clear how these practices can be re-introduced in agricultural systems of the developed world whilst still sustaining or increasing crop production. These practices also have a major contribution to microbiome community structure and function (Oberson et al., 1993; Mäder et al., 2002; Dossa et al., 2012; Debenport et al., 2015; Reganold and Wachter, 2016; Hartman et al., 2017, 2018; Wang Y. et al., 2017). Therefore, they might be important to consider when designing microbiome field experiments or testing commercial field applications of microbial inoculants.

Crop Rotation, Intercropping, and Use of Local Plant Varieties

Some ancient agricultural practices started to become less important around the 1940s, since monocropping and synthetic fertilizer applications significantly increased crop yields. These massive agricultural changes were part of the Green Revolution with the intention of feeding an increasingly growing human population. Together with the development of input-intensive agricultural systems for various single cash crops, other agricultural practices were no longer practical in developed countries. However, under the current global scenario of land degradation, fertilizer shortage or global warming, developing sustainable agriculture solutions face the challenge of feeding the still growing human population with minimal ecological and economic impact.

Land management has significant impacts on soil and root microbial community structure and stability and consequently on microbiome-associated functions (Hartman et al., 2017, 2018). Crops grown in monoculture or short rotations often suffer yield decline, due to an enrichment of pathogenic relative to beneficial microbes (Bennett et al., 2012; Hilton et al., 2013; Santhanam et al., 2015). In response, in a field setting, inoculation with native root-associated bacterial isolates can significantly decrease the emergence of diseases associated with continuous cropping (Santhanam et al., 2015) illustrating the potential for employing local microbial resources to increase plant yield and fitness in sustainable agriculture. As mentioned earlier, since soil disease suppression is lost when a different host plant is grown, this

property also seems to be directly related to continuous cropping of the same species. These two opposite outcomes for agricultural production do not only reflect the enormous impact that plant hosts have on the soil microbiome, but also how the latter can impact on plant species that can successfully colonize an environment in natural conditions.

In addition to general edaphic factors, different plant species (Rovira, 1969), plant ecotypes (Micallef et al., 2009), or even different locations (microenvironment) of a root system (Pinton et al., 2007) result in the release of distinct root exudates. Therefore, soil microbial communities are shaped differently depending on the plant species grown. Intercropping was an ancient agricultural practice that was abandoned due to the development of modern intensive agricultural systems. However, intercropping is still a common practice in developing countries, where different plant species are grown in close proximity. Intercropping experiments performed in the Sahel region (Africa) have shown that this practice increases crop yield, soil organic carbon levels and community diversity of both bacteria and fungi (Debenport et al., 2015). Moreover, the co-cultivation with indigenous shrubs improves soil quality and N conservation (Dossa et al., 2012) highlighting the importance of using local species that have already adapted strategies to exploit the natural resources of an ecosystem. Intercropping has been suggested as an alternative for sustainable agriculture production. However, for it to become a common practice in developed countries, multiple challenges would need to be addressed, such as the development of cropping systems adapted to this agricultural practice.

In terms of microbiome research, more studies are required to comprehend how different cropping practices have such a relevant impact in the soil microbiota and whether both cropping practices and microbiome engineering could contribute to sustainable agriculture in the long term.

Tillage and Soil Farming

Land tilling is extended in modern agriculture since it minimizes weed growth and creates a seedbed that is adapted to the machinery commonly used in the field. Since the introduction of plant growth regulators in the 1940s (Bagavathiannan and Davis, 2018), no-tillage systems have been explored as a practice in conservation agriculture. However, no tilling systems require the use of cover crops and especially higher amounts of herbicides, which puts off many consumers and farmers. In turn, this practice minimizes soil particle disturbance, increases organic carbon soil content and enhances soil aggregation as well as water infiltration (Álvaro-Fuentes et al., 2008; Hobbs et al., 2008; Li et al., 2017; Wang Y. et al., 2017). Long-term no tillage and organic input management practices impact soil pH (being slightly higher in organic systems) (Mäder et al., 2002) and nutrient flux from the soil matrix to the soil solution. In terms of microbiome research, no-tilling and organic farming practices correlate with increases in soil microbial diversity, biomass and microbial community stability (Oberson et al., 1993; Mäder et al., 2002; Reganold and Wachter, 2016; Wang Y. et al., 2017). These positive effects on the soil microbiota are likely due to the increase of organic matter (acting as food resources for the microbial community), the decrease of physical perturbations (Wang Y. et al., 2017) and the increase in soil aggregate stability (Siegrist et al., 1998).

CONCLUSION

The generation of microbial communities with customized (beneficial) activities has the potential to serve as a powerful approach to enhance sustainable agricultural production by increasing crop health, through combatting plant diseases and reducing the application of fertilizers. To reach this goal a fundamental understanding regarding the functioning of the plant microbiome through microbe–microbe and plant–microbe interaction is required, as well as a deeper understanding of the soil microbial community structure over time (long-term studies) and its plasticity and response to the environmental changes. Also, since individual microbes are key for the regulation of microbial community structure and stability, more comprehensive studies investigating community dynamics using these individual microbes and their soil microbial communities would assist in advancing the field. This knowledge could help to fully understand the impact that these keystone microbes have on crop yields, disease resistance and global nutrient cycles, but also to reveal strategies for microbiome engineering.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture

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The use of excess conventional Phosphorus (P) fertilizers to improve agricultural productivity, in order to meet constantly increasing global food demand, potentially causes surface and ground water pollution, waterway eutrophication, soil fertility depletion, and accumulation of toxic elements such as high concentration of selenium (Se), arsenic (As) in the soil. Quite a number of soil microorganisms are capable of solubilizing/mineralizing insoluble soil phosphate to release soluble P and making it available to plants. These microorganisms improve the growth and yield of a wide variety of crops. Thus, inoculating seeds/crops/soil with Phosphate Solubilizing Microorganisms (PSM) is a promising strategy to improve world food production without causing any environmental hazard. Despite their great significance in soil fertility improvement, phosphorus-solubilizing microorganisms have yet to replace conventional chemical fertilizers in commercial agriculture. A better understanding of recent developments in PSM functional diversity, colonizing ability, mode of actions and judicious application should facilitate their use as reliable components of sustainable agricultural systems. In this review, we discussed various soil microorganisms that have the ability to solubilize phosphorus and hence have the potential to be used as bio fertilizers. The mechanisms of inorganic phosphate solubilization by PSM and the mechanisms of organic phosphorus mineralization are highlighted together with some factors that determine the success of this technology. Finally we provide some indications that the use of PSM will promote sustainable agriculture and conclude that this technology is ready for commercial exploitation in various regions worldwide.

Keywords: mineralization, phosphorus, soil nutrient management, soil microbes, solubilization

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INTRODUCTION

Phosphorus (P) is one of the essential elements that are necessary for plant development and growth; it makes up about 0.2% of a plant's dry weight. It is second only to nitrogen among mineral nutrients most commonly limiting the growth of crops (Azziz et al., 2012; Tak et al., 2012). On average, the phosphorus content of soil is about 0.05% (w/w); however, only 0.1% of this phosphorus is available for plant use (Zhu et al., 2011). Traditionally, the challenge of soil

Abbreviations: PSM, phosphate solubilizing microorganisms.

phosphorus deficiency is addressed by the application of phosphorus fertilizers. However, the majority of the applied fertilizer phosphorus is not available to plants and the addition of inorganic fertilizers in excess of the amount that is commonly employed to overcome this effect can lead to environmental problems such as, groundwater contamination and waterway eutrophication (Kang et al., 2011). It is therefore of great interest to investigate management strategies that are capable of improving phosphorus fertilization efficiency, increase crop yields and reduce environmental pollution caused by phosphorus loss from the soil.

Soil microorganisms enhance plant nutrient acquisition. They are involved in a wide range of biological processes including the transformation of insoluble soil nutrients (Babalola and Glick, 2012a). Some are capable of solubilizing and mineralizing insoluble soil phosphorus for the growth of plants. Apart from chemical fertilization, microbial P-solubilization and mineralization is the only possible way to increase plant-available phosphorus. In the natural environment numerous microorganisms in the soil and rhizosphere are effective at releasing phosphorus from total soil phosphorus through solubilization and mineralization (Bhattacharyya and Jha, 2012). This group of microorganisms are referred to as Phosphorus Solubilizing Microorganisms (PSM). Many species of soil fungi and bacteria are able to solubilize phosphorus *in vitro* and some of them can mobilize phosphorus in plants (Zhu et al., 2011). PSM increases the bioavailability of soil insoluble phosphorus for plant use (Zhu et al., 2011). They solubilize insoluble inorganic (mineral) phosphorus and mineralize insoluble organic phosphorus (Sharma et al., 2013). The salt-tolerant or halophilic soil microorganisms that also exhibit the ability to solubilize insoluble phosphorus facilitate the development of saline-alkali soil-based agriculture (Zhu et al., 2011).

The inoculation of soil or crop with phosphate solubilizing/mineralizing microorganisms is therefore a promising strategy for the improvement of plant absorption of phosphorus and thereby reducing the use of chemical fertilizers that have a negative impact on the environment (Alori et al., 2012).

PHOSPHORUS SOLUBILIZING MICROORGANISMS (PSM)

A large number of microbial organisms including bacteria, fungi, actinomycetes, and algae exhibit P solubilization and mineralization ability. Soil bacteria that have been reported to mobilize poorly available phosphorus via solubilization and mineralization include *Pseudomonas spp., Agrobacterium spp., and Bacillus circulans* (Babalola and Glick, 2012b). Other phosphorus solubilizing and mineralizing bacteria include various strains of *Azotobacter* (Kumar et al., 2014), *Bacillus* (Jahan et al., 2013; David et al., 2014), *Burkholderia* (Mamta et al., 2010; Zhao et al., 2014; Istina et al., 2015), *Enterobacter, Erwinia* (Chakraborty et al., 2009), *Kushneria* (Zhu et al., 2011), *Paenibacillus* (Fernández Bidondo et al., 2011), *Ralstonia*, *Rhizobium* (Tajini et al., 2012), *Rhodococcus, Serratia, Bradyrhizobium, Salmonella, Sinomonas,* and *Thiobacillus* (Postma et al., 2010; David et al., 2014).

The microbial fungi that function similarly include strains of *Achrothcium, Alternaria, Arthrobotrys, Aspergillus, Cephalosporium, Cladosporium, Curvularia, Cunninghamella, Chaetomium, Fusarium, Glomus, Helminthosporium, Micromonospora, Mortierella, Myrothecium, Oidiodendron, Paecilomyces, Penicillium, Phoma, Pichia fermentans, Populospora, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Sclerotium, Torula, Trichoderma,* and *Yarrowia* (Srinivasan et al., 2012; Sharma et al., 2013).

Soil fungi have been reported to be able to traverse long distances within the soil more easily than bacteria and may be more important to the solubilization of inorganic phosphate in soils as they typically produce and secrete more acids, such as gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric and acetic acid, than bacteria (Sharma et al., 2013). In addition, approximately 20% of actinomycetes could solubilize P, including those in the genera *Actinomyces*, *Micromonospora,* and *Streptomyces*. Algae such as cyanobacteria have also been reported to show P solubilization activity (Sharma et al., 2013).

BENEFITS OF PHOSPHORUS SOLUBILIZING MICROORGANISM

For better utilization of the phosphorus accumulated in soils, PSMs that are capable of transforming insoluble phosphorus to soluble forms can function as biofertilizers. This increases the soluble phosphorus content (Zhu et al., 2012). The use of phosphorus biofertilizers is a promising approach to improving food production through enhancing agricultural yield as it is better to use an environmentally friendly approach (that is, a paradigm that emphasizes the use of biological soil amendments in place of chemicals) to solve the problems of infertile soil (Babalola and Glick, 2012a). **Figure 1** shows the effect of inoculation with a PSM (*Pseudomonas* sp.) on a maize plant. The growth of maize that was inoculated with PSM was improved compared to the control that was not inoculated. PSM act as biofertilizers by making otherwise unavailable P available to growing plants. Phosphorus solubilizing bacteria may also aid the growth of plants by stimulating the efficiency of biological nitrogen fixation, synthesizing phytohormones and enhancing the availability of some trace elements such as zinc and iron (Wani et al., 2007).

Many PSM inoculation studies have shown both improved plant yield and increased phosphorus uptake both in pot experiments and under field conditions. In a pot experiment where *Aspergillus niger* was used as a biofertilizer (using wheat husks with 20% perlite as carrier material) the soil colonization rate was 5.6×10^6 spores g^{-1} soil (Wang et al., 2015). The benefits of adopting microbial management of the rhizosphere for sustainable agriculture production includes enhancing the bioavailability of phosphate to crops, stimulated roots and shoots

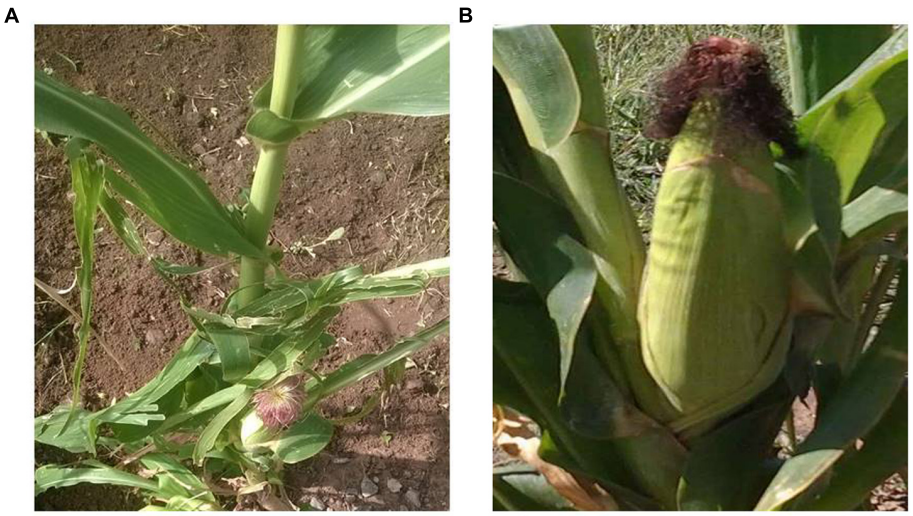


FIGURE 1 | Biofertilizer effect of *Pseudomonas* sp. on maize. **(A)** Maize not inoculated with a PSM. **(B)** Maize inoculated with a PSM *Pseudomonas* sp.

growth, improved root and shoot length, and increased fresh and dry shoot weights, P-labeled phosphate uptake, and significant improvement of grain and dry matter yields (Rodríguez and Fraga, 1999). **Table 1** shows the effect of some PSM on a variety of crops.

Phosphate Solubilizing Microorganisms have considerable synergistic effect on the growth and development of crops (Tallapragada and Gudimi, 2011). Besides solubilizing P, some PSM also demonstrate potential as biocontrol agents against some plant pathogens. PSM manage the pathogens by producing antifungal compounds (such as PAL, phenolics and flavonoids), siderophores, antibiotics, hydrogen cyanide and lytic enzymes all of which enhance inhibition of the growth of plant pathogens.

Phosphate Solubilizing Microorganisms technology improves the fertility and agricultural use of saline-alkaline soil without causing any environmental or health hazard that accompanies the continuous use of synthetic fertilizers. *Kushneria* sp. YCWA18, a strain that is capable of solubilizing both inorganic phosphorus and organo-phosphorus has also demonstrated moderate

TABLE 1 | Effects of some PSM on crops.

PSM	Test crop	Result	Source
Aspergillus *niger*	Wheat	Improved growth	Xiao et al., 2013
Serratia sp.	Wheat	Increased growth	Swarnalakshmi et al., 2013
*Aspergillus awamori*S29	Mung bean	Increased plant growth, total P content, and plant biomass	Jain et al., 2012
Burkholderia gladioli	Sweetleaf	Increased plant growth	Mamta et al., 2010
Pseudomonas aeruginosa	Chinese cabbage	Increased total weight and total length	Wang et al., 2010
*P. putid*a	Moss	Increased growth	Tani et al., 2011
Azotobacter chroococcum, Saccharomyces cerevisiae, and *Bacillus megaterium*	*Moringaoleifera*	Increased shoot and root lengths, increased shoot and root dry weights, increased vitamin C and protein content g/g dry weight leaves	Zayed, 2012
Burkholderia gladioli	Oil palm	Increased growth and phosphate uptake	Istina et al., 2015
Aspergillus niger Penicillium aculeatum	Chinese cabbage	Increased growth	Wang et al., 2015
Bacillus sp. and *Pseudomonas* sp.	Sesame	Increased seed yield	Jahan et al., 2013
Bacillus thuringiensis	Rice	Increased shoot length	David et al., 2014
Pseudomonas striata and *Glomus fasciculatum*	Soybean-wheat	Better root property and increased grain yield	Mahanta et al., 2014
Burkholderia cepacia	Maize	Improved plant growth	Zhao et al., 2014
Azotobacter chroococcum and *Bacillus subtilis*	Wheat	Enhanced productivity of wheat	Kumar et al., 2014
P. favisporus TG1R2	Soybeans	Increased dry biomass	Fernández Bidondo et al., 2011
Rhizobium tropici CIAT899	Beans	Enhanced increased; nodule number, nodule mass, shoot dry weight, and root growth	Tajini et al., 2012

halophilic properties and can be used in the development of saline-alkaline based agriculture (Zhu et al., 2011). *Aerococcus* sp. strain PSBCRG1-1, *Pseudomonas aeruginosa* strain PSBI3-1, *A. terreus* strain PSFCRG2-1 and *Aspergillus* sp. strain PSFNRH-2 were all shown to solubilize tricalcium phosphate at different NaCl concentrations (Srinivasan et al., 2012). The PSM *Burkholderia cepacia* promoted the growth of maize plants in the presence of NaCl concentrations of up to 5% (Zhao et al., 2014). These organisms all have potential as biofertilizers in saline-alkaline soil based agriculture. In one set of experiments, for bacterial solubilization, increases in NaCl concentration up to 0.8 M resulted in an increase in the percentage of phosphorus released but it declined thereafter. On the other hand, with increases in NaCl concentration the amount of P released among phosphate solubilizing fungi was found to decrease throughout the incubation periods (Srinivasan et al., 2012).

MECHANISMS OF INORGANIC PHOSPHATE SOLUBILIZATION BY PSM

A number of theories explain the mechanism of inorganic phosphate solubilization. As observed in many experiments, the principal mechanism is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions and CO2 (Rodríguez and Fraga, 1999; Sharma et al., 2013). Organic acids produced as described in **Figure 2** together with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release P (Seshachala and Tallapragada, 2012); The organic acids are produced in the periplasmic space by the direct oxidation pathway (Zhao et al., 2014). The excretion of these organic acids is accompanied by a drop in pH that results in the acidification of the microbial cells and the surroundings, hence, P ions are released by substitution of H^+ for Ca^{2+} (Goldstein, 1994). Surprisingly, Asea et al. (1988) discovered that no correlation exists between the pH and the amount of P solubilized. Hence Illmer and Schinner (1995) proposed the theory of acidification by H^+. They explained that H^+ released is associated with cation assimilation. For example, assimilation of $NH_4{}^+$ together with H^+ excretion brings about P solubilisation (Illmer and Schinner, 1995). An alternative mechanism to organic acid production for solubilization of mineral phosphates is the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase (Rodríguez and Fraga, 1999). It was also reported that the assimilation of $NH4^+$ within microbial cells is accompanied by the release of protons and this results in the solubilization of phosphorus without the production of any organic acids (Sharma et al., 2013). Of all the organic acids, gluconic acid is the most frequent agent of mineral phosphate solubilization; it chelates the cations bound to phosphate, thus making the phosphate available to plants. Gram-negative bacteria solubilize mineral phosphate by direct oxidation of glucose to gluconic acid (Goldstein, 2000). Pyrroloquinoline quinone (PQQ) acts as a redox cofactor in glucose dehydrogenases (GDH) resulting in phosphate solubilisation (Rodríguez et al., 2000).

Other mechanisms of mineral phosphate solubilization by microorganisms are the production of inorganic acids (such as sulphuric, nitric, and carbonic acids) and the production of chelating substances. It has, however, been reported that the effectiveness of the inorganic acids and the chelating substances in the release of phosphorus in soil is less than that of the organic acids. Kim et al. (1997b) therefore reiterate that organic acid production in P solubilization by PSM is not the sole reason for the increase in P concentration into culture medium. Furthermore, Mycorrhizal fungi effectively extend plant roots, aiding crop phosphorus nutrition by increasing the volume of soil from which phosphate may be absorbed (Browne et al., 2009).

Another mechanism of microbial phosphate solubilization reported in the literature is the liberation of enzymes or enzymolysis, the mechanism of P solubilization by PSM in a medium containing lecithin where the increase in acidity is caused by enzymes that act on lecithin and produce choline (Zhu et al., 2011).

MECHANISMS OF ORGANIC PHOSPHORUS MINERALIZATION

The major source of organic phosphorus in soil is the organic matter. The values of organic phosphorus in soil can be as high as 30–50% of the total P and soil organic P is largely in the form of inositol phosphate (soil phytate). Other organic P compounds that have been reported are: phosphomonoesters, phosphodiesters, phospholipids, nucleic acids, and phosphotriesters (Rodríguez and Fraga, 1999). In addition, large quantities of xenobiotic phosphonates (pesticides, detergent additives, antibiotics, and flame retardants) that are regularly released into the environment also contain organic P. Most of these organic compounds are high molecular-weight materials that are generally resistant to chemical hydrolysis and must therefore be bio-converted to either soluble ionic phosphate (Pi, $HPO_4{}^{2-}$, $H_2PO_4{}^-$), or low molecular-weight organic phosphate, to be assimilated by the cell (Peix et al., 2001).

Phosphorus mineralization refers to the solubilization of organic phosphorus and the degradation of the remaining portion of the molecule. One important theory proposed by Halvorson et al. (1990) for the solubilisation of organic P is the sink theory. This refers to continuous removal of P that result in the dissolution of Ca-P compounds. Consequently, the decomposition of P in organic substrates is consistently correlated with the P content in the biomass of PSM (Dighton and Boddy, 1989). This biological process plays an important role in phosphorus cycling. Different groups of enzymes are involved in this. The first groups of enzymes are those that dephosphorylate the phosphor-ester or phosphoanhydride bond of organic compounds. They are non-specific acid phosphatases (NSAPs). The most studied among these NSAPs enzymes released by PSM, are the phosphomonoesterases also referred to as phosphatases (Nannipieri et al., 2011). These enzymes can either be acid or alkaline phosphomonoesterases (Jorquera et al., 2011). The pH of most soils where phosphate activities were

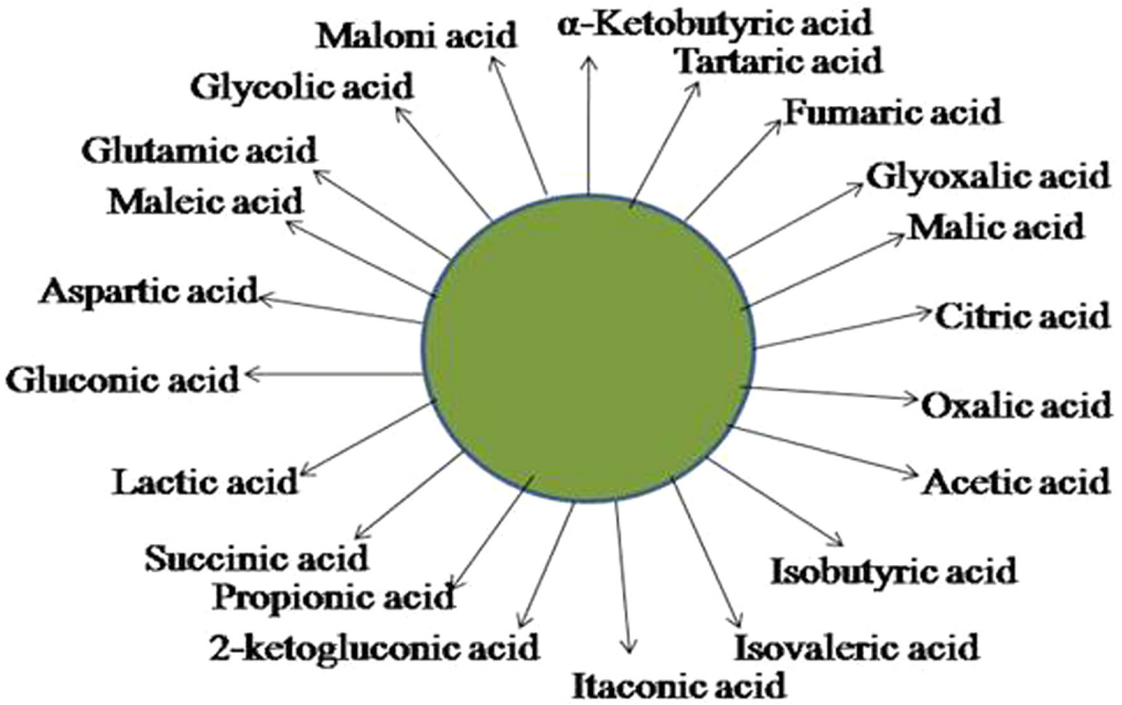


FIGURE 2 | Schematic representation of the organic acids that may be produced by PSM and used to solubilize inorganic forms of phosphate.

reported ranges from acidic to neutral values. This signifies that acid phosphatases play the major role in this process (Rodríguez and Fraga, 1999).

Another enzyme produced by PSM in the process of organic P mineralization is phytase. This enzyme is responsible for the release of phosphorus from organic materials in soil (plant seeds and pollen) that are stored in the form of phytate. Phytate degradation by phytase releases phosphorus in a form that is available for plant use. Plants generally cannot acquire phosphorus directly from phytate, however, the presence of PSM within the rhizosphere may compensate for a plant's inability to otherwise acquire phosphorus directly from phytate (Richardson and Simpson, 2011).

FACTORS INFLUENCING MICROBIAL PHOSPHATE SOLUBILIZATION

The ability of PSM to transform insoluble organic and inorganic phosphorus is associated with, the nutritional richness of the soil, and the physiological and growth status of the organism. PSM from soils from environmental extremes such as saline-alkaline soils, soil with a high level of nutrient deficiency, or soil from extreme temperature environments have the tendency to solubilize more phosphate than PSM from soils from more moderate conditions (Zhu et al., 2011). There has been a conflicting report on the influence of temperature on phosphorus solubilization by microbes. White et al. (1997) found 20–25°C as the optimum temperature for maximum microbial phosphorus solubiliztion while 28°C was reported by Kang et al. (2002), and Varsha (2002). In addition, others including Kim et al. (1997a), Rosado et al. (1998), Johri et al. (1999), and Fasim et al. (2002), have recorded 30°C as the best temperature for P solubilization. Nahas (1996) and Nautiyal et al. (2000) reported P solubilization at extreme temperature of 45°C in desert soil while Johri et al. (1999) reported solubilization at a low temperature of 10°C.

Among other factors influencing microbial phosphate solubilization are interactions with other microorganisms in the soil, the extent of vegetation, ecological conditions, climatic zone soil types, plant types, agronomic practices, land use systems, and the soil's physicochemical properties such as organic matter and soil pH (Seshachala and Tallapragada, 2012). Phosphorus is solubilized faster in warm humid climates and slower in cool dry climates. A well-aerated soil will more readily permit rapid phosphorus solubilisation compared to a saturated wet soil. The land use system is the use that the farmland has been previously committed to, such as cropping or livestock activities or even mixed use. Recently, Zhang et al. (2014) reported that adding small amounts of inorganic phosphorus to the rhizosphere could drive phytic acid mineralization by bacteria and thereby improve plant phosphorus nutrition. Lime and compost, used as a soil improver, also had positive effects on phosphate solubilizers. Phosphorus Solubilizing Bacteria population richness and diversity, according to Azziz et al. (2012), were more abundant and diverse following crop rotation. Soil rich in organic matter will favor microbial growth and therefore favors microbial phosphorus solubilisation. Soil pH values between 6 and 7.5 are

best for P-availability, this is because at pH values below 5.5 and between 7.5 and 8.5 limits P from becoming fixed by aluminum, iron, or calcium, and hence, not being available for plant use. A negative correlation was observed between the amount of phosphate solubilized by *B. cepacia* SCAUK0330 and the pH drop that is associated with this process. The pH drop leads to an increase in phosphate solubilization. At pH 3.12, 452 $\mu g \cdot mL^{-1}$ of phosphorus was solubilized, and when 154 $\mu g \cdot mL^{-1}$ of P was solubilized the pH value was 4.95 (Zhao et al., 2014). Research has also shown that microbial phosphate solubilization largely depends on the kinds of metabolite produced and its rate of release (Zhu et al., 2011).

FUTURE PROSPECTS

As additional insights are gained regarding PSM and the mechanisms that they use, there is every reason to believe that the use of PSM as biofertilizers will likely improve their use, as effective and important components in the establishment of sustainable soil management systems. The focus of consumers of agricultural produce is on the health, quality and nutritional value of those products. Thus, the employment of PSM as biofertilizers is an option that can increase food production without imposing any health hazard, and at the same time conserve the environment. It is essential that researchers continue to learn more about PSM and, immediately, translate this knowledge into a form that can readily be used by farmers.

CONCLUSION

This review has shown that phosphate-solubilizing microorganisms have tremendous potential as Bio-fertilizers. Mobilizing soil inorganic phosphate and increasing its bioavailability for plant use by harnessing soil PSM promotes sustainable agriculture, improves the fertility of the soil, and hence increases crop productivity. The use of PSM as microbial inoculants is a new horizon for better plant productivity. PSM technology can contribute to low-input farming systems and a cleaner environment. However, there is need to develop PSB technologies specific to various regions and this should be communicated to farmers in a relatively short time.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Chemistry of Plant–Microbe Interactions in the Rhizosphere and the Potential for Metabolomics to Reveal Signaling Related to Defense Priming and Induced Systemic Resistance

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Plant roots communicate with microbes in a sophisticated manner through chemical communication within the rhizosphere, thereby leading to biofilm formation of beneficial microbes and, in the case of plant growth-promoting rhizomicrobes/-bacteria (PGPR), resulting in priming of defense, or induced resistance in the plant host. The knowledge of plant–plant and plant–microbe interactions have been greatly extended over recent years; however, the chemical communication leading to priming is far from being well understood. Furthermore, linkage between below- and above-ground plant physiological processes adds to the complexity. In metabolomics studies, the main aim is to profile and annotate all exo- and endo-metabolites in a biological system that drive and participate in physiological processes. Recent advances in this field has enabled researchers to analyze 100s of compounds in one sample over a short time period. Here, from a metabolomics viewpoint, we review the interactions within the rhizosphere and subsequent above-ground 'signalomics', and emphasize the contributions that mass spectrometric-based metabolomic approaches can bring to the study of plant-beneficial – and priming events.

Keywords: chemical communication, induced resistance, metabolites, metabolomics, plant–microbe interactions, priming, signalomics

Abbreviations: ABA, Abscisic acid; AHL, *N*-acyl-homoserine lactone; ET, Ethylene; ETI, Effector-triggered immunity; GC-MS, Gas chromatography mass spectrometry; ISR, Induced systemic resistance; JA, Jasmonic acid; LC-MS, Liquid chromatography mass spectrometry; MAMP, Microbe-associated molecular pattern; MeJA, Methyl jasmonic acid; MeSA, Methyl salicylic acid; MTI, MAMP-triggered immunity; LP, Lipopeptide; PGPR, Plant growth-promoting rhizo-microbes/-bacteria; PRR, Plant pattern recognition receptors; QqQ, Triple quadrupole; QS, Quorum sensing; qTOF, Quadrupole time-of-flight; RMPP, Rhizomicrobe-induced plant priming; SA, Salicylic acid; TOF, Time-of-flight; UHPLC-MS, Ultra-high performance liquid chromatography coupled to mass spectrometry; VOC, Volatile organic compound.

INTRODUCTION: SUSTAINABLE PRODUCTION OF FOOD PLANTS

The world is facing a concerning challenge to produce sufficient food in a sustainable manner, with an increasing global population and decreasing food resources. Food plant production is hampered by a plethora of biotic stresses such as pathogens and herbivores (Iriti and Faoro, 2009; Gust et al., 2010; Thakur and Sohal, 2013). To defend themselves, plants rely on innate immunity of which the success in fighting disease infections or herbivore feeding depends on how rapid and strong an activated immune can be deployed. To combat plant diseases and limit the use of pesticides and herbivore agrochemicals, genetic modification has been used (Bhandari, 2014). However, the use of such strategies has caused major debates citing environmental – (Aktar et al., 2009; Bhandari, 2014) and consumer concerns (Ferreira et al., 2012); hence, the need for new eco-friendly strategies. In the context of plant protection, priming refers to a stimulus or treatment for improved responses to upcoming environmental challenges. Colonization of plant roots by beneficial microbes in the rhizosphere is such a stimulus since it may result in ISR which have a positive effect on the ability of the plant to defend itself against attack by pathogens infecting the leaves (Hilker et al., 2015). Here, we highlight chemical communication in the rhizosphere (plant roots interacting with plant-beneficial rhizobacteria and – fungi) and ISR or RMPP as an environmentally friendly method to combat pathogens and herbivores, as investigated through the use of LC coupled to MS-based metabolomics.

PRE-FORMED BARRIERS AND PLANT IMMUNE RESPONSES: POTENTIAL OBSTACLES FOR INTERACTIONS WITH RHIZOMICROBES

Plants use preformed defense mechanisms aimed at preventing both pathogen entrance and herbivore feeding (**Figure 1**). Failure hereof, either below- or above-ground (De Coninck et al., 2015), leads to plant activation of an immune response termed microbe/pathogen-associated molecular pattern (MAMP)-triggered immunity (MTI) which relies on the detection of conserved microbial signature molecules (MAMPs) *via* extracellular transmembrane receptors or PRRs (Jones and Dangl, 2006; Conrath et al., 2009; Sanabria et al., 2009; Deslandes and Rivas, 2012; Denancé et al., 2013; Gao et al., 2013). Some pathogens are capable of down-regulating MTI by the secretion of effector molecules, thereby leading to effector-triggered susceptibility (ETS). To overcome this, plant resistance (R) proteins recognize these molecules and activate a second line of defense which is a rapid and robust response termed ETI (Pieterse et al., 2009; Gao et al., 2013; De Coninck et al., 2015), and which is associated with the hypersensitive response (HR). The MTI and ETI sections of induced immunity are complementary, and signaling interactions occur between MTI and ETI at very early stages. Furthermore, MTI and ETI share many biochemical features, but differ in the intensity or amplitude of the host responses (Zipfel, 2008; Zhang and Zhou, 2010; Dempsey and Klessig, 2012). Damage-associated molecular patterns (DAMPs) are molecules arising from necrotic, damaged or stressed cells, e.g., cutin monomers, small peptides, and cell wall fragments. Plants recognize these molecules in a similar manner as MAMPs and respond by activating defense signaling cascades (Herman et al., 2008; Yamaguchi et al., 2010; Liu et al., 2013; De Coninck et al., 2015). These plant defense responses are strictly regulated in order to minimize resource expenditure and fine-tune the signaling cascades. This crucial role is fulfilled by phytohormones like SA, JA, and ET as essential signaling molecules (Bartoli et al., 2013) for both local and systemic responses. It is important to note that this basic signaling defense is more complex because of other phytohormones including ABA, auxins, cytokinins, gibberellins, and brassinosteroids (BRs) that interplay in the background. Recently, even more plant signaling molecules such as azelaic acid (AZA), pipecolic acid (PIP), and strigolactones have been reported (Pieterse et al., 2009; Denancé et al., 2013; Vos et al., 2013). In order to establish an effective symbiotic relationship between plants and PGPR, these preformed barriers and innate immunity defenses have to be bypassed through chemical communication between plant and microbe (**Figure 1**).

Recent findings indicate that symbionts and pathogens deploy similar molecular strategies to dampen and overcome immune responses, and that the MAMP/PRR recognition system is also engaged in cooperative plant-microbe interactions with beneficial microbial communities that can lead to root colonization. This suggests a multifaceted management role by microbial communities of the innate immune system for controlled accommodation of beneficial microbes *vs.* pathogen elimination (Hacquard et al., 2017).

CHEMICAL COMMUNICATION WITHIN THE RHIZOSPHERE

The rhizosphere is one of the most complex ecosystems on earth and is inhabited by various organisms including nematodes, fungi, bacteria, and arthropod herbivores (Venturi and Keel, 2016). Compared to bulk soil, the rhizosphere is associated with increased bacterial abundance and activity, but lower diversity. Plants are known to effect a selective pressure on the microbial community found in the rhizosphere and community-level analysis have revealed differential microbial communities associated with different plant species. This suggests a definite role of plant-derived metabolites in the microbiome assemblage in the rhizosphere (Hacquard et al., 2017; Yang et al., 2017; Zhang et al., 2017). The common PGPR genera in the rhizosphere includes: *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, *Arthrobacter*, and *Paenibacillus* (Finkel et al., 2017; Sasse et al., 2017; Zhang et al., 2017).

Recent knowledge advancement in plant-beneficial microbe interactions has led to the development and commercialization of microbial inoculation (either one or a consortium) to improve plant health. These inoculants are natural or synthetic microbial communities (Johns et al., 2016). This is done in one of the

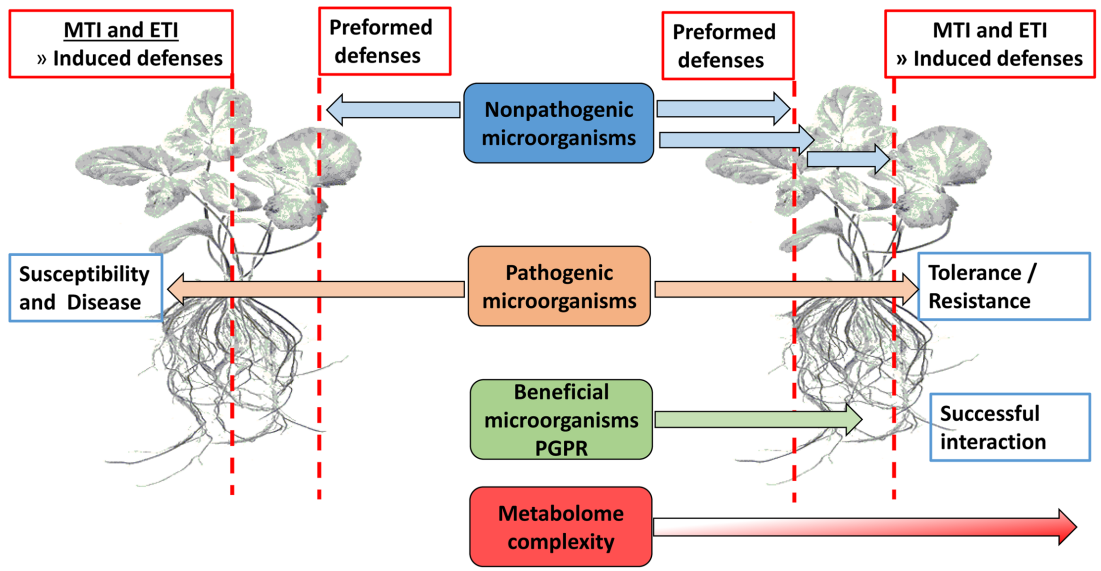


FIGURE 1 | Overview of physical barriers (waxes, suberin, callose, lignin, etc.) and innate immunity defenses (MTI and ETI) that presents obstacles to potential microorganisms in establishing a beneficial interaction with plant roots.

following ways: (1) introduction of new microbes into the soil, (2) manipulation of environmental factors (temperature, nutrients, moisture level, etc.), and (3) growing plants that will influence the soil microbe community (Finkel et al., 2017; Pineda et al., 2017).

These organisms interact with each other and with the plant in a sophisticated manner, achieved by chemical communication established in the rhizosphere (**Figure 2**). In response to altered gene expression, plants subsequently release an array of metabolites (primary and secondary). It is through such communication that mutual relationships are established that are vital for root–root interactions (Mommer et al., 2016), nutrient availability, microorganism accumulation, and biofilm formation of soil microbial communities (Rosier et al., 2016; Sasse et al., 2017), as well as inhibition of soil–borne pathogens (Bertin et al., 2003; Li et al., 2013). In this regard, metabolomic approaches have enabled researchers to identify and quantify compounds secreted by the microorganisms as well as profiling the metabolite 'blends' present in root exudates that play a vital role in this mutual interaction (**Figure 3**). The term 'signalomics' describes these metabolomics approaches employed to decipher the chemical communications occurring within the rhizosphere.

Bacteria-to-Bacteria Communication

Soil bacteria present in the microbiome assemblages produce an array of signaling metabolites that affect gene expression within the host plants, and these compounds have become an important and interesting subject for researchers. Here, VOCs are the well-documented signaling molecules within bacterial communities (**Figure 3**). These are low-molecular weight lipophilic compounds synthesized from different metabolic pathways and serve as a chemical window in which information is released (Kanchiswamy et al., 2015). Recently is has been shown that VOCs play a greater role in microbial communication than the non-volatile counterparts. Rhizobacteria produce numerous VOCs comprising alkanes, alkenes, alcohols, ketones, terpenoids and sulfur compounds. Furthermore, the metabolite complexity of the volatile profiles is attributed to species – or genotype – specific metabolism (Kanchiswamy et al., 2015; Tyc et al., 2015; Kai et al., 2016).

Colonization of plant roots by PGPR involve QS, a cell-to-cell communication mechanism through the release of signals to cognate receptors, thereby influencing gene expression in correlation to bacterial population density (González and Marketon, 2003; Hong et al., 2012; Helman and Chernin, 2015). These signals, also referred to as autoinducers, allows both intra- and inter-bacterial communication between different species (González and Marketon, 2003; Hassan et al., 2016).

Bacteria-to-Plant Communication

To establish a symbiotic relation with plants, rhizobacteria either secrete or emit molecules beneficial to the plant. These molecules, originating from the rhizosphere, are able to trigger specific changes or adjustments to the plant transcriptome. While phytohormones are growth – and defense regulators produced by plants, PGPR are also able to produce these compounds that include auxins, cytokinins, gibberellins, ABA, SA, and JA, among others (**Figure 3**) (Fahad et al., 2015). VOCs produced by PGPR are involved in maintaining soil health, plant growth modulation and resistance induction (Wei-wei et al., 2008; Kai et al., 2009). Certain plants are responsive toward various known VOCs produced by PGPR such as 2-heptanol, 2-endecanone, and pentadecane. For example, co-cultivation of *Arabidopsis thaliana* and two PGPR strains (*Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a) in Petri dishes (allowing diffusion of

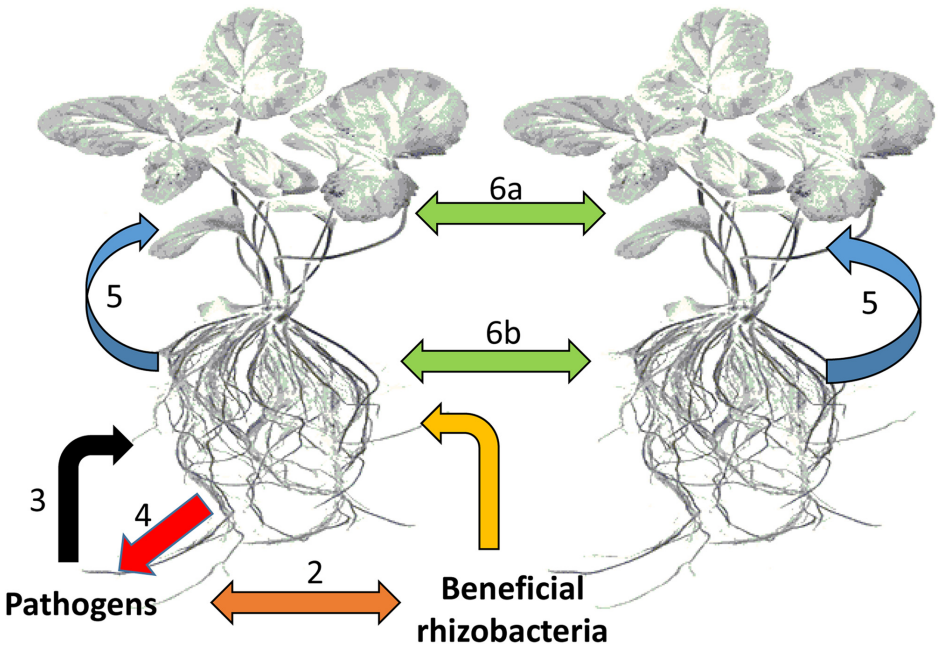


FIGURE 2 | Types of chemical-based interactions affecting plants. The plant host plus all its symbiotic microbes can be regarded as a community or an ecological unit (holobiont). (1) Interactions between plant roots and beneficial bacteria within the rhizosphere, (2) competitive interactions between beneficial bacteria and potential pathogens, (3) attack by potential pathogens on plant roots, (4) counter defense responses against pathogen attack, (5) communication between plants roots and leaves, and (6) interplant communication through leaves (6a) and roots (6b).

bacterial volatiles from one side to another) resulted in enhanced growth of *A. thaliana*. Here, 3-hydroxy-2-butanone (acetoin) and 2,3 butanediol were the common VOCs between the two strains (Ryu et al., 2003).

Quorum sensing is a population density mechanism used by bacterial communities to communicate and sense their environment. AHLs are the well documented QS signals frequently produced by Gram-negative bacteria (González and Marketon, 2003; Hassan et al., 2016). AHLs are perceived by plants and contribute to the establishment of a bacterial–plant symbiotic relationship (Schikora, 2016). UHPLC-MS (ultra-high performance liquid chromatography coupled to MS) methodology as described in the Section "Metabolomics: A Tool for Analysis of Plant Interactions with Rhizomicrobes" can precisely detect and quantify AHLs as well as the *N*-acyl homoserine degradation products, thereby enabling the study of signaling dynamics in QS (Rothballer et al., 2018).

Plant-to-Bacteria Communication

The chemical complexity of root exudates is dependent on a number of external factors such photosynthesis activity, plant size, and soil conditions. These secreted metabolites (**Figure 3**) are species- or genotype-specific and can be differentially modified depending on the secreting source. Given this strong complexity and specificity, root exudates have the potential to overlay a much more detailed layer of information about the communication events in the rhizosphere (Mommer et al., 2016; Sasse et al., 2017). Also, the chemical compositions of root exudates have a direct effect on the rhizosphere communities and it has been shown that specific plant species use these compounds to select soil microbe communities. For example, citric acid identified from cucumber root exudates attracted *B. amyloliquefaciens* SQR9 and cause biofilm formation. In addition, the banana root exudate fumaric acid attracted *B. subtilis* N11 and stimulated biofilm formation (Zhang et al., 2014). Studies have also shown that strain growth and antifungal activity of certain *Pseudomonas* spp. is dependent on organic acids and sugars isolated from tomato root exudates (Kravchenko et al., 2003).

Another class of compounds found in the root exudates are flavonoids (i.e., 2 phenyl-1,4-benzopyrone derivatives) which induce bacterial *nod* genes, thus leading to lipo-chitooligosaccharides (LCOs) that initiate nodule formation in the roots. Interestingly, LCO also plays a role in interactions between arbuscular mycorrhizal fungi and plants. Furthermore, these flavonoids are able to mimic bacterial QS molecules, thus influencing bacterial metabolism (Hassan and Mathesius, 2012). QS plays an important role in bacterial genotype and phenotype regulation for successful root colonization (Rosier et al., 2016). Different types of low carbon molecules are also present in the root exudates; these molecules serve as precursors for biosynthesis of PGPR phytohormones. Tryptophan, which is a precursor for indole-3-acetic acid, is concentrated in the root tip region (Haichar et al., 2014). In addition, the ET precursor, aminocyclopropane-1-carboxylic acid (ACC), also exudes from plants and can be used as a source of nitrogen and carbon by PGPR (Haichar et al., 2012).

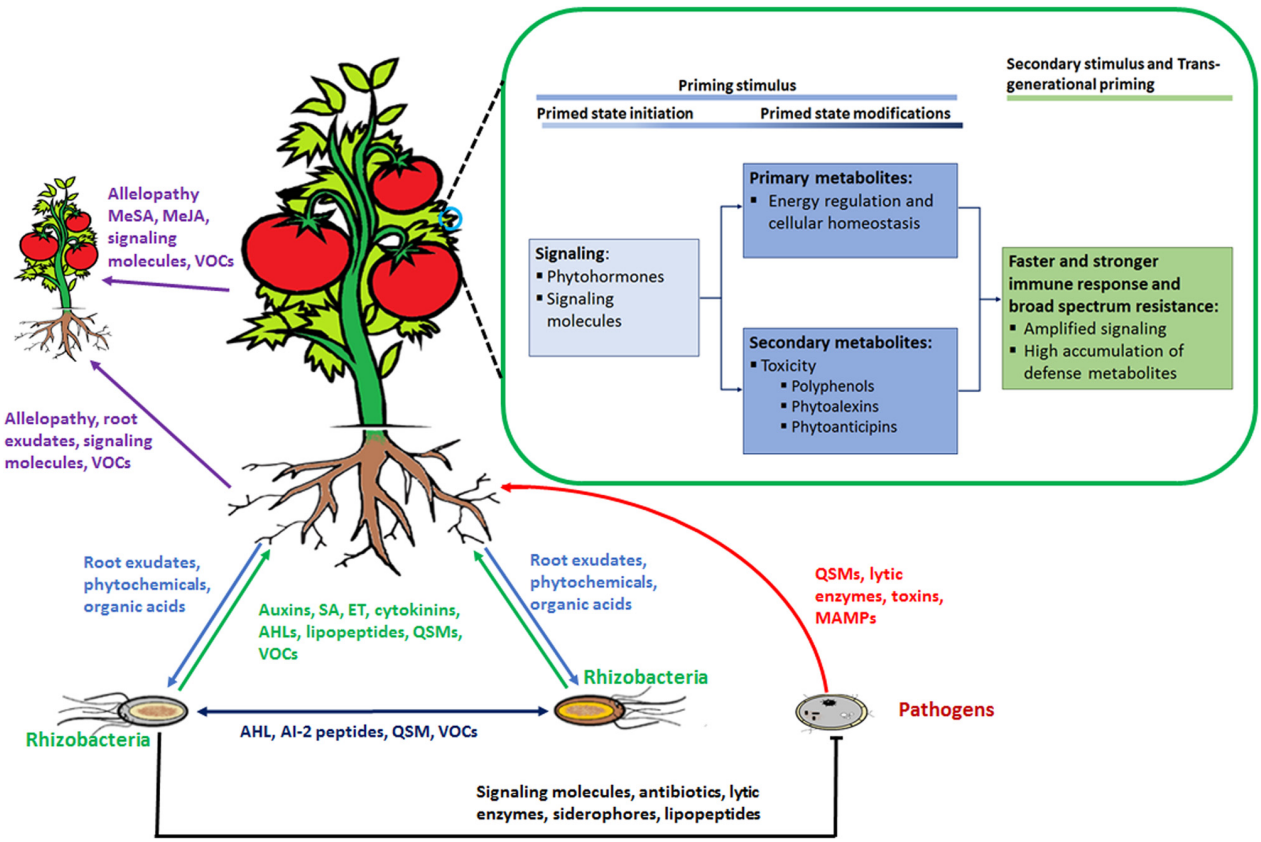


FIGURE 3 | Rhizosphere plant and microbial 'signalomics'. Plants and rhizomicrobes secrete compounds beneficial to each other to establish mutual relationships. This below–ground interaction, in turn, primes plants against various environmental stimuli that includes abiotic as well as biotic stresses. Perception of the priming stimulus leads to activation of signaling molecules, primary metabolism regulation and gene activation of enzymes involved in the production of secondary defense metabolites. When a secondary stimulus is detected the same process as in the priming stage takes place but at an enhanced level to minimize impact on the plant. Plants are able to pass on the induced primed state to their progeny in a process known as *trans*-generational priming. In addition, plants communicate with each other using allelopathic molecules. Abbreviations: volatile organic compounds (VOCs), quorum sensing molecules (QSM), *N*-acyl homoserine lactones (AHL), SA, methylsalicylic acid (MeSA), methyljasmonic acid (MeJA) ET.

Plant-to-Plant Communication

Communication between plants occurs below- and above-ground (**Figure 2**), either through secretion/release of certain signaling molecules (Mommer et al., 2016). Root exudates released into the rhizosphere contain a blend of signaling molecules that are transmitted to neighboring plants (Badri and Vivanco, 2009). However, root–root interactions are mostly studied in the context of species competition and invasive plants. Allelopathy is the most prominent probability, in which plants release phytotoxins such as catechin (a flavan-3-ol flavonoid). This compound is able to mediate intraspecific and interspecific interactions, and to inhibit establishment and growth of neighboring plants, thus reducing competition and increasing nutrient availability (Thorpe et al., 2009; Mommer et al., 2016). Compounds with allelopathic effects belong to one of the following chemical classes: benzene-derived compounds, phenolics, hydroxamic acids, and terpenes (Badri and Vivanco, 2009; Massalha et al., 2017). On the other hand, VOCs are the most studied allelochemicals in plant-plant interactions. VOC-mediated signaling in the rhizosphere is believed to occur through common mycorrhizal networks between plants, protecting them against degradation and enhancing plant-to-plant transmission. Beside rhizosphere signaling, plants do secrete their own VOCs into the air that are carried to neighboring plants (**Figure 3**).

RHIZOSPHERE DEFENSE AND PRIME-INDUCING COMPOUNDS

Over the years, PGPR have been extensively studied for plant growth promotion and ISR induction, and are promising alternatives to chemical fertilization, pesticides, and herbicides (Kloepper et al., 2004; Gupta et al., 2015). PGPR effect beneficial properties through direct mechanisms (i.e., nitrogen fixation, mineral solubilization and biosynthesis of phytohormone and siderophore production) and indirect mechanisms (production of antibiotics, hydrolytic enzymes, siderophores, LPs, and ISR) (Beneduzi et al., 2012; Garcia-Fraile et al., 2015; Gupta et al., 2015). Here, we look at the major classes of molecules secreted

by PGPR that are involved in plant protection against soil-borne pathogens and induction of ISR/RMPP.

Antibiotics and related molecules are secreted by certain bacteria and have the ability to inhibit pathogen growth at low concentrations (**Figure 3**). Such compounds from *Bacillus* and *Pseudomonas* genera are the best studied in disease management (Haas and Défago, 2005; Saha et al., 2012). For example, 2,4 diacetylphloroglucinol (2,4 DAPG) is an antibiotic produced by *P. fluorescens* that has a 75% inhibition effectiveness against the soil-borne pathogen *Sclerotium rolfsii* (Asadhi et al., 2013). Phenazine-1-carboxylic acid (PCA) is another antimicrobial compound secreted by the same organism and causes oxidation-reduction and accumulation of superoxides in target cells. This molecule is effective against wheat disease caused by *Gaeumannomyces graminis* var. *tritici* and *S. rolfsii*, causing stem rot in groundnut (Lohitha et al., 2016). Another novel antibiotic from *B. subtilis* is zwittermicin which is effective against a spectrum of soil-borne pathogens (Saraf et al., 2014). Several bacteria secrete hydrolytic enzymes, e.g., proteases, glucanases, chitinases, lipases and amylases. These enzymes degrade numerous cell wall components of fungi and oomycetes (Bull et al., 2002; Saraf et al., 2005, 2014).

Various PGPR such as *Bacillus* spp. and others, produce LPs (either linear or cyclic LPs) that act as antibiotics. These are classified into three families: iritin, fengycin and surfactin depending on the branching fatty acid (Saha et al., 2012; Saraf et al., 2014), and have antagonistic effects against a wide range of soil-borne pathogens. Besides being antagonistic to pathogens, LPs such as fengycin, surfactin and iturin are capable of inducing immune responses in plants by acting as bacterial determinants (Ongena et al., 2007; Romero et al., 2007). The role of these molecules in ISR/RMPP has been studied on various plants. In bean and tomato both purified and compounds from producing strains were found to induce immune responses or prime plants (Ongena et al., 2007). *B. subtilis* S499 can prime cucumber plants against *Colletotrichum lagenarium*. However, plants treated with semi-purified LPs were susceptible to *C. lagenarium* (Ongena et al., 2005a). Recent studies on LPs involvement in immune responses strongly show that these molecules are involved in ISR or RMPP. For example, cyclic LPs purified from *B. amyloliquefaciens* subsp. *plantarum,* isolated from the lettuce rhizosphere, primed plants against *Rhizoctonia solani* (Chowdhury et al., 2015).

Siderophores are low molecular weight compounds synthesized by microorganisms under iron limiting conditions. With high membrane permeability, siderophores act as ferric ion transport vehicles into microbial cells (Butler and Theisen, 2010). The common iron-binding substances in these compounds include hydroxamic acid, hydrocarboxylic acid, and catechols, as well as other related structures (Pattus and Abdallah, 2000; Butler and Theisen, 2010; Ahmed and Holmström, 2014). Siderophore production is beneficial to plants (directly supply iron to plants) and is implicated in soil-borne disease suppression (reducing competitiveness of soil-borne pathogens) (Tank et al., 2012). A mutant of *P. putita* over-expressing siderophores was more effective against *Fusarium* wilt in tomato when compared to a siderophore-deficient mutant of *P. aeruginosa* which lost its biocontrol ability. Furthermore, *B. subtilis*-produced siderophores exhibit antagonistic effects against wilt and dry root rot- causing fungi in chickpea (Patil et al., 2014). Also, purified siderophores had similar disease suppression activity to those observed from the producing strains.

Several reports have demonstrated that AHLs can influence plant physiological processes such as root elongation (Bai et al., 2012), plant perception (Han et al., 2016), and induce a broad spectrum resistance (Schikora, 2016). Plant priming by AHLs has recently been documented with reports that even commercial available pure AHLs also induce priming in plants (Schenk et al., 2014). For examples, both short and long chain AHLs produced by *Serratia liquefaciens* strain MG1 and *P. putida* strain IsoF primed tomato plants against *A. alternata* via SA and ET defense pathway (Schuhegger et al., 2006). Also, in barley endophytic *Acidovorax radicis* N35 rhizobacteria producing 3-hydroxy-decanoyl-homoserine lactone induced defense responses and caused accumulation of flavonoids such as saponarin and lutonarin (Han et al., 2016).

Among the metabolites produced by PGPR, volatiles are small molecules that can effectively promote plant growth, induce resistance and inhibit growth of pathogenic organisms (Ryu et al., 2004; Beneduzi et al., 2012; Song and Ryu, 2013). For example, volatiles emitted by different rhizobacterial isolates were reported to inhibit mycelial growth of *Rhizoctonia solani* (Kai et al., 2007). High vapor pressure volatiles are able to diffuse in the soil (Insam and Seewald, 2010), which gives these compounds an advantage to act at distance. *In vitro*, volatiles from four *Bacillus* and *Paenibacillus* spp. showed intensive antagonistic activities against soil-borne pathogens including *Ascochyta cutrillina*, *Alternarai solani*, and *A. brassicae*. From GC-head space analysis, four metabolites namely 2,4 decadienal, oleic acid, diethyl phthalate, and *n*-hexadecanoic acid showed overlapping presence among the strains (Wei-wei et al., 2008).

Plants rapidly recognize both potential pathogens and PGPR (**Figure 1**) in a similar manner based on MAMPs such as lipopolysaccharide (LPS) and flagellin, and secondary metabolites. MAMPs from beneficial microbes are known to activate MTI, but in this case, the activated defenses do not ward off the beneficial microbes (Van Wees et al., 2008). This is not fully understood, but might involve the nature of the complex chemical communication involved in rhizobacteria-plant interactions. As mentioned, PGPR produce plant signaling molecules such as auxins, cytokinins, gibberellins, ABA, SA, ET, and JA (Fahad et al., 2015). It is well known that SA, ET, and JA cross-communicate to fine-tune the defense response, depending on the detected stimulus (Dempsey and Klessig, 2012; Derksen et al., 2013). SA is an interesting signaling molecule produced by certain PGPR. For example, several *Pseudomonas* spp. produce SA under low iron conditions which is channeled toward SA-containing siderophores (Mercado-Blanco and Bakker, 2007). However, SA produced by *P. aeruginosa* (siderophore producing mutant KMPCH) was shown to induce systemic resistance (Audenaert et al., 2002; Verhagen et al., 2010). Thus, both MAMPs and SA are involved in RMPP.

In response to different stimuli, plants emit numerous VOCs with signaling and inhibitory properties. These within-plant VOCs signaling leads to induction and priming of plant defense (Heil and Silva Bueno, 2007). Among other volatiles profiled in head-space experiments, MeSA, MeJA, and *cis*-jasmone (CJ) are well documented volatile signaling molecules (Heil and Silva Bueno, 2007). These were found to induce plant defense and priming against herbivore-feeding in wild lima beans. CJ has been tested on various plants and it has been shown to induce production of defense-related VOCs such as (*E*)-ocimene, 6-methyl-5-hepten-2-one and (*E*)-(*1R,9S*)-caryophyllene (Pickett et al., 2007). Also, (*Z*)-3-hexen-1-ol was found to have a two-fold priming effect and modulation of herbivorous insect behavior (Wei and Kang, 2011). Even though progress has been made in understanding the involvement of plant VOCs in signaling, attraction of predators and pathogen inhibition, there is no knowledge on plant VOCs induced in response to ISR/PGPR-priming by rhizomicrobes. However, metabolomic studies have shown that regardless of the perceived stimulus, similar metabolic pathways are activated (Pastor et al., 2014; Balmer et al., 2015; Mhlongo et al., 2016a,b). Thus, such studies suggest that the blend of signaling VOCs is the same/similar, leading to production of defense metabolites within the producing plant as well as in distal plants.

ISR/RHIZOMICROBE PLANT PRIMING (RMPP)

In the rhizosphere, a complex relationship exists among plants, soil microbes, and soil (Van Dam and Bouwmeester, 2016). The microbial diversity (population and activity) in this zone is influenced by physical, chemical and biological properties of the root-associated soil (Barea et al., 2002). The rhizosphere is inhabited by both deleterious and beneficial microbes (**Figure 2**) that can significantly influence plant growth and crop yield (Beneduzi et al., 2012; Vacheron et al., 2013; Garcia-Fraile et al., 2015). The beneficial microbes include symbiotic bacteria, free-living bacteria, actinomycetes, and mycorrhizal fungi that increase nutrients/plant growth enhancer availability and suppress soil-borne pathogens (Garcia-Fraile et al., 2015). Diverse genera of PGPR dominated by *Bacillus* and *Pseudomonas* spp. have been identified, and are the most desirable beneficial group for their variable qualities such as plant growth promotion, disease control and bioremediation. The mechanisms utilized by PGPR to suppress diseases and herbivores as well as priming of plants, have been critically studied and reviewed over the last few years (Saraf et al., 2005, 2014; Pineda et al., 2010; Beneduzi et al., 2012). PGPR may either directly (inhibition of metabolism) or indirectly (through competition) reduce soil-borne pathogen infections. Some PGPR such as *Bacillus* and *Pseudomonas* spp. synthesize antibiotics that are active against various bacterial and fungal pathogens, toxins against insect pests, lytic enzymes that inhibit soil-borne pathogen growth, and siderophores. Production of cyanogenic compounds have also been shown to repel both root and leaf herbivores. Lastly, PGPR present in the rhizosphere may prevent plant diseases by competing for available nutrients, preventing contact between the pathogen and the plant root, or by interfering with the mechanisms leading to plant infection (Saraf et al., 2005, 2014).

The concept of plant priming dates back to 1901 when Beauverie and Ray showed that plants infected by a pathogen developed an enhanced defense response against secondary infections. This lead to the realization that plants can be sensitized/primed to produce an enhanced defense response, thereby making the plants more resistant to secondary environmental stresses. While it is evident that plant defense can be induced and may lead to less resource expenditure (reduced fitness cost), the success depends on the appropriate activation of defenses that can be faster, earlier, more sensitive, or stronger. These timeous activation of suitable defense responses in primed plants can save plants from becoming diseased or consumed, thus adding a benefit of off-set the cost of establishing the primed condition (Conrath et al., 2009, 2015; Tanou et al., 2012; Hilker et al., 2015).

Studies using PGPR have identified genes associated with ISR/RMPP. For example, transcriptome analysis of *P. fluorescens* WCS417r-ISR hosting plants showed systemic expression of defense genes when compared to the control, and *P. syringae* infection led to identification of genes (mostly JA- and ET-regulated genes) with more enhanced expression than non-ISR expressing plants (Bakker et al., 2007; Van Wees et al., 2008; Conrath et al., 2009; Segarra et al., 2009; Vacheron et al., 2013). Also, ISR/RMPP can be induced by PGPR volatiles without the organisms being in contact with the roots. *Bacillus* spp. producing volatiles such as 3-hydroxy-2-butanone and (2R,3R)-(-)-2,3-butanediol were found to prime *Arabidopsis* plants against pathogen infections and herbivore attack (Conrath et al., 2001; Farag et al., 2013; Song and Ryu, 2013; Yi et al., 2013).

Priming can also be a result of epigenetic changes from small interfering RNA (siRNA) or DNA recombination caused by environmental stresses (Bruce et al., 2007; Pastor et al., 2012). This form of protection is present in the genetic material of the species and would last longer in plants compared to accumulation of metabolites. Since plants are not capable of communication with their progeny, a mechanism is required to alert against possible stresses that may be encountered in nature (Holeski et al., 2012). It was not until the early 1980s when *trans*-generational studies were conducted showing that inoculation of a plant with a disease-causing agent induces resistance in their progeny not only to the administered agent, but to a wide spectrum of pathogens (Pieterse, 2012; Slaughter et al., 2012). In addition, other studies showed that plants that have been infected by a pathogen produce seeds with higher levels of phytoalexins than controls. Epigenetic changes or *trans*-generational priming can be inherited by the progeny, where it then controls expression of defense genes (Holeski et al., 2012). In a study where *Arabidopsis* plants were primed with β-aminobutyric acid (BABA) or by MAMPs from *P. syringae,* the progeny showed high levels of defense gene expression *via* the SA-dependent pathway and was resistant to *P. syringae* and *Hyaloperonospora arabidopsidis.* These progenies also had a stronger priming phenotype than

the parents. *Trans*-generational priming is achieved through defense response memorization and propagation (in both meiosis and mitosis) by the parents (Luna et al., 2012; Pastor et al., 2012; Slaughter et al., 2012; Po-Wen et al., 2013). However, since there are many mechanisms associated with priming and research aiming at these are still underway, it is not clear how this memorization occurs. The involvement of chromatin modifications adds to the other metabolite-based mechanisms since it is directly linked to gene expression patterns that can be inherited by the offspring.

KEY METABOLIC EVENTS IN DEFENSE PRIMING

The priming ability of PGPR is associated with cell wall modification, expression of defense genes, primary metabolite modification and biosynthesis of secondary metabolites (Conrath et al., 2009). As shown in **Figure 3**, priming can be divided in to three major events: (1) perception of the priming stimulus, (2) secondary stimulus, and (3) *trans*-generational priming. The early stages of priming involve signaling by phytohormones and other signaling molecules. Phytohormones are well-documented plant metabolites involved in different stages (**Figure 3**) of plant defense responses or plant priming (Dempsey and Klessig, 2012; Denancé et al., 2013). For example, JA and ET are major hormones in ISR/PGPR priming induction, while SA is the major hormone involved in systemic acquired resistance (SAR). Other phytohormones such as cytokinins, auxins, ABA, gibberellins, and brassinosteroids are reported to play a role in plant resistance but the significance of these molecules is not well understood (De Vos et al., 2005; Koornneef and Pieterse, 2008; Pieterse et al., 2009; Naseem and Dandekar, 2012; Denancé et al., 2013; Uhrig et al., 2013). These hormones interact either antagonistically or synergistically with the SA-JA-ET signaling backbone and reprogram the defense output (Koornneef and Pieterse, 2008; Verhage et al., 2010; Naseem and Dandekar, 2012).

Using *P. fluorescens* as inducer, a total of 50 metabolites were differentially regulated in ISR-induced *Arabidopsis* plants. Amongst these, amino acids and sugars were the differentiated primary metabolites (van de Mortel et al., 2012). ISR/PGPR priming studies are mostly based on molecular rather than metabolomics approaches. Hence, knowledge about metabolome changes during ISR/PGPR priming and the significance thereof, is limited. However, the metabolic events in priming in response to chemical elicitation are more similar, despite the use of different stimuli (Pastor et al., 2014; Balmer et al., 2015; Mhlongo et al., 2016a,b). As such, metabolic studies employing other agents may be used to explain the role of both primary and secondary metabolites in plant priming (Djami-Tchatchou et al., 2017).

The main role of primary metabolism during plant defense is to supply energy for the initiation of plant priming and in the synthesis/activation of phytohormones, phytoanticipins, and phytoalexins. Here, the energy referred to is required for different processes such as defense gene expression of various defense pathways, plant metabolism regulation and resource re-channeling toward defense. As a result, plant priming responses are associated with minor fitness costs when compared to naïve plants. Thus, priming activation leads to temporal down regulation of other metabolic pathways. Recently it has been shown that both signaling molecules (Mhlongo et al., 2017) and secondary metabolite conjugates accumulated during the priming stage (Mhlongo et al., 2016a,b), and can be converted to their active forms when a secondary stress is detected. Glycosylated signaling molecules, specifically that of AZA, SA, and MeSA, were found to accumulate during LPS-induced priming of tobacco cells (Mhlongo et al., 2017). Also, glycosylation of hydroxycinnamic acids was observed in tobacco cells treated with both chemical and pathogen-derived priming agents (Mhlongo et al., 2016a,b). Besides sugar conjugation, the respiratory cycle and tricarboxylic acid cycle (TCA) are also affected by priming activation (Gamir et al., 2014). In this regard, TCA intermediates (citrate, malate, 2-oxalate) were found to over-accumulate in BABA-induced priming. Furthermore, amino acids serve as building blocks for many secondary metabolites such as SA, polyamines, tyramine, alkaloids, and phenylpropanoids.

Secondary metabolites play an important role in plant defense systems and environmental adaptation, and their presence fluctuates in response to different environmental stimuli (Dörnenburg, 2004). As discussed above, PGPR are able to trigger secondary metabolism by means of different chemical molecules. Many studies have shown that mycorrhizal or rhizobacterial root colonization quantitatively modify phenolic compounds, alkaloids, terpenoids, and essential oils in plants (Toussaint et al., 2007; Araim et al., 2009; Ramos-Solano et al., 2015). Using nine PGPR strains on blackberry plants, Ramos-Solano et al. (2015) showed that phenolics, flavonoids, and anthocyanins were the modified secondary metabolites associated with delayed post-harvest fungal growth on berries. Other secondary metabolites such as coumarins and flavonoids also quantitatively changed in plants associated with PGPR (van de Mortel et al., 2012). In maize significant changes in benzoxaninones were observed in plants associated with mycorrhizal or rhizobacterial colonization (Song et al., 2011). Also, maize root inoculation with *P. putita* KT2440 induced metabolic changes and systemic resistance in the plants. The early responses were *via* JA- and ABA-dependent pathways, and phospholipids were highlighted as the important metabolites in the KT2440 interaction. Lastly, benzoxaninones were differentially abundant in roots after 3 days (Planchamp et al., 2014).

Microbial compounds such as LPs and AHLs can also prime plants through modification of secondary metabolites (Ongena et al., 2005a; Schenk et al., 2014; Chowdhury et al., 2015; Han et al., 2016). LP-overproducing *Bacillus* activated the lipoxygenase enzyme (LOX) regulated pathway (Blée, 2002). In potato tuber cells, fengycin treatments resulted in activation of phenylpropanoid pathway metabolism (Ongena et al., 2005b). Moreover, AHLs stimulated callose deposition and accumulation phenolics, oxylipins and SA in several plant species (Schenk et al., 2014; Schikora, 2016).

Plants are capable of maintaining the primed state throughout their life cycle and passing it on to the next generation (*trans*-generational priming) (**Figure 3**) (Luna et al., 2012; Pieterse,

2012; Munné-Bosch and Alegre, 2013; Mauch-Mani et al., 2017). Epigenetic modification is the well-documented *trans*-generational priming mechanism (Gamir et al., 2014; Mauch-Mani et al., 2017). The few reports available on metabolomics related to *trans*-generational priming suggest that phytohormone levels are not modified in the progeny of primed plants (Luna et al., 2012). However, Mandal et al. (2012) showed that progeny resistant to tobacco mosaic virus (TMV) had enhanced levels of primary metabolites, particularly sucrose, glucose, and fructose and the amino acids; ala, val, ser, thr, gln. Despite the lack of documented metabolomic work describing *trans*-generational priming, Gamir et al. (2014) suggested that this process is highly dependent on the characteristics of the pathogen. For example, biotrophic stimuli mainly impact primary metabolism while insects and necrotrophic fungi trigger secondary metabolism *via* JA/ET-dependent pathways.

METABOLOMICS: A TOOL FOR ANALYSIS OF PLANT INTERACTIONS WITH RHIZOMICROBES

Metabolomics, an array of advanced bio-analytical techniques in conjunction with chemometrics and bioinformatics tools, enables characterization of the perturbations to the metabolomes of interacting organisms (Tugizimana et al., 2013) (**Figure 4**). As stated, the rhizosphere can contain a spectrum of different microbial communities, constituting very complex chemical environments. Metabolomics, as a data-driven, hypothesis-generating scientific approach with the aim to detect and quantify 100s of compounds per analysis (Lloyd et al., 2015), is ideally suited to the analysis of complex interactions and promises to facilitate the modeling of reciprocal responses between plants and organisms within the rhizosphere.

Conceptually, and following a reductionist approach, the tritrophic interaction between plant, rhizomicrobe, and pathogen can be studied separately and in isolation. For example, a co-culture metabolomics approach has been proposed (Allwood et al., 2010) to assess the intracellular metabolomes (metabolic fingerprints) of both host and pathogen and their extruded (extracellular) metabolites (metabolic footprints). However, in order to fully evaluate the changes occurring in the host plant due to these tritrophic interactions under conditions relevant to disease and resistance, there is a need for combining the information provided by different techniques, including metagenomics and metametabolomics (Heinken and Thiele, 2015; Jorge et al., 2016; Ofaim et al., 2017). This novel approach to metabolomics analyses of host–pathogen interactions will facilitate a greater understanding of both their independent metabolism and the metabolic cross-talk which represents the interactome.

Recent advances on both analytical instrumentation and –analysis with high selectivity, accuracy, and robustness, and combined with data processing software developments and availability of public databases, have facilitated this endeavor. Thus, these progressions have enabled researchers not only to study one aspect of a biological system, but also the interaction with the surroundings (Rochfort, 2005; Lloyd et al., 2015; Tenenboim and Brotman, 2016; Van Dam and Bouwmeester, 2016). Below we summarize the main events in an adaptable metabolomics workflow suitable for the study of plant–microbe interactions and highlight some analytical advances (**Figure 5**).

Sample Preparation

The sample preparation method(s), to a large extent, determines the type of compounds to be detected. Sample preparation for any metabolomics study comprises several steps mostly dictated by the chosen analytical platform. The main steps include material harvesting at a specific time and quenching to minimize metabolic turnover rates. Next, metabolite extraction with organic solvents or solid phase extraction is performed, taking the matrix in which the metabolites occur into account. This is followed by pre-analytical sample preparation (concentration, purification or derivatization) (Tugizimana et al., 2013; Jorge et al., 2016).

Separation and Detection

Gas chromatography, liquid chromatography (LC), and capillary electrophoreses (CE) coupled to MS have developed into the preferred bio-analytical platforms used in metabolomics (Naz, 2014). GC-MS is usually coupled to a quadrupole (Q), qTOF, and QqQ mass analyzers. In recent years, TOF analyzer interest has grown due to the ability to provide high mass accuracy, higher duty cycles and fast data acquisition in comparison to Q analyzers (Lei et al., 2011; Jorge et al., 2016). QqQ analyzers enable easy compound identification and quantification, and overcome analyte co-elution due to the ability to perform multiple reaction monitoring (MRM) (Gomez-Gonzalez et al., 2010; Lei et al., 2011; Dzier et al., 2012). Recently, GC-MS analysis using stable isotope probing (SIP) has enabled the elucidation of rate limiting steps in metabolic pathways (You et al., 2014). The innovation of GCxGC, using two different stationary phases, provides high separation efficiency and peak capacity, and the generated narrow peaks require a fast scanning mass analyzer such as TOF or semi-fasts scan Q (Adahchour et al., 2005; Jin et al., 2015). One major setback of GC-MS is that it only analyses volatile and thermally stable compounds. To overcome this, derivatization (chemical modification) of molecules with -OH, -COOH, -NH, and -SH functional groups by silylation reagents is employed (Villas-Bôas et al., 2011; Abbiss et al., 2015).

LC-MS column chemistry selection and retention mechanisms makes it the technique most used to complement GC-MS. Most LC-MS applications use reverse phase (RP) and normal phase (NP) stationary phases with eluates eluted with a mobile phase mixture (e.g., organic solvents and water) (Haggarty and Burgess, 2017). Other column chemistries include hydrophilic interaction (HI), ion-exchange (IE), and porous graphitic carbon (PGC) (West et al., 2010). Advances in column dimension and particle size (core-shell and monolithic) has enabled researchers to analyze a wide range of different analytes with high separation efficiency at a high speed (Sanchez et al., 2013; Hayes et al., 2014; Preti, 2016; Urio and Masini, 2015). These column developments lead to the expansion of ultra-high performance liquid chromatography (UHPLC). This chromatography format

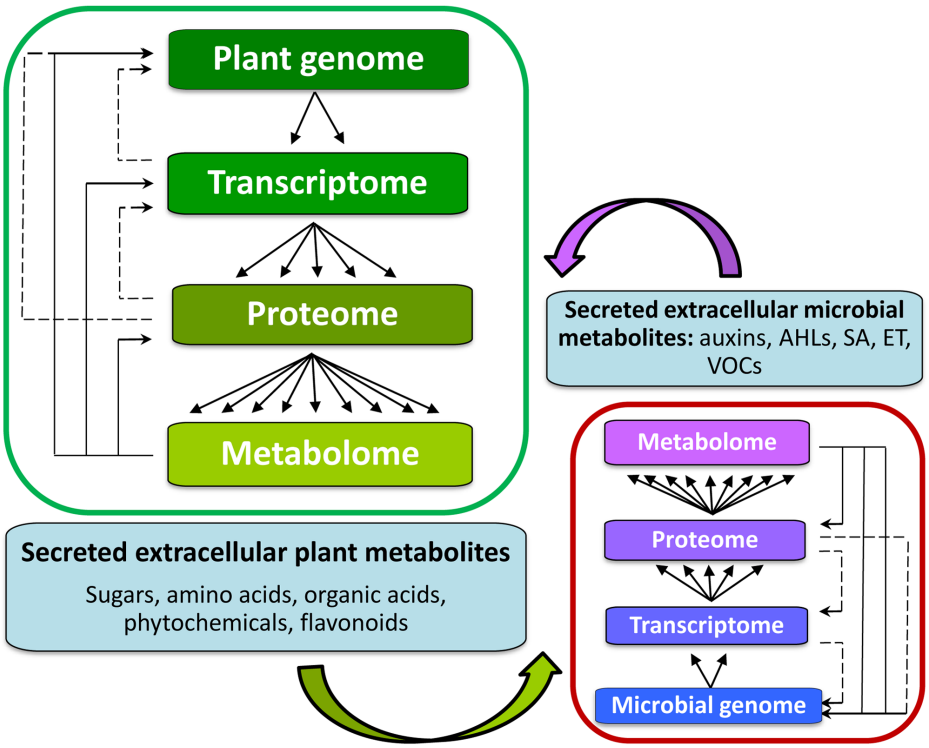


FIGURE 4 | Background for metabolomics studies of signaling in the rhizosphere between plant hosts **(left)** and microorganisms within the rhizosphere **(right)**. The inter-organismal communication affects the biological information flow from genome to metabolome. The metabolome is complementary to the transcriptome and proteome, captures the functional, or physiological state of the cell, and provides a communications link between genotype and phenotype. Metabolites also form part of the regulatory systems in an integrated manner (solid lines indicating regulatory loops). Altered gene expression is ultimately reflected in changes in the pattern and/or concentration of metabolites. It is through these interactions amongst the members of the central dogma components, that a cell acquires its full functionality of its cellular metabolism.

is similar to HPLC except that it uses a column with particle size $\leq$2 μm, small column diameter (1–1.2 mm) and operates at high pressure (Sanchez et al., 2013; Fekete et al., 2014; Walter and Andrews, 2014). Electron spray ionization (ESI) is the most popular ionization method preferred in biochemical analysis. This is because it is a soft ionization technique with little internal energy, thus allowing accurate mass determination. Alternatively, by increasing the collision energy, fragmentation can be obtained leading to structural information (Hird et al., 2014; Madala et al., 2014; Ncube et al., 2014). Collision-induced dissociation (CID) with inert gases (He, Ar, or N_2) is used to obtain more structural information and this is referred to as tandem MS^n experiments (Nizkorodov et al., 2011; Madala et al., 2014). Tandem MS^n instruments either perform tandem MS^s in-time [Ion Trap, Orbitrap, Fourier-transform-ion cyclotron resonance MS (FT-ICR-MS) or in-space (qTOF and QqQ)]. In-time refers to the ability to perform multiple stages of MS achieved by allowing ions from the ion source into the ion trap followed by fragmentation to generate diagnostic information. On the other hand, in-space refers to instruments with two mass analyzers separated by a collision cell which allows two MS stages (Hird et al., 2014).

CE separates compounds based on charge and size, and offers high resolving power. CE-MS is mainly used for intermediate primary metabolic pathways (glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway) and is usually coupled to a TOF mass analyzer (Ramautar et al., 2015, 2016).

In recent years, MS imaging (MSI) has been advanced and applied in different metabolic studies. MSI is a new imaging technique that provides the distribution of compounds on the surface (cells, tissue, or specific sections). Here, a two or three dimensional image is created by taking measurements across an individual pixel basis (Wheatcraft et al., 2014; Heyman and Dubery, 2016; Rao et al., 2016). Compared to other traditional molecular imaging techniques, MSI allows a greater amount of information to be obtained by providing well-resolved feature distribution for a wide range of metabolites (Schwamborn, 2012). MSI techniques are divided into non-ambient and ambient approaches. Non-ambient approaches such as matrix assisted laser desorption ionization (MALDI) MS (Heyman and Dubery, 2016) and TOF secondary ion MS (TOF SIMS) have high sensitivity and spatial resolution (Fletcher et al., 2013; Park et al., 2015). These approaches are, however, time-consuming due to the extensive sample preparation and may introduce errors (Park et al., 2015; Heyman and Dubery, 2016; Rao et al., 2016). On the other hand, ambient approaches (desorption electrospray ionization (DESI) MSI, laser ablation electrospray ionization (LAESI) MSI, air-flow-assisted

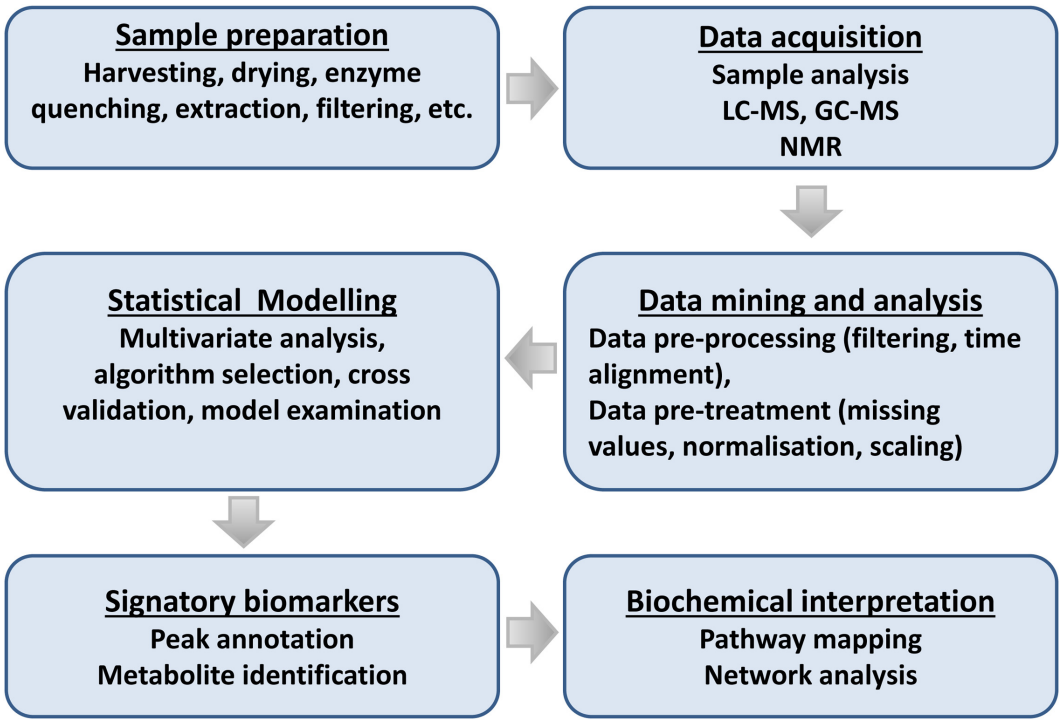


FIGURE 5 | Flowchart for plant metabolomic studies. The three main steps of a metabolomic analysis are sample preparation, data acquisition, and data mining. These three steps are interrelated and lead to the discovery of signatory biomarkers, metabolite annotation, and biochemical interpretation.

desorption electrospray ionization (AFADESI)-MSI, and nano-DESI MSI), requires less sample preparation and thus produce images of a native state. However, this native state analysis comes with low sensitivity and resolution compared to non-ambient approaches (He et al., 2015; Rao et al., 2016). Recently, single cell analysis (SCA), also referred to as single cell MS (SCMS), has received more attention due to the ability to provide chemical composition of biological samples at cellular level. SCA uses non-ambient, ambient and direct extraction (live single-cell video MS) approaches (Fujii et al., 2015; Onjiko et al., 2015; Rao et al., 2016).

Data Analysis and Visualization

Metabolomics generates large amounts of complex datasets that require both storage and data processing tools (reduction of data complexity) (Okazaki and Saito, 2012; Berg et al., 2013; Tugizimana et al., 2014). This can be achieved by using free statistical tools such as MarVis1, Mzine, XCMS, MAVEN, Metaboanalyst, MetAlign (Benton et al., 2008) as well as commercial software such as Markerlynx (Waters), Profiling solutions (Shimadzu), Mass profiler pro (Agilent) and Metabolic profiler (Bruker). Such tools focus on homogenous information generation for further statistical analysis. Each calculated *m/z* ion is defined by the same variables that only correspond to it. Recently, Kuich et al. (2015) developed a software (Maui-VIA) specifically for GC-MS data processing. The second step of data analysis involves the application of multivariate statistical tools to reduce data dimensionality, variables discrimination and to reveal shared features among samples (sample clustering). The widely used chemometric methods are unsupervised clustering [principal component analysis (PCA)] and supervised [orthogonal projection to latent structures discriminant analysis (OPLS-DA)] (Trivedi and Iles, 2012; Worley and Powers, 2013).

Metabolite Annotation and Identification

Metabolite identification is the ultimate goal of any untargeted metabolomics study. Over the years, databases incorporating mass spectra, compound names and structures, statistical models and metabolic pathways have been developed. Such databases complement each other, however, a restricting factor is that the information is scattered and limited by the number of identified metabolites (Fukushima and Kusano, 2013; Sakurai et al., 2013, 2014). Recently, a number of databases incorporating MS or nuclear magnetic resonance (NMR)-based metabolomics and statistical tools have been developed, i.e., MeRy-B, MeltDB, and SetupX (Ferry-Dumazet et al., 2011; Fukushima and Kusano, 2013). Also, a number of integrated databases (e.g., PlantMetabolomics.org) are also emerging (Bais et al., 2010). These include full annotation of metabolites, metabolic profiling and statistical tools. This indicates that integrated databases will facilitate metabolomic developments and advances in biological systems.

Metabolomics Data Storage and 'Omics' Data Integration

Initiatives for metabolic data production, storage, dissemination, and analysis to encourage data sharing among researchers have

been attempted. MetaboLights is an open access database that contains data, including meta- and raw data, from GC-MS and LC-MS published metabolomics work (Steinbeck et al., 2012).

An integrative study is driven by two purposes: (1) gene function prediction, and (2) systemic interaction characterization of biological systems (Rochfort, 2005; Fukushima et al., 2009; Fernie and Stitt, 2012). '-Omics' analysis produces enormous data sets describing cellular components, their interaction and state of biological networks. Thus, computational methods are needed to reduce this dimension across the wide spectrum of '-omics' data (Blazier and Papin, 2012; Conesa and Herna, 2014). Metabolic network construction is an advantageous platform for '-omics' data integration. It is a manually curated, computational framework that explains gene–protein reaction relationships, assembled from annotated genomes, biochemical reactions, and cell phenotypes (Herrgård et al., 2006; Blazier and Papin, 2012). Thus, to systematically investigate complex host–microbial interactions, a systems biology approach is required that integrates high-throughput data and computational network models. For example, Heinken and Thiele (2015) proposed a constraint-based modeling and analysis approach, that enables the prediction of mechanisms behind metabolic host-microbe interactions on the molecular level.

CONCLUSION AND OUTLOOK

Recent studies have highlighted the complexity of the rhizosphere as an interlinked ecosystem consisting of different microorganisms that can enhance plant growth through different mechanisms. Chemical communication plays an important role in establishing a mutual relationship between plant roots and PGPR. In addition, both plants and PGPR determine the community of PGPR found in the rhizosphere. In attempts to unravel rhizosphere signalomics, several metabolites, both primary and secondary, have been identified to be the major messengers between plant roots and PGPR. Here, root exudates and PGPR metabolites (non-volatile and volatile) play major roles in establishing a mutual relationship. PGPR are also capable of interfering with phytohormone-linked signaling to inhibit or limit defense responses. PGPR do not only enhance plant growth, but also prime plants against infection by different phytopathogens. ISR/PGPR priming is a result of the complex rhizosphere interaction between plant roots and PGPR, leading to pre-conditioning of plants for an enhanced defense response against secondary stimuli. Most studies done on ISR/PGPR priming are gene- or transcription-based with very few on metabolomics. However, the limited studies available suggest that the early stages involve biosynthesis of signaling molecules followed by modulation of both primary - and secondary metabolism. When secondary stimuli are subsequently perceived, triggered events occur in an enhanced manner. These different physiological states (naïve, primed and primed and triggered) are reflected in changes to the metabolomes and can be investigated through targeted and untargeted metabolomics approaches. However, such studies generally focus on single organisms rather than studying the more complex system consisting of plant, rhizomicrobes and pathogen. Through increased technological advances, both biologically and chemically, we are now better able to study in detail the chemical changes which are associated with microbe-plant interactions and the biochemical mechanisms behind them. Metametabolomics, targeted at the phytobiome would therefore be a future approach aimed at unraveling the complexity of chemical communication in the rhizosphere.

AUTHOR CONTRIBUTIONS

Conceived and designed the research: MM and ID. Contributed to the paper and revised it critically for important intellectual content: MM, LP, NM, NL, and ID. All authors gave approval to the final version.

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Inner Plant Values: Diversity, Colonization and Benefits from Endophytic Bacteria

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One of the most exciting scientific advances in recent decades has been the realization that the diverse and immensely active microbial communities are not only 'passengers' with plants, but instead play an important role in plant growth, development and resistance to biotic and abiotic stresses. A picture is emerging where plant roots act as 'gatekeepers' to screen soil bacteria from the rhizosphere and rhizoplane. This typically results in root endophytic microbiome dominated by Proteobacteria, Actinobacteria and to a lesser extent Bacteroidetes and Firmicutes, but Acidobacteria and Gemmatimonadetes being almost depleted. A synthesis of available data suggest that motility, plant cell-wall degradation ability and reactive oxygen species scavenging seem to be crucial traits for successful endophytic colonization and establishment of bacteria. Recent studies provide solid evidence that these bacteria serve host functions such as improving of plant nutrients through acquisition of nutrients from soil and nitrogen fixation in leaves. Additionally, some endophytes can engage 'priming' plants which elicit a faster and stronger plant defense once pathogens attack. Due to these plant growth-promoting effects, endophytic bacteria are being widely explored for their use in the improvement of crop performance. Updating the insights into the mechanism of endophytic bacterial colonization and interactions with plants is an important step in potentially manipulating endophytic bacteria/microbiome for viable strategies to improve agricultural production.

Keywords: biocontrol bacteria, endophytic bacteria, plant defense signaling, plant growth promotion, plant microbiome

INTRODUCTION

It has been projected that the world's population will increase to 9.1 billion by 2050 (FAO, 2009). Increasing agricultural productivity is of the upmost priority for governments around the globe. However, the pathway to achieving this goal is becoming progressively difficult. Reduced arable land through urban sprawl, climate change and poor land management practices has led researchers and practitioners to explore non-traditional farming practices

(Smith et al., 2016). The purposeful use of plant growth promoting bacteria (PGPB) as biofertilizers in agriculture is a promising technology to provide effective and environmentally friendly solutions with the potential to ensure food security (Glick, 2014). However, to achieve this, scientists still need to forge a deeper understanding of the mechanisms underlying plant growth benefits by PGPB (e.g., beneficial endophytes).

Millions of years of evolution have led plants to develop a diverse range of mechanisms to cope with biotic and abiotic stresses. Establishing a continuing relationship with bacteria has evidently enhanced their capability to cope with stresses as well as to facilitate their growth and development. Endophytes are non-pathogenic organisms that live inside plant tissues for at least part of their life cycles (Rosenblueth and Martínez-Romero, 2006). Some endophytic bacteria are able to systemically prime the plant's immune system. Primed plants do not display major changes in defense-related gene expression in the absence of a pathogen, but mount an accelerated defense response upon pathogen or insect attack, providing broad-spectrum resistance (Pieterse et al., 2014; Conrath et al., 2015). Recently, several studies demonstrated the effectiveness of endophytic bacteria in protecting plants from a series of abiotic stresses including drought (Rolli et al., 2015; Sheibani-Tezerji et al., 2015), low temperature (Su et al., 2015; Subramanian et al., 2015) and salinity (Ali et al., 2014). In addition, it was also observed that *Agave tequilana* plants directly digested endophytic bacteria for a nitrogen (N) source for their growth (Beltran-Garcia et al., 2014). Therefore, a potential to explore the development of endophytic bacteria in agricultural practices and in variable climatic conditions should not be ignored.

The interaction of the drivers of plant root microbial habitat and diversity have been explored in comprehensive reviews such as; Bulgarelli et al. (2013), Gaiero et al. (2013), and Reinhold-Hurek et al. (2015). The influence of climate change on these microbial communities and how they react and adapt to these changes as well as the use of endophytic bacteria in agriculture have been identified as potential research priorities. In order to fully understand these influences, the mechanisms behind the techniques used and potential drivers of inovation need to be explored. An underlining goal of this review is to give a brief understanding of the biodiversity, distribution and elements of endophytic bacteria to lay a platform for exploring their potential benefical use in agricultural practices. Exploring the plant growth promoting traits of endophytic bacteria, such as boosting plant nutrient uptake or buffering capacity from abiotic stress is a relatively novel but promising area for the development of sustainable agriculture (Glick, 2014; Santoyo et al., 2016).

In this review, the authors aim to discuss key issues within the scope of plants and endophytic bacteria interactions. Based on the findings in most recent studies on endophytic bacteria, we explore (1) which bacteria live in plant endophytic habitats, (2) how do endosphere bacterial communities respond to plant stresses and environmental stimuli, (3) where exactly do endophytic bacteria colonize plants, (4) what are the traits that enable bacteria to successfully invade and persist into standing heterogeneous communities, (5) how do bacterial endophytes deal with the plant's immune system, (6) how does the plant host influence endophytic colonization via hormone signaling pathways, and (7) what are the traits of endophytic bacteria that deliver plant phenotypes and therefore may hold promise for use in agriculture. We believe that understanding the interactions between endophytic bacteria and their plant hosts will assist in the design of new strategies for productive and sustainable practices in agriculture.

BIODIVERSITY AND ACQUISITION OF ENDOPHYTIC BACTERIA

Different plant organs are associated with different endophytic bacterial communities in terms of diversity and composition. The microbiome in the root endosphere is significantly less diverse than microbiomes in the rhizosphere and bulk soil (Liu et al., 2017). The number of bacterial cells within root endophytic environments reaches c. 10^4–10^8 per gram of root tissues, which is considerable less when compared with bulk soil (c. 10^6–10^9 bacterial cells g^{-1} soil) and rhizosphere (c. 10^6–10^9 bacterial cells g^{-1} plant tissue) (Bulgarelli et al., 2013). This suggests that roots are effective habitat filters, restricting community membership to progressively more narrowly defined lineages as environments deviate from soil to roots (Bulgarelli et al., 2012). Root endophytic bacterial communities are typically dominated by Proteobacteria (∼50% in relative abundance), Actinobacteria (∼10%), Firmicutes (∼10%) and Bacteroidetes (∼10%) (Supplementary Table S1). Other bacterial phyla, including but not limited to Chloroflexi, Cyanobacteria, Armatimonadetes, Verrucomicrobia, Planctomycetes, and Nitrospirae are common in the root endosphere, but represent a smaller fraction of the community (Supplementary Table S1) (Sessitsch et al., 2012; Edwards et al., 2015). Archaea, Acidobacteria and Gemmatimonadetes appear to be either absent or rare (<1%) in the root endosphere despite being significant representatives of bulk soil microbial communities (Bates et al., 2011; Sessitsch et al., 2012).

Understanding the differences, if any, between plant root and leaf/shoot endosphere microbiomes is the first step of many in developing a clear pathway to beneficial development of biofertilizers in agriculture. There is evidence that plant root bacterial endophytes are mainly recruited from soil, which then ascend to stems and leaves via the apoplast in xylem vessels (Chi et al., 2005). Therefore, it is not surprising that plant leaf/shoot endosphere microbiomes have significant overlaps with those in roots at both, the taxonomic and functional levels (Bodenhausen et al., 2013; Bulgari et al., 2014; Bai et al., 2015). Consistently, the work of de Oliveira Costa et al. (2012) showed that Proteobacteria, Actinobacteria and Firmicutes are the dominant groups in the leaf microbiome of common bean plants (*Phaseolus vulgaris*), which was revealed by a culture-based analysis.

Recent studies observed that the plant root endosphere could be dominated by only a few bacterial groups, which provides further evidence of the active and robust selection of bacteria from soil to plants. Examples for this are Gammaproteobacteria of the genera *Enterobacter*, *Pseudomonas* and *Stenotrophomonas*

that constitute the core bacterial operational taxonomic units (OTUs) in root endosphere of both, the sweet potato IPB-137 (Gammaproteobacteria, 47%) (Marques et al., 2015) and rice (Gammaproteobacteria, 30–98%) (Sessitsch et al., 2012; Ferrando and Scavino, 2015; Ren et al., 2015a). In some cases, it was found that only one or two bacterial OTUs dominated the endosphere of plant tissues. These are a *Pseudomonas*-like OTU (34%) in the roots of *Populus deltoids* (Gottel et al., 2011) and two OTUs affiliated to *Pseudomonas* (52%) and *Enterobacter* (35.5%) in sugarcane stems (Magnani et al., 2013). Members of Actinobacteria, especially the genus *Streptomycetes* are well known for their efficient synthesis of antibiotic compounds that suppress a diverse range of phytopathogens (Palaniyandi et al., 2013). Metagenomic surveys using 16S rRNA phylogenetic marker gene showed that the *Streptomycetaceae* family typically dominated the Actinobacteria in the root endosphere of both *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012) and wheat seedlings (Liu et al., 2017). Collectively, the abovementioned studies demonstrate that the microbiome in the plant endosphere is much simpler than that in the adjacent soil, and that it harbors distinct assemblages rather than random subsets of the soil microbiome.

This leads to the question of how plants manage to recruit 'good bugs' that they might use while expelling those that do not provide benefits. In recent reviews by Bulgarelli et al. (2013) and Reinhold-Hurek et al. (2015), two- and three-step models were proposed for describing the dynamics of the root-associated microbiome across the three niches (rhizosphere, rhizoplane, and endosphere), which highlighted a screening role of each of the root compartments in the acquisition process of the endosphere microbiome. The rhizosphere as the 'growth chamber' is the first compartment that profoundly influences the soil microbiome. The distinct physicochemical and biological conditions caused by the carbon-rich molecules and antimicrobial compounds in the rhizosphere may favor the growth and reproduction of some soil bacterial groups while suppressing some others. Secondly, the root rhizoplane plays a 'critical gating role' (Edwards et al., 2015). Those bacteria that are attracted to the rhizosphere but lack adhesion ability are not permitted to enter the endosphere due to their inability to bind to root surfaces (Reinhold-Hurek et al., 2015). Therefore, only a small subset of the rhizosphere microbiome enters the endosphere. Lastly, the plant immune system actively excludes some specific bacterial groups (Lundberg et al., 2012). As observed in many cases, Acidobacteria, Gemmatimonadetes and Archaea consecutively decrease while Proteobacteria (especially the Gammaproteobacteria) significantly increase in relative abundance from bulk soil to root endosphere (Edwards et al., 2015; Liu et al., 2017). The underlying mechanisms and the ecological rationale behind this phenomenon are still poorly understood. Despite being still speculative, distinct pH and nutrient conditions (Naether et al., 2012) as well as the high [O_2] (Blossfeld et al., 2011) in the root interior may be major factors leading to the rare presence of Acidobacteria in plant roots. It is worth pointing out, nevertheless, that the bacterial microbiome in the plant endosphere is not likely to be simply assembled by the plant, but is also the result from complex microbial interactions (e.g., microbial competition and cooperation). Overall, more research into how the endosphere microbiota are assembled is necessary to shed light onto strategies for recruiting, maintaining and monitoring them for the provision of benefits to sustainable agriculture.

DETERMINANTS OF MICROBIAL COMMUNITY ASSEMBLY IN THE ENDOSPHERE

The bacterial components in plant interior are mostly harmless or beneficial to their host and they are dynamic (Rosenblueth and Martínez-Romero, 2006). The changes of their composition and diversity are driven by the ecology of the plant and soil, which are highly dependent on the plant's geographic location, endogenous host interactions and exogenous environmental factors (Edwards et al., 2015). Soil that harbors an immensely rich pool of bacterial species is the microbial 'seed bank' for roots, and its properties may affect plant physiology and root exudation profiles which in turn profoundly influence the structure of the root endosphere microbiome (Philippot et al., 2013). Studies performed on the endosphere microbiome of different plants, using high-throughput amplicon sequencing, have revealed that host plant species (Shen and Fulthorpe, 2015; Ding and Melcher, 2016), genotype (Marques et al., 2015; Rodriguez-Blanco et al., 2015), plant organ type (Hameed et al., 2015), developmental stage (e.g., seedling or mature plant) (Ren et al., 2015a; Yu et al., 2015; de Almeida Lopes et al., 2016), growing season (e.g., of trees) (Shen and Fulthorpe, 2015; Ding and Melcher, 2016), geographical location (field conditions) (Edwards et al., 2015), soil type (Edwards et al., 2015), host plant nutrient status (Hameed et al., 2015), cultivation practice (Edwards et al., 2015) and fertilization (Rodriguez-Blanco et al., 2015) are among the observed factors that significantly influence the plant endosphere microbiome.

It was observed that transgenic glyphosate-resistant cultivars of soybean had a higher diversity and abundance of culturable endophytic bacteria than wild-type plants (de Almeida Lopes et al., 2016). Marques et al. (2015) depicted that the plant genotype affected the functional diversity of endophytic bacteria, as IAA-producing strains were predominantly isolated from one of the three genotypes of sweet potato studied. The work of Kõiv et al. (2015) also demonstrated that plant diseases can influence the composition of endophytic bacterial communities. An anaerobic pectolytic *Clostridia* population was particularly enriched in soft rot disease- (caused by *Pectobacterium atrosepticum*) infected potato (*Solanum tuberosum*) tubers, and this change occurred possibly due to oxygen depletion inside the tubers (Kõiv et al., 2015).

In addition to soil and host properties, fluctuations of environmental CO_2 and temperature modulate endophytic bacterial communities. In the context of climate change and given the importance of endophytic bacteria for plant growth and health, understanding how endophytic bacteria respond to an elevated CO_2 or temperature can aid in future decision-making policies around environmental issues. Ren et al. (2015b)

demonstrated that leaf endophytic bacteria appear to be more vulnerable to climate change than soil bacterial communities. The community structure of endophytic bacteria in rice leaves was influenced by elevated CO_2 levels at the tillering and filling stages, but not during maturity, and this influence also correlated with N fertilization levels (Ren et al., 2015a). Moreover, Ren et al. (2015b) showed that endophytic communities inhabiting leaves at different locations (upper or lower leaf) in the plant respond differentially to elevated CO_2. Oxygen availability also exerts effects on endophytic bacterial communities in rice, especially on diazotrophs. Ferrando and Scavino (2015) observed that diazotrophic community composition in rice roots shifted significantly after flooding stress, with Gammaproteobacteria and Betaproteobacteria being the predominant groups in the endosphere before and after flooding. These results are intriguing as they indicate a restructuring of microbe populations in the endosphere by plants upon changes in specific environmental factors. However, the challenge remains to discover whether there is a link between corresponding microbiome variation and plant physiological conditions/health.

In order to address the question of 'which bacteria live in the endosphere?', taxonomy-based approaches were deployed. By contrast, function-based metagenomics, metatranscriptomic, and metaproteomic analyses which represent the functional variations of endophytic communities are able to answer 'what can they do in the endosphere?' Current investigations on the functional dynamics of endophytic communities using metagenomic analyses have been performed to a much lesser extent than phylogeny-based analyses. Recently, a functional study conducted on tomato plants revealed that bacterial endophytes colonizing roots were significantly affected by root-knot nematodes (Tian et al., 2015). Genes involved in plant polysaccharide degradation, carbohydrate/protein metabolism and N_2 fixation were increased in abundance, which indicates that bacteria inside roots may start proliferative growth and become saprophytic after infection by root-knot nematodes. This study also provides evidence to suggest that particular functional attributes of endophytic bacteria are induced in plants suffering from stress.

DISTRIBUTION OF ENDOPHYTIC BACTERIA AND COLONIZATION PATTERNS

Bacterial colonization patterns in plant endophytic compartments have thus far been mainly studied in grasses (e.g., rice and kallar grass) using cultivated model strains. Some of the most popular approaches to enumerate and visualize colonization of bacteria in plant tissues include fluorescence *in-situ* hybridization (FISH) and using reporter gene- (e.g., *gfp* or *gus*) modified bacterial strains combined with microscopy. In plants, emerging lateral roots break through the epidermis, cortex, endodermis, casparian strip (band around endodermis) and pericycle, thereby naturally forming a 'highway' for bacteria to enter at these sites. From there, bacteria can further enter the phloem and xylem vessels that transport photosynthates (phloem), nutrients and water (xylem) (a schematic illustration is shown in **Figure 1**) (Compant et al., 2010). Bacteria colonizing inside the root conductive tissues can further translocate to shoots and leaves driven by plant transpiration (Compant et al., 2010). Endophytic infection can also occur at wounds (e.g., leaf scars, root ruptures) as a result of herbivore or other mechanical damage (Compant et al., 2010).

Typical hot spots for bacterial colonization are lateral root emergence sites, outer cell layers, root cortex, phloem and xylem, which can occur both intracellularly and in the apoplast (Reinhold-Hurek et al., 2006) (**Figure 1**). For instance, PGPB *Burkholderia* sp. strain PsJN colonized root rhizodermis cells, internal tissues, particular internodes and leaves of grapevine (Compant et al., 2005, 2008). The work of Anand and Chanway (2013) and Anand et al. (2013) also supports this in their findings for the diazotrophic bacterial strain *Paenibacillus polymyxa* P2b-2R which extensively colonized the surface and interior of roots, stems and needles of lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.). An unusual colonization strategy has been recently discovered for a facultative intracellular symbiont of *Methylobacterium extorquens* strain DSM13060, which aggregated around the nucleus of the living cells of Scots pine (*Pinus sylvestris* L.) shoot tips (Koskimäki et al., 2015). Broadly speaking, the plant parts in or close to soil are inclined to harbor more bacteria than the uppermost plant organs (Fisher et al., 1992).

Regarding bacteria in the phylosphere, there are indications that bacterial endophytes are derived from soil by screening soil bacteria via rhizosphere and root systems (Lamb et al., 1996). Alternatively, these bacteria can be from phyllosphere epiphytes through natural openings (e.g., stomata, hydathodes), wounds and cracks generated by wind, insect and pathogen attacks (Vorholt, 2012). Specifically, in a leaf, bacteria can colonize in upper epidermis cells, palisade mesophyll cells, xylem vessels as well as spaces between spongy mesophyll layer cells (Olivares et al., 1997) (**Figure 2**). Bacteria have also been detected in plant reproductive organs, such as flowers, fruits and seeds, but in small numbers (Rosenblueth and Martínez-Romero, 2006; Compant et al., 2011; Truyens et al., 2015). Consistent with this, Compant et al. (2011) visualized the colonization of *Pseudomonas* spp. and *Bacillus* spp. in grapevine using FISH and found these bacteria colonizing epidermis and xylem of the ovary, intercellular spaces of pulp cells and along cell walls inside seeds. Another example is *Streptomyces mutabilis* strain IA1 that controls the fungal pathogen *Fusarium culmorum* in wheat seedlings and colonizes the area inside the caryopsis, up to the endocarp layer of wheat seeds (Toumatia et al., 2016).

Colonization of endophytic bacteria can be also categorized into 'obligate,' 'facultative' and 'passive' depending on if they require plant tissue to live and reproduce (for a review on this topic see Hardoim et al., 2008). Obligate endophytic bacteria are derived from seeds and cannot survive in soils. Facultative endophytic bacteria widely exist in soil, and they carry out colonization and infection when conditions are suitable. Most facultative endophytic bacteria remain within the cortex but some also enter the central phloem and xylem (Compant et al., 2010). Bacteria lacking the capability to colonize and infect can

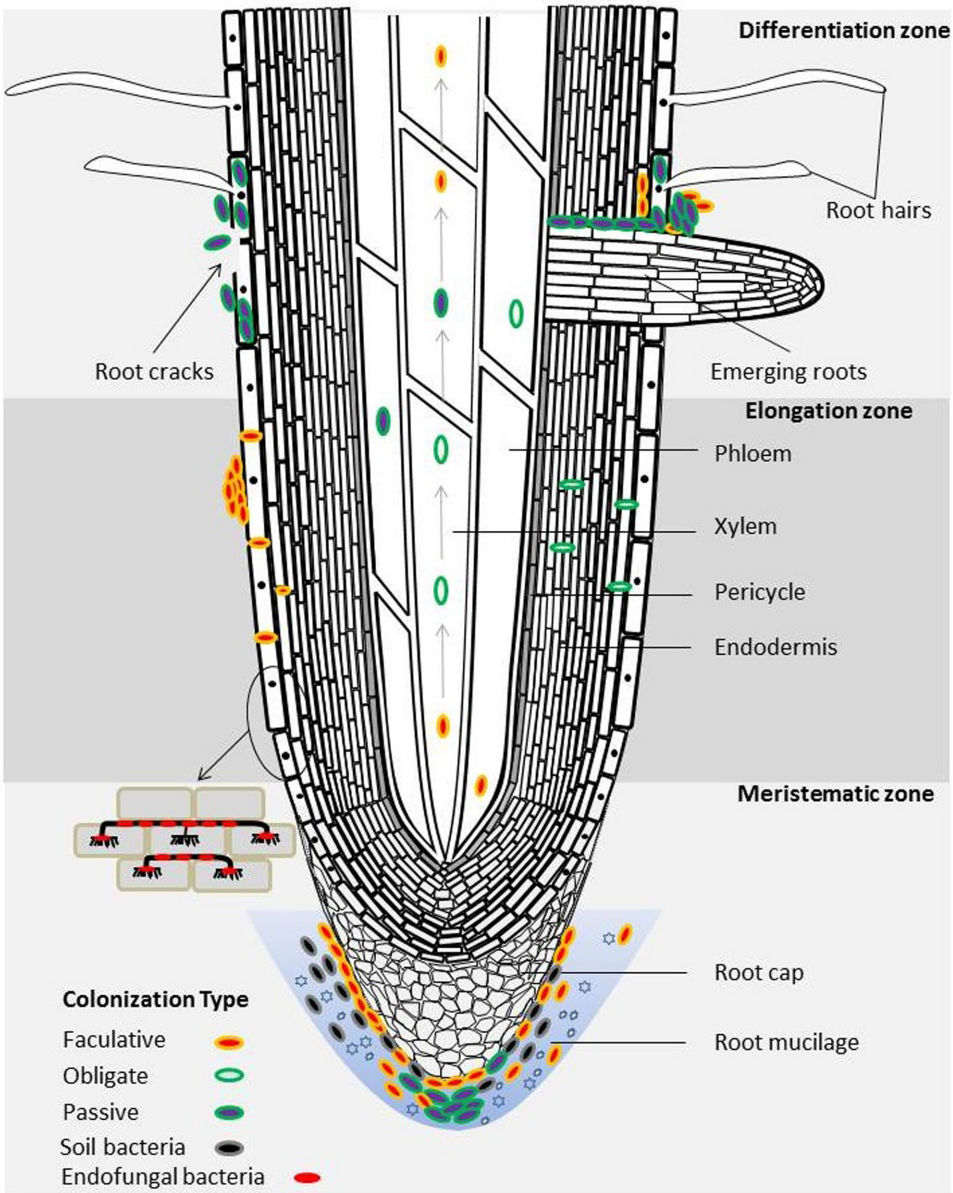


FIGURE 1 | Schematic representation of the bacterial distribution and colonization patterns in the endosphere of a plant root. The emerging sites of lateral roots are among the hotspots of bacterial colonization. Arrows represent the translocation of bacteria inside the xylem and phloem. Endophytic bacteria may engage in different life styles as depicted by different colored ovals. This illustration was inspired by studies of Compant et al. (2005, 2008) and Glaeser et al. (2016).

enter plant endophytic niches via wounds and cracks on the plant, which is documented as the passive mode of endophytic colonization (Christina et al., 2013) (**Figure 1**). Current evidence reveals that some bacteria live in symbiosis with plant endophytic fungi (Desirò et al., 2015; Glaeser et al., 2016). Interestingly, particular endofungal bacteria colonize plants in a similar fashion as their fungal host (**Figure 1**). For instance, the endofungal bacterium *Rhizobium radiobacter* F4 hosted by the fungus *Piriformospora indica* colonizes plant roots and forms aggregates of attached cells and dense biofilms at the root surface (Glaeser et al., 2016).

In summary, it is evident that bacteria are able to colonize both intracellularly and extracellularly the interior of plants. Despite having been detected in all plant parts, roots that have the most intimate contact with soil may function as the first avenue for the recruitment of endophytic bacteria. Endophytic bacteria may have a genetic basis to their different colonization and infection patterns, which may further correlate to their interaction patterns within plants. In the following section, we provide details on traits that enable endophytic bacteria to successfully establish in their plant hosts.

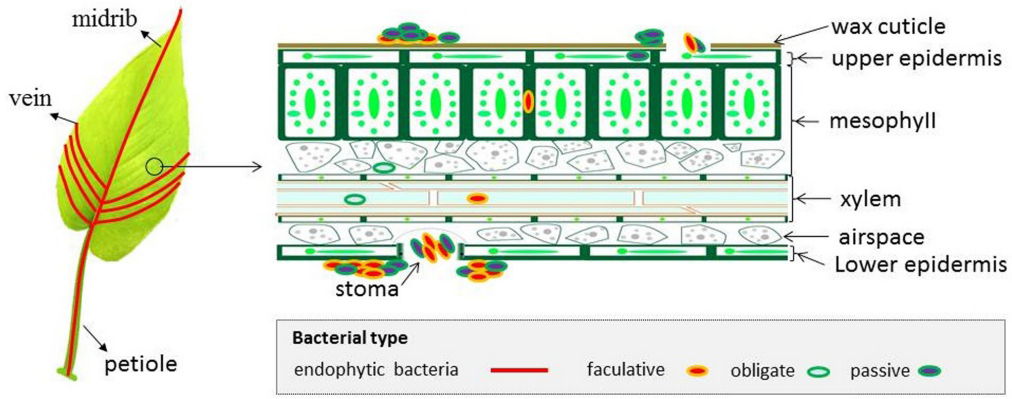


FIGURE 2 | Schematic representation of bacterial colonization patterns in a leaf. The picture shown on the left demonstrates that the presence of bacteria has been detected in the leaf petiole, midrib and veins. The picture shown on the right is a magnified leaf cross-section, which demonstrates that endophytic bacteria may not only colonize the apoplast but are also present intracellularly. Endophytic bacteria are believed to be able to ascend from roots to leaf via the vascular tissues of xylem and phloem.

TRAITS FOR SUCCESSFUL INVASION, COLONIZATION AND TRANSLOCATION OF ENDOPHYTES

During millions of years of coevolution with plants, bacteria have been equipped with necessary traits that enable them to invade, colonize and translocate in the plant's interior. Motility, chemotaxis, production of cell-wall degrading enzymes and lipopolysaccharide formation are among the observed traits for bacteria to infect and adapt to life inside plants (Piromyou et al., 2015). The importance of these traits has been confirmed by comparative genomics, metagenomics and transcriptomic analyses, combined with mutational studies (Böhm et al., 2007; Straub et al., 2013; Sheibani-Tezerji et al., 2015). Bacteria may adjust gene expression when infecting and colonizing plants (Coutinho et al., 2015; Piromyou et al., 2015). This can be demonstrated by genes encoding proteins related to bacterial motility, chemotaxis and adhesion that were induced in *Burkholderia kururiensis* M130 in the presence of rice plant extracts (Coutinho et al., 2015). The bacterial flagellum that often acts as a potent microbe-associated molecular pattern (MAMP) for recognition by the innate immune system may also mediate endophytic competence by enabling bacterial chemotactic movement and anchoring to plant surfaces (Buschart et al., 2012). The five endophytic bacteria examined by Straub et al. (2013) all contain the entire flagella machinery and a flagella-deficient mutant was hampered in colonization efficiency of wheat roots (Croes et al., 1993). Additionally, adherence to the root surface is also a crucial step for bacteria to infect plants. This is exemplified by the fact that genes encoding Type IV pili (TFP), the crucial virulence factor formed by pilin subunits, exist in the genome of endophytic *B. phytofirmans* PsJN bacteria (Mitter et al., 2013). Moreover, mutant analysis has demonstrated the essential role of TFP-dependent adhesion for the establishment of *Azoarcus* sp. inside rice roots (Dörr et al., 1998). It was further revealed that TFP retraction protein-mediated twitching motility is essential for N_2-fixing bacteria *Azoarus* sp. strain BH72 to establish inside rice roots but this was not important for the colonization on the root surface (Böhm et al., 2007).

Cell-wall degrading enzymes are important for plants to break plant cell walls and translocate compounds to the apoplast. Genes encoding cell-wall degrading enzymes widely exist in the genomes of endophytic bacteria (Straub et al., 2013). For example, genes encoding plant polymer-degrading cellulases, xylanases, cellobiohydrolases, endoglucanase and cellulose-binding proteins were detected in high copy numbers in the metagenome of rice root endophytic bacterial communities (Sessitsch et al., 2012). *In vitro* assays confirmed that endoglucanases are crucial for *Azoarcus* sp. to colonize inside rice roots (Reinhold-Hurek et al., 2006). To be able to ingress intracellularly and translocate within the symplast, endophytic bacteria may also secrete pectinases to degrade the middle lamella between plant cells. It was found that pectinase is an important determinant modulating early infection of the PGPB *Bradyrhizobium* sp. SUTN9-2 on rice, which originally formed symbiotic relationships with the leguminous weed *Aeschynomene americana* (Piromyou et al., 2015). Moreover, pectin esterase expression in this bacterium was up-regulated after being inoculated on rice seedlings (Piromyou et al., 2015). In addition to the above-mentioned traits, Kost et al. (2014) found that oxalotrophy, the capacity of utilizing oxalate as a carbon source, is required for the successful colonization of *B. phytofirmans* PsJN on lupin and maize plants. Oxalotrophy was reported to be only associated with plant-beneficial *B. phytofirmans* species, while plant pathogenic or human opportunistic pathogenic species of the *Burkholderia* genus are not able to use oxalate (Kost et al., 2014). This study suggests a role of oxalate in plant selection for beneficial endophytes, while avoiding pathogenic bacteria from the complex soil bacterial communities. Overall, the traits discussed above seem to be required for the active invasion and systemic transmission of endophytic bacteria within plants.

BACTERIAL ENDOPHYTES CIRCUMVENT HOST DEFENSE

Plants highly rely on their sophisticated defense systems to counteract attacks from phytopathogens (Jones and Dangl, 2006). MAMP-triggered immunity (also known as horizontal resistance) that has pattern-recognition receptors as a surveillance system to perceive conserved MAMPs equips plants with a first line of basal defense that is able to halt the growth of most pathogens. During the coevolution with plants, pathogens developed the strategy of injecting effectors into plants to suppress or circumvent MAMP-triggered immunity. In response, plants developed a second line of defense called effector-triggered immunity (also known as vertical resistance). Within the latter strategy, plants have developed receptors that recognize the effectors of pathogens. In the case of biotrophic or hemibiotrophic pathogens that depend on living cells for nutrient uptake, a hypersensitive response (HR) may be activated leading to programmed cell death (PCD) of cells under attack (Jones and Dangl, 2006). However, this rapid defense response must be suppressed in the case of necrotrophic pathogens (nutrient uptake from dead or degraded plant tissues) or beneficial microbes, including beneficial endophytes. In many plants, including *Arabidopsis*, the salicylic acid (SA) defense signaling pathway targets biotrophic pathogens, while the jasmonic acid (JA) pathway suppresses the SA pathway playing a role in defense against necrotrophic pathogens, but also insects and beneficial plant-microbe interactions (Pieterse et al., 2012).

The plant immune system can therefore play a major role to influence the colonization and multiplication of plant bacterial endophytes. There is growing evidence that, to avoid antagonistic effects, endophytic bacteria produce their own MAMPs (unlike phytopathogens), which generally do not elicit significant plant immune responses, such as the expressions of pathogenesis-related (PR) proteins (Vandenkoornhuyse et al., 2015). This therefore avoids bacteria being eliminated by the plant's immune system. Many cell surface components of endophytic bacteria are distinct from those of phytopathogens. For example, the flagellin-sensing system flg22-Flagellin Sensing 2 (FLS2) in grapevine differentially recognizes the flagellin-derived epitopes of endophytic PGPB *B. phytofirmans* from those of a bacterial pathogen such as *Pseudomonas aeruginosa* or *Xanthomonas campestris* (Trdá et al., 2014). This suggests that the flagellin of endophytic bacteria may have evolved to circumvent recognition by the plant's immune system. Bacterial protein secretion systems (SSs) are another group of important cell surface components with a role in host immune modulation. SSs are composed of large protein complexes that transverse the cell envelope and contain a channel mediating the translocation of proteins or protein-DNA complexes (Green and Mecsas, 2016). Eight (Type I SS∼ Type VI SS and Sec, and Tat) and six (Sec, Tat, secA2, Sortase, Injectosome and Type VII SS) different protein SS have been described for Gram-negative and Gram-positive bacteria, respectively (Tseng et al., 2009; Green and Mecsas, 2016).

Among the SSs, T3SS and T4SS are pivotal for pathogens to deliver effector proteins into the plant, which can induce effector-triggered immunity (Green and Mecsas, 2016). T3SS and T4SS may be either absent or present in low abundance in endophytic bacteria and therefore, these bacteria do not seem to elicit significant plant defense responses (**Figure 3**). Krause et al. (2006) sequenced the genome of *Azoarcus* sp. strain BH72 and described it as 'disarmed' due to the lack of both T3SS and T4SS as well as other important cell surface components that are usually present in pathogens. Similarly, the genomic inventory of five grass endophytic bacterial strains, including *Herbaspirillum frisingense* GSF30(T), *Gluconacetobacter diazotrophicus* PAI5, *Azoarcus* sp. BH72, *Klebsilla pneumoniae* 342 and *Azospirillum* sp. B510 characterized in biomass grasses completely lack T3SS (Straub et al., 2013). Further, a metagenomic survey demonstrated the rare presence of T3SS- and T4SS-encoding genes in the genomes of eleven endophytic bacterial strains (Reinhold-Hurek and Hurek, 2011). All the endophytic *Herbaspirillum* strains examined so far lack the T4SS that functions in virulence (Juhas et al., 2008; Straub et al., 2013). However, this comes with an exception that T3SS and T4SS are crucial components for *Bradyrhizobium* sp. SUTN9-2 (isolated from the leguminous grass *Aeschynomene americana* L.) to colonize the roots of rice seedlings (Piromyou et al., 2015). With regard to T6SS, their functions are largely unknown but they may also be important for plant-bacterial endophyte interactions (Sessitsch et al., 2012; Mitter et al., 2013). Taken all together, endophytic bacteria tend to lack T3SS and T4SS that in pathogens are related to induction of defense responses but some rhizobium-type endophytic bacteria may require T3SS to establish in the plant endosphere (**Figure 3**).

Production of a range of reactive oxygen species (ROS) is typically a non-specific tactic for plant defense to induce HR and PCD against biotrophic pathogens (Apel and Hirt, 2004). Interestingly, it was observed that colonization of endophytic bacteria also elicited an oxidative burst in rice and the traditional Chinese medicine plant *Atractylodes lancea* (Alquéres et al., 2013; Han et al., 2015; Zhou et al., 2015). To detoxify the initial ROS produced by plants, the endophytic bacteria may resort to ROS-scavenging enzymes (**Figure 3**). A high number and diversity of genes encoding ROS-scavenging enzymes such as superoxide dismutase (SOD) and glutathione reductase (GR) are represented in the metagenome of the endophytic bacterial communities in rice roots (Sessitsch et al., 2012). Genes encoding enzymes involved in ROS-scavenging were also detected in the genome of *Enterobacter* sp. 638 (Taghavi et al., 2010). ROS-scavenging enzymes are reported to be involved in the biological N fixation of *Gluconacetobacter diazotrophicus* and are essential for its successful colonization in endophytic rice roots (Alquéres et al., 2010, 2013). The transcript levels of ROS-scavenging enzyme-encoding genes were upregulated in *G. diazotrophicus* strain PALS when they colonized the plant's interior (Alquéres et al., 2010, 2013). In summary, endophytic bacteria have evolved a wide range of strategies to avoid, circumvent or cope with the antagonistic effects of plant defenses.

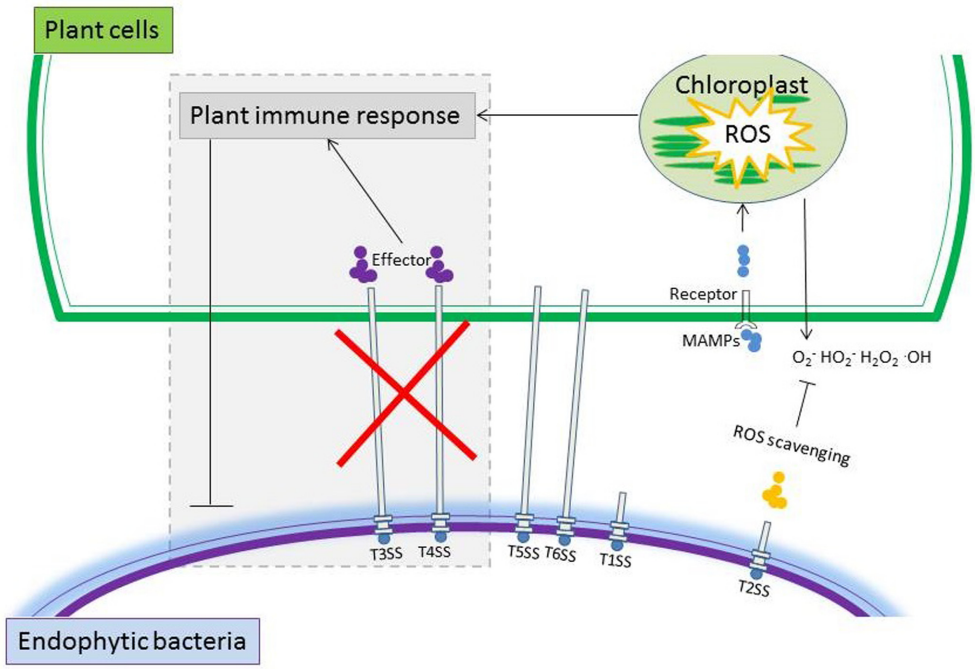


FIGURE 3 | Schematic representation summarizing typical properties that may be employed by endophytic bacteria to cope with the plant's immune system. Genes encoding secretion systems including T2SS, T5SS, and T6SS are normally detected in high copy numbers in endophytic bacteria. The rare presence of T3SS and T4SS that generally elicit significant plant defense, and the production of scavenging enzymes of endophytic bacteria may have contributed to their successfully colonization in plants.

PLANT HORMONE SIGNALING PATHWAYS INFLUENCE ENDOPHYTIC BACTERIAL COLONIZATION

Given the critical role of phytohormones in plant defense, it is important to determine whether the microbiome is influenced by host plant defense signaling pathways, which is important for at least two reasons. Firstly, these pathways can be induced by external stimuli and have the potential to provide a mechanism to alter the microbiome structure toward plant-beneficial interactions. Secondly, this may help illustrate the role of plant-associated microbiomes in plant nutrition and plant defense against biotic attacks. Several studies have investigated how plant defense signaling regulates the colonization of bacteria inside plants. The activation of the ethylene (ET) signaling pathway suppressed the endophytic colonization of *Medicago truncatula* by the PGPB *Klebsiella pneumoniae* 342 (Kp342) and the human enteric pathogen *Salmonella enterica* serovar Typhimurium (Iniguez et al., 2005). Furthermore, an ET-insensitive *M. truncatula* mutant was 'hyper-colonized' by Kp342 compared with wild-type plants (Iniguez et al., 2005). In line with this study, the activation of JA signaling was found to suppress rice root colonization by *Azoarcus* sp. strain BH72 (Miché et al., 2006). The activation of JA signaling also strongly suppressed early stage nodulation in *Lotus japonicus* (Nakagawa and Kawaguchi, 2006). These studies indicate that enhanced plant signaling may restrict the colonization of specific endophytic bacteria or rhizobium in the plant endosphere (**Figure 5**). The suppression of bacterial colonization may be a strategy of the plant's immune system to control the abundance of hosted bacteria and to maintain the most 'plant-favorable' bacterial density in the inner tissues. The potential use of plant hormones for the suppression of specific plant endophytic bacteria warrants further investigation [e.g., to control human pathogens present in food, such as *Salmonella* strains in vegetables (Iniguez et al., 2005)].

The diversity of bacterial communities in the endosphere may correlate to plant defense capabilities. This is supported by the higher bacterial diversity in the root endosphere of wilt-resistant tomato cultivar *Arka Abha* than that of the susceptible cultivar *Arka Vikas* (Upreti and Thomas, 2015). Moreover, bacteria isolated from the wilt-resistant cultivar were more likely to employ antimicrobial strategies (e.g., production of siderophores and HCN) than those from the wilt-susceptible cultivar (Upreti and Thomas, 2015). These findings highlight the importance of investigating how the diversity of the endosphere microbiome is affected by plant defense signaling pathways. Our recent study revealed that an activated JA signaling pathway reduced bacterial diversity in the endosphere of wheat roots, while the microbiome in the rhizosphere and shoot endosphere were not influenced (Liu et al., 2017). Similar reports documented that the diversity of endophytic bacterial communities in *Arabidopsis* leaves decreased by the activation of SA signaling, but the

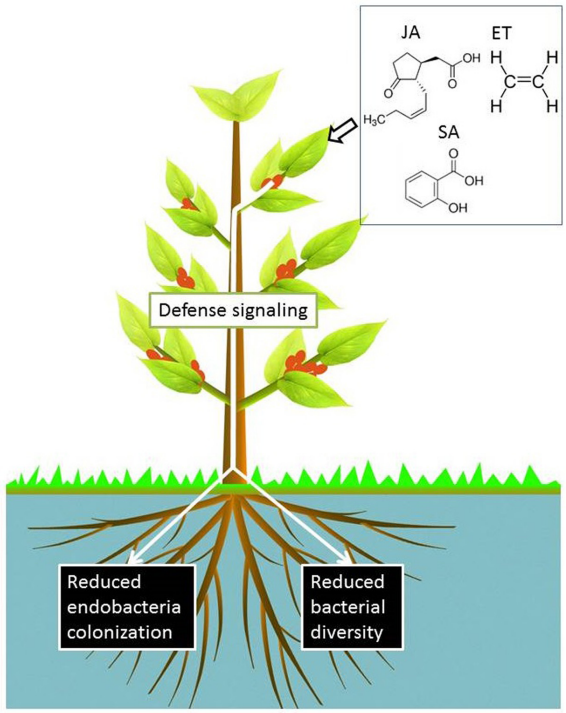


FIGURE 4 | Activation of SA, JA, and ET signaling pathways by exogenous treatments suppresses the colonization of particular bacterial inoculants in the root endosphere. In contrast, defense signaling effects on the plant endosphere-associated microbiome could be small and variable (Iniguez et al., 2005; Miché et al., 2006; Kniskern et al., 2007; Lebeis et al., 2015), but a reduced bacterial diversity has been observed in the roots of wheat seedlings upon activation of JA signaling (Liu et al., 2017).

communities were not influenced by the activation of the JA-dependent defense pathway (Kniskern et al., 2007). A recent study by Lebeis et al. (2015) provides evidence to suggest that plant roots differentially sculpt their endophytic bacterial communities in different isogenic *Arabidopsis* defense signaling mutants. This observation was based on analysis at the family level and therefore, community profiling at lower taxonomic ranks, that is at genus and species level is required. ET signaling also influences bacterial communities in the plant's endosphere. It was observed that the diversity of culturable root bacterial communities in isogenic transformed *Nicotiana attenuate* plants impaired in ET biosynthesis (*ir-aco1*) or perception (*35S-etr1*) was lower than that of wild-type plants (Long et al., 2010). Overall, plant signaling defense pathways appear to influence the diversity of endophytic bacteria, although changes could be variable and small (**Figure 4**).

PLANT GROWTH-PROMOTING TRAITS

Cropped soils are often deficient in macro and micronutrients and are prone to contain decimating soil-borne pathogens such as *Fusarium*, *Pythium* and *Phytophthora* ssp (Dixon and Tilston, 2010; Weil et al., 2016). These comprise enormous constrains to plant production worldwide. To obviate this problem and obtain crop yield increase, agricultural production has become increasingly reliant on the use of chemical fertilizers, herbicides, fungicides and insecticides for either supplementing soils with macro and micronutrients or to kill pathogens and insects. However, it is necessary to re-examine many of these approaches due to the potential human and environmental hazards, the intensive energy processes and the depletion of non-renewable resources involved in the industrial production of these agrochemicals (Aktar et al., 2009). Biofertilizers using PGPB is a possible approach to effectively provide plants with nutrients, mediate phytostimulation and therefore reduce the need for chemicals in agriculture, possibly launching a green revolution if better understood and consistent results can be obtained (Lugtenberg and Kamilova, 2009). Endophytic bacteria are capable of promoting plant growth through a wide variety of direct and indirect mechanisms. The direct mechanisms of plant growth promotion include providing plants with nutrients/substrates (e.g., phosphorous, nitrogen and iron) and producing various plant hormones (Santoyo et al., 2016). Indirect beneficial effects of endophytic bacteria on plants are mainly derived from their antagonistic effects toward phytopathogens (Compant et al., 2010). The involved mechanisms for this include production of cell-wall degrading enzymes (e.g., chitinase and β-1,3-glucanase) and antimicrobial compounds, lowering endogenous stress-related ET in plants, induction of induced systemic resistance (ISR) in host plants, quenching the quorum sensing (QS) of phytopathogens and competition for niche and resources (Compant et al., 2010; Glick, 2014; Santoyo et al., 2016). A single endophytic bacterial strain or bacterial community may have more than one of these plant growth-promoting traits (PGPTs) (Rolli et al., 2015; Tsurumaru et al., 2015; Miliute et al., 2016) (**Figure 5**). Bacterial strains with plant growth-promoting functions continue to be discovered but as of yet a clear path to developing PGPTs for agricultural purposes has not been developed (Dey et al., 2014). One reason for the current inconsistency when using bioinocula is that too little is known about the specific interactions that is influenced by the host and microbe genotypes/phenotypes, the environment and whether and how beneficial microbes and microbiomes can be attracted, maintained and adapted to the plant's requirements. Below, we review the increasingly recognized or novel PGPTs of endophytic bacteria and discuss their potential applications in agriculture.

Phytohormone Production

Producing phytohormones is a common feature of endophytic bacteria to boost plant growth and increase plant stress tolerance (Pieterse et al., 2009). Genes encoding proteins for biosynthesis of indole acetic acid (IAA) (Zúñiga et al., 2013), cytokinins (CKs) (Bhore et al., 2010) and gibberellins (GAs) (Shahzad et al., 2016) are often present in the metagenome of plant endophytic bacterial communities; e.g., four pathways of IAA biosynthesis were detected in the metagenome of the tomato root gall-associated microbiome (Tian et al., 2015). Inoculation with endophytic bacteria may benefit plants via the production or suppression of phytohormones. For instance, the endophytic bacterium *Sphingomonas* sp. LK11 enhanced tomato growth, which may

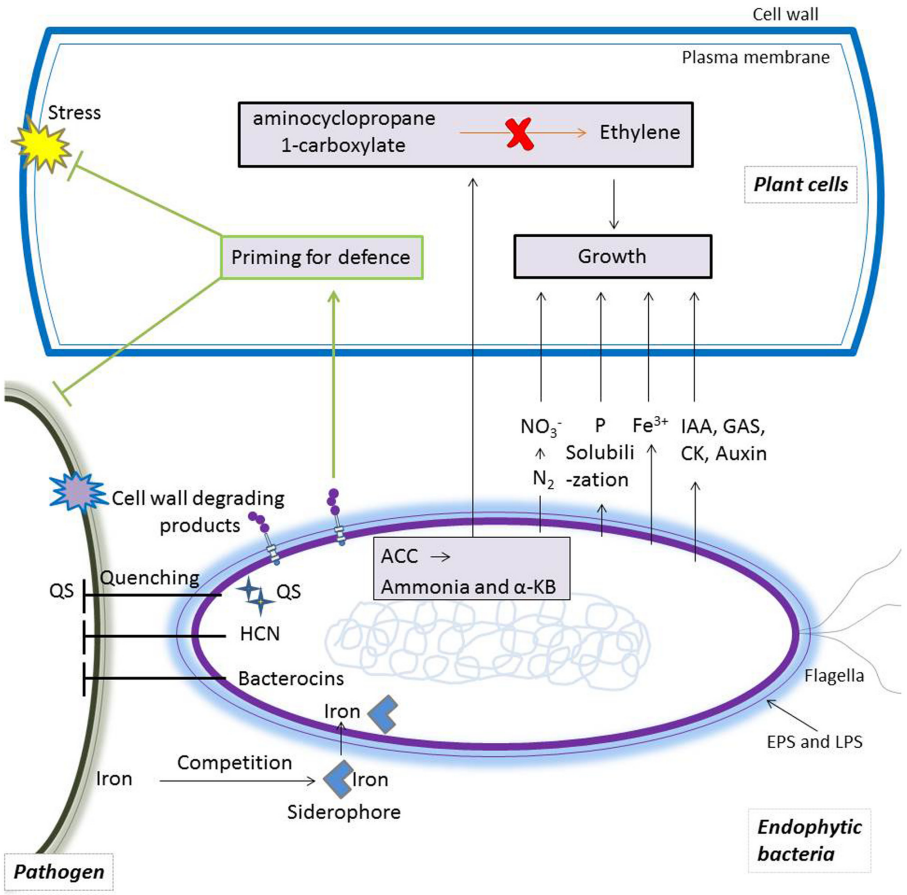


FIGURE 5 | Schematic representation summarizing plant growth promoting traits (PGPTs) of endophytic bacteria. Some endophytic bacteria are able to improve plant growth by reducing the synthesis of stress ethylene in plants, producing growth-promoting phytohormones and providing plants with macro- and/or micro-nutrients such as phosphate, nitrogen and Fe^{3+}. Endophytic bacteria can also benefit plants indirectly by suppressing the growth and reproduction of phytopathogens via multi-antagonistic effects, including quenching quorum sensing (QS), competing for nutrients, producing cell-wall degrading products and antimicrobial compounds. In this graph, arrows denote plant-bacteria interactions and '⊥' indicates inhibition. IAA, indoleacetic acid; ACC, 1 aminocyclopropane-1-carboxylic acid; GAs, gibberellins; CK, cytokinin, EPS, extracellular polymeric substance; LPS, lipopolysaccharide; αkb, α-ketobutyrate.

have been mediated by the production of GAs and IAA (Khan et al., 2014). Additionally, *S. mutabilis* strain IA1 isolated from a Saharan soil was able to produce IAA and GA3. Inoculation of wheat seedlings with this bacterium reduced the progression and severity of *F. culmorum* infection (Toumatia et al., 2016). Another study showed that *Luteibacter* sp. promoted the IAA production by its fungal host, the foliar fungal endophyte *Pestalotiopsis* aff. *neglecta* (Hoffman et al., 2013). This study highlights that there are important indirect plant microbial interactions that promote plant growth that are rarely considered and await discovery. Overall, there is a body of evidence which suggests that enhancing phytohormone production via endophytic bacteria for increased crop production in agriculture is a viable strategy.

1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase

The production of ET in stressed plants may lead to decreased plant growth or even cell death when present at high concentrations (Glick, 2014). Some microbes including bacterial endophytes use 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ET, as a carbon and nitrogen source by producing ACC deaminase (Zhang et al., 2011; Karthikeyan et al., 2012; Ali et al., 2014; Glick, 2014). Production of ACC deaminase is arguably the most efficient function for PGPB to reduce the various deleterious environmental effects on plants (Glick, 2014). Increasing global warming, desertification, soil salinization as well as extreme weather events of drought, flood and cold may exert greater stress on plants leading to reduced crop yields (Miraglia et al., 2009). Plants exposed to these stresses accumulate ACC in roots, which systematically spreads to shoots and leaves via the xylem where it is converted to stress ET by ACC-oxidase that is already present in leaves (Tudela and Primo-Millo, 1992). Inoculation with bacterial ACC deaminase producers may decrease the endogenous ACC level in plant roots and therefore increases plant tolerance to stresses (Glick, 2014). A recent study found that bacteria isolated from the endosphere of halophytic *Limonium sinense* (Girard) possessed efficient ACC

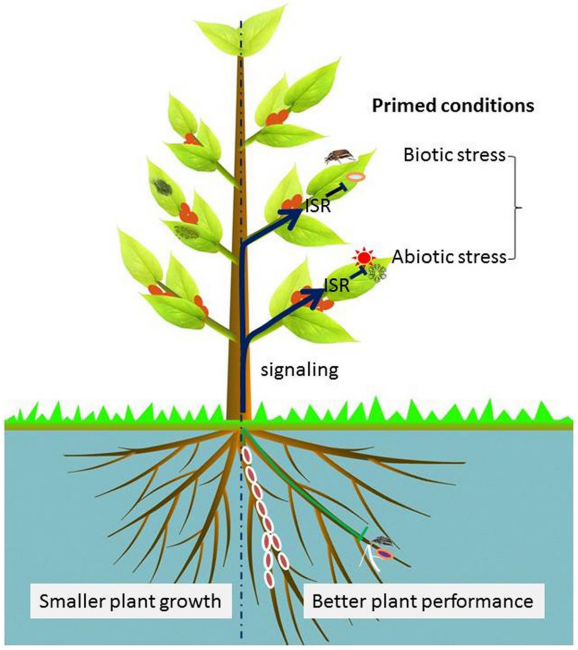


FIGURE 6 | Visualization of endophytic bacteria-induced systemic resistance (ISR). The right half of this illustration presents the elicitation of plant-primed conditions by endophytic bacteria. Endophytic bacteria-mediated ISR may be modulated by either one or combined signaling cascades of SA, JA, and ET in an endophytic bacteria-dependent manner. Some beneficial effects may include changes in root architecture relative to uninoculated plants as shown by the left half of the plant.

deaminase activity that were able to increase seed germination, root and shoot length, leaf area and numbers of *L. sinese* seedlings under salinity stress (Qin et al., 2014). While desirable results are often obtained under laboratory conditions, it should be noted that exploration of ACC deaminase producers can only occur in an agricultural context if these bacteria are able to colonize plants persistently. Development of transgenic plants overexpressing ACC deaminase genes also represents a promising strategy to overcome stress ET in plants under stress conditions.

Cold and Drought Stress Tolerance

The mechanisms underlying endophytic bacteria-mediated improvements of plant resistance to abiotic stress are starting to be elucidated. Tomato plants inoculated with psychrotolerant endophytic bacteria *Pseudomonas vancouverensis* OB155 and *P. frederiksbergensis* OS261 were able to better cope with cold stress (10–12°C) (Subramanian et al., 2015). Less membrane damage and increased antioxidant activity relative to the control plants were observed. Additionally, cold acclimation genes (*LeCBF1* and *LeCBF3*) were induced in bacteria-inoculated plants (Subramanian et al., 2015). Similarly, inoculation of the endophytes *Burkholderia phytofirmans* strain PsJN on *Arabidopsis* led to increased *Arabidopsis* growth and a strengthened cell wall, and thereby an increased cold stress resistance (Su et al., 2015). Endophytic bacteria were also able to increase plant tolerance to drought. Using a transcriptomics approach, it was found that endophytic *B. phytofirmans* PsJN displayed a diverse range of functionalities when inoculated on potato plants (Sheibani-Tezerji et al., 2015). Transcripts involved in transcriptional regulation, cellular homeostasis and ROS detoxification were upregulated in *B. phytofirmans* PsJN in drought stress-affected potato. This suggests that endophytes sense physiological changes in plants and adjust gene expression to adapt to the new environments. Endophytic bacteria have therefore the potential to be used as protective agents in agricultural systems under extreme climatic environments as they can influence plant physiological responses to stresses.

Boosting Plant Nutrient Uptake

Siderophore Production

Although iron is essential for all living organisms, its bioavailability in soil is limited. The production of siderophores by microbes assists plant growth, since these compounds chelate iron in the soil and generate soluble complexes that can be absorbed by plants (Ahmed and Holmström, 2014). We previously found that plants lacking soil bacteria suffered from iron deficiency (Carvalhais et al., 2013). Therefore, this mechanism helps plants to thrive in low iron soils. A great potential for rice root microbiomes in assisting plants in iron uptake has been suggested, given the considerable amount of gene copies encoding proteins in siderophore biosynthesis, siderophore reception and iron storage being detected in the rice root endosphere (Sessitsch et al., 2012). A key role for siderophore production has also been shown for endophytic *Streptomyces* sp. GMKU 3100 (Rungin et al., 2012). Its beneficial properties for rice plants have been established via studying a siderophore-deficient mutant. In addition, siderophores are also involved in plant protection as they deprive phytopathogens of iron by binding to the bioavailable forms of iron first (Verma et al., 2011; Aznar et al., 2015) (**Figure 5**).

Nitrogen Metabolism

Nitrogen is crucial for plant growth and health. Approximately 30–50% of the N in crop fields results from biological fixation of N_2 by soil microorganisms (Gourion et al., 2015). A considerable number of microbial genes involved in N cycling were found in the metagenome of rice roots, which indicates that the rice-related nitrification and ammonia oxidation processes might be subjected to the influence of the endophytic root microbiome (Sessitsch et al., 2012). Some endophytic bacteria possess both, nitrogen fixation (e.g., *nifH*) and denitrification genes (Straub et al., 2013). The importance of endophytic bacteria in N cycling is also supported by the evidence that N_2 fixation by foliar endophytic bacteria has occurred in many subalpine conifer species (Moyes et al., 2016). For instance, the N fixing isolate *Paenibacillus polymyxa* P2b-2R obtained from lodgepole pine tissue was able to colonize both, the rhizosphere and endosphere compartments, of maize plants and to promote maize growth (Puri et al., 2016). N_2-fixation by endophytes may provide long-lived conifers with a low-cost and stable way for N supply. However, to which extent do the bacterial endophytes contribute to the whole plant N pool is yet to be investigated.

Biocontrol of Plant Diseases

Given the similar colonizing patterns as phytopathogens and the intimate contact with plants, bacterial endophytes hold tremendous potential for being used as biocontrol agents in agriculture (Santoyo et al., 2016). For example, biocontrol practices using endophytic bacteria may be achieved either by direct inhibition of pathogens or by indirect strengthening of the plant immune system that in turn halts the growth and development of pathogens in plants (**Figure 5**). Direct inhibition of pathogens is mainly mediated by the synthesis of inhibitory allelochemicals such as antibiotics, hydrogen cyanide (HCN), iron-chelating siderophores and antifungal metabolites (Compant et al., 2010). Quenching QS by degrading autoinducer signals of pathogens is also among the direct modes of biocontrol activity of endophytic bacteria (Miller and Bassler, 2001). Indirect biocontrol mechanisms of endophytic bacteria include the induction of plant systemic resistance that inhibits a broad spectrum of phytopathogens (Niu et al., 2011; Conrath et al., 2015). In this section, we briefly summarized the main biocontrol traits within the abovementioned mechanisms to facilitate the use of endophytic bacteria to combat disease.

Primed Plants for Enhanced Defense at Low Physiological Costs

Bacterial endophytes have been reported to prime plants for faster and more intense defense responses upon pathogen attacks at low physiological cost to the plant (Martinez-Medina et al., 2016). This process depends on either JA, SA, ET or a combination of these signaling pathways (Pieterse et al., 2014). Typical priming is triggered by exposing plants to a low dose of JA, SA or ET as well as by beneficial plant-microbe interactions (Conrath et al., 2015) (**Figures 5**, **6**). For instance, the study by Brock et al. (2013) revealed that *Enterobacter radicincitans* DSM 16656, a highly competitive colonizer of the endophytic environment of various crops, is able to induce priming in *Arabidopsis* via SA- and JA/ET- dependent pathways. Similarly, the endofungal bacterium *R. radiobacter* F4 is able to colonize plant roots without specificity and it is able to increase plant resistance against the bacterial leaf pathogens *Xanthomonas translucens* pv. *translucens* and *Pseudomonas syringae* pv. tomato DC3000. Mutational analysis indicated that the resistance was mediated by ISR via a JA-dependent pathway (Glaeser et al., 2016). All these examples add to a growing number of findings that are paving the way to strategies that use bacterial endophytes to boost plant immunity. However, it remains unclear whether bacteria which colonize the root surface or endosphere contribute to priming and ISR.

Antimicrobial Components of Endophytic Bacteria

The antimicrobial compounds produced by endophytic bacteria represent a promising alternative protection to plants against phytopathogens (Brader et al., 2014) (**Figure 5**). For instance, a series of isoforms of iturins were purified from *Bacillus amyloliquefaciens* (Han et al., 2015). Exogenous treatment with the purified iturins induced MAMP-triggered immunity defense in cotton plants; in addition to triggering ROS burst, disrupting cell-wall integrity and affecting fungal signaling pathways (Han et al., 2015). Endophytic bacteria are also able to produce resistance-conferring volatile organic compounds (VOCs) (Chung et al., 2016). Maize plants inoculated with endophytic *Enterobacter aerogenes* that produce VOC 2,3-butanediol (2,3-BD) showed enhanced resistance against the northern corn leaf blight whose causative agent is the fungus *Setosphaeria turcica* (D'Alessandro et al., 2014). The endophytic *Pseudomonas poae* strain RE*1-1-14 that was originally isolated from sugar beet roots was able to suppress the fungal pathogen *Rhizoctonia solani* (Zachow et al., 2015). A novel lipopeptide poaeamide produced by this bacterium may relate to its suppression toward *R. solani* and its establishment in sugar beet roots. Despite the potential scope and impact that these biocontrol traits could have in agriculture, the understanding required for identification of antimicrobial components of bacteria and their application under field conditions is still in its infancy.

Interruption of Quorum Sensing of Plant Pathogens

Quorum sensing is a crucial strategy for bacteria to survive in complex ecological niches. It regulates the physiological activities of bacteria, involving cell-to-cell communication, reproduction, biofilm formation, competence and adaptation (Miller and Bassler, 2001). Certain endophytic bacteria employ QS quenching as an antivirulence strategy to control phytopathogens (**Figure 5**). For instance, certain endophytic bacterial strains in *Cannabis sativa* L. disrupt cell-to-cell communication of the biosensor strain *Chromobacterium violaceum* via quenching its QS signals (Kusari et al., 2014). A similar mechanism could be deployed in an agricultural context. For example, diffusible signal factor (DSF) is necessary for the virulence of several *Xanthomonas* species and *Xylella fastidiosa* (Newman et al., 2008). Thereof, *Bacillus* and *Pseudomonas* complemented with *carAB*, a gene required for the fast DSF degradation in *Pseudomonas* spp. strain G, can possibly be used to biocontrol these DSF producing pathogens.

However, a lack of persistence in the context of soil to establish a compatible interaction with plants may mostly make the deployment of endophytic bacteria difficult at field settings (Le Cocq et al., 2017). A much more profound understanding of novel/untapped mechanism of PGPTs in delivering beneficial plant-associated phenotypes is needed to ensure their practicality in the field. Furthermore, genome sequencing of strain collections might foster a faster and less labor-intensive method to screen for sets of PGPTs that are readily detected in genomes of endophytic bacteria.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Insights into the microbial ecology of the plant's endosphere have been greatly expanded in the era of high-throughput DNA sequencing. Phylogenetic marker gene sequencing surveys and

meta'omic analyses have enabled scientists to probe microbial community composition in a high-resolution and culture-independent manner. Based on these techniques and culture-dependent methods, solid proofs have been obtained that plants sculpt their root endophytic microbiome, and roots have an effective 'gate-keeping' role in this process. Although the inoculation of beneficial bacterial endophytes can notably improve plant growth and yield, it remains unclear if it is essential for these bacteria to colonize internally to generate beneficial effects on plants. Similarly, there are pending questions regarding to which extent endophytic microbiomes support plant growth and defense. For example, how much the nitrogen fixed by endophytic diazotrophs contributes to the overall plant nutrition? Also, whether/how microbial fluctuations in the endosphere correlate to plant health and behavior?

The use of gnotobiotic plants (grown either under sterile conditions or with known microbes) would allow the elucidation of the importance of the endosphere microbiota in plant growth and health. Additionally, it will be of great interest in the future to reveal the mechanisms and ecological rationales behind the rare presence of Acidobacteria, Gemmatimonadetes and Archaea in the plant's endosphere. These microorganisms are still prohibitively difficult to be cultured. Besides taxonomic surveys, recently emerging techniques like single-cell isolation and sequencing should provide alternatives that circumvent the necessity for cultivation and thereby give steps forward to obtain more comprehensive pictures about their lifestyles and interactions with plants (Gawad et al., 2016). Furthermore, there are some prohibitive technical obstacles for studying the endosphere microbiome. Many laboratories still have difficulties in optimizing DNA samples of surface-sterilized plant tissues (e.g., leave, shoot, seeds) for microbiome sequencing purposes. This is due to largely the abundance of bacterial DNA in non-root tissues being much smaller, relative to plant DNA. Besides optimizing the PCR conditions, a non-biased enrichment of endophytic bacterial cells from plant tissues may circumvent this problem (Dos-Santos et al., 2017).

Although there is a wealth of literature on culture-dependent and independent characterization of endophytic bacterial diversity and the associated *in vitro* mechanisms for plant growth promotion, reports on successful use of endophytic bacteria in plants under field conditions are extremely scarce. Nevertheless, the effect of the *Burkholderia phytofirmans* strain PsJN has been demonstrated to increase biomass and promote growth in switchgrass (*Panicum virgatum* L.) especially in low fertility soils (Lowman et al., 2015). Furthermore, systemic resistance was induced in pepper infected with *Xanthomonas axonopodis* pv. vesicatoria (causal agent of bacterial spot) in the field by an additive effect of the endophyte *Bacillus pumilus* INR7 combined with the chemical inducer benzothiadiazole (Yi et al., 2013).

Proteobacteria, Actinobacteria and Firmicutes are core phyla in the plant endosphere, which are also those groups harboring commensal plant growth-promoting bacteria. Given the diverse PGPTs, the intimate interactions with plants and similar colonizing patterns as phytopathogens, bacterial endophytes bear a great potential to be used for developing biocontrol agents and biofertilizers. Increasing agricultural production by harnessing the plant-associated microbiome is a tantalizing prospect. Prior to this, ways to change the composition and function of microbiomes need to be identified ("microbiomes engineering"). As mentioned, research efforts have been made on manipulating plant microbiomes by inducing the plant's signaling defense pathways using exogenous phytohormone treatments. Nevertheless, the contradicting results obtained from different studies suggest that it is still challenging to manipulate the plant's endosphere microbiome. Future studies need to be conducted to bridge the knowledge gaps of how microbial function of the endosphere is affected by plant immunity. A combination of multi 'omics' such as metagenomics, proteomics and metabolomics and the advancing computational data-mining approaches should be able to reveal a more comprehensive picture of the endosphere microbiome, therefore transforming the way we understand bacterial endophytes and their interactions with plant hosts. It must, however, be addressed that culture-dependent methods are still important because they provide the indispensable materials for identifying bacterial physiological characteristics and allow the predication of the metabolic potential and biogeochemical function of a lineage by using genomic surveys. Promising areas to develop are efforts to breed for endophyte-optimized crops, endophytic microbiomes engineering and a better understanding how key endophytes can be attracted, maintained and adapted to benefit plants at various growth stages. While substantial basic and applied work remains to be done, it is envisioned that in the not too distant future bacterial endophytes can be at least partial substitutes for chemical fertilizers and pesticides and their targeted applications on crops may push forward a paradigm shift in agriculture.

AUTHOR CONTRIBUTIONS

HL did the writing and drew the graphs. LC, MC, ES, PD, CP, and PS revised this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02552/full#supplementary-material

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