PLANT MICROBIOME: INTERACTIONS, MECHANISMS OF ACTION, AND APPLICATIONS, VOLUME II

EDITED BY: Alok Kumar Srivastava, Prem Lal Kashyap, George Newcombe and Gustavo Santoyo

PUBLISHED IN: Frontiers in Microbiology

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88976-416-7
DOI 10.3389/978-2-88976-416-7

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PLANT MICROBIOME: INTERACTIONS, MECHANISMS OF ACTION, AND APPLICATIONS, VOLUME II

Topic Editors:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India
Prem Lal Kashyap, Indian Institute of Wheat and Barley Research (ICAR), India
George Newcombe, University of Idaho, United States
Gustavo Santoyo, Universidad Michoacana de San Nicolás de Hidalgo, Mexico

Citation: Srivastava, A. K., Kashyap, P. L., Newcombe, G., Santoyo, G., eds. (2022). Plant Microbiome: Interactions, Mechanisms of Action, and Applications, Volume II. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-416-7

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frontiers | Frontiers in Microbiology

EDITORIAL
published: 31 May 2022
doi: 10.3389/fmicb.2022.915684

Editorial: Plant Microbiome: Interactions, Mechanisms of Action, and Applications, Volume II

Alok Kumar Srivastava[1]*, *Prem Lal Kashyap*[2]*, *Gustavo Santoyo*[3]* and *George Newcombe*[4]*

[1] National Bureau of Agriculturally Important Microorganisms, Indian Council of Agricultural Research (ICAR), Mau, India, [2] Indian Institute of Wheat and Barley Research, Indian Council of Agricultural Research (ICAR), Karnal, India, [3] Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mexico, [4] Department of Forest, Rangeland and Fire Sciences, University of Idaho, Moscow, ID, United States

Keywords: microbiome, symbionts, biostimulants, metabolites, arbuscular mycorrhizal fungi (AM fungi), rhizosphere, root-knot nematodes, abiotic stress

OPEN ACCESS

Edited and reviewed by:
M. Pilar Francino, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO), Spain

***Correspondence:**
Alok Kumar Srivastava
aloksrivastva@gmail.com
Prem Lal Kashyap
plkashyap@gmail.com
Gustavo Santoyo
gustavo.santoyo@umich.mx
George Newcombe
georgen@uidaho.edu

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *08 April 2022*
Accepted: *26 April 2022*
Published: *31 May 2022*

Citation:
Srivastava AK, Kashyap PL, Santoyo G and Newcombe G (2022) Editorial: Plant Microbiome: Interactions, Mechanisms of Action, and Applications, Volume II. Front. Microbiol. 13:915684. doi: 10.3389/fmicb.2022.915684

Editorial on the Research Topic

Plant Microbiome: Interactions, Mechanisms of Action, and Applications, Volume II

In a recent article, Suzanne Simard (Simard, 2022) writes of trees that they are "in constant communication with one another through an underground biological neural network made of mycorrhizal fungi." Networked plants also potentially link other symbionts. Since plants comprise 82% of all living biomass on Earth (Bar-On et al., 2018), their microbiome-enabled network has global reach ecologically. Plant symbionts may mostly be bacteria or fungi but animals (e.g., nematodes), protists, viruses, and even archaeans (Müller et al., 2015) can also integrate into the community. Moreover, when we consider that humanity must depend on this microbially diverse, plant-centered network for food, fiber, optimized carbon sequestration, and conservation of known and unknown biodiversity, it is not difficult to make the case for its importance. Even though we may not yet have seen all the moving parts in action, so to speak, our current studies of facilitated and direct interactions steadily add to our understanding. From a current focus on single plant species, particularly their rhizospheres, we may eventually move to a broad understanding of the collective microbiome of the global plant community.

So, here in Vol. II of the "Plant Microbiome: Interactions, Mechanisms of Action, and Applications" we add 19 articles that collectively address some of the key questions that we can pose and answer today. A total of 15 of the 19 represent reports on original research. The remaining four represent original reviews. The original research articles focus primarily on a range of agricultural crop plants with their associated microbes: cassava, sugarcane, tea, soybean, rice, cotton, Chinese chives, licorice, and tomato. However, also included among the research articles are reports on the effects of symbionts on *Lolium arundinaceum* (tall fescue), *Oxytropis glacialis* (a locoweed species of the Tibetan plateau), and *Ulva fasciata* (a marine green alga). Another research article addresses the interactions of nematophagous fungi and root-knot nematodes, and how those interaction outcomes affect plant health (Kassam et al.).

The four original reviews in this new volume are diverse. In the first, Fadiji et al. provide evidence for specific microbes that can help to mitigate the abiotic stresses of climate change. These microbial biostimulants might be called adaptogenic, since they can help plants to adapt to extreme weather events that are becoming more common with continuing climate change. Although the COVID pandemic temporarily reduced anthropogenic emissions of

greenhouse gasses, deepening concern over the effects of climate change is warranted (Depledge et al., 2022). Excessive heat, untimely frosts, salinity and drought, and flooding threaten the viability of agriculture everywhere. Those microbes singled out for discussion of their protection of crop plants against extreme heat and cold are primarily bacterial taxa, although species of *Glomus*-forming arbuscular mycorrhizae are also included. The same is true for protection against drought and salinity stress, although, *Penicillium* spp. are included with *Glomus* spp. in the fungal category.

The second review by Suman et al. is complementary to that of Fadiji et al.. Microbial bioinoculants are discussed as essential adjuncts to sustainable agriculture. Sustainability is assumed to include an adaptive response to climate change mediated by microbes. Again, bacteria are emphasized in terms of their ability to stimulate growth of their hosts, along with their ability to defend plants. Some fungal examples are also covered in this article review by Fadiji et al..

The third review by Mishra et al. focuses on metabolomic insights into endophyte-derived compounds that affect plant performance. Early in their review, the authors emphasize that although plants themselves produce important secondary metabolites, their endophytes do as well. The list of these dual-source metabolites includes medically important compounds such as taxol, vincristine, vinblastine, camptothecin, and hypericin. Additionally, metabolites in terms of their functions in plant defense have also been discussed.

In another review, Chot and Reddy focus on the growth of ectomycorrhizal (ECM) plants on land contaminated with heavy metals. For many years, phytoremediation has included the effects of symbionts (Newman and Reynolds, 2005). The authors stated that this particular subject (i.e., remediation with ECM plants) is "highly under-studied," but that the probable mechanisms include the following: (1) physical intervention (presumably of the ECM mantle) such that metals in soil do not contact plant tissue; (2) enhanced antioxidant activity and metal sequestration; and (3) mycorrhiza-altered nutrient uptake that increases tolerance of metals.

With respect to the reports of original research, we turn first to endophytes of *Oxytropis glacialis* (a locoweed species of the Tibetan plateau). Locoweeds are defined as plant species that contain a toxic alkaloid, swainsonine, that can cause locoism in grazing animals; species of both *Astragalus* and *Oxytropis* have been implicated. Braun et al. (2003) were the first to show that endophytes in locoweeds could produce swainsonine. In this volume, Cao et al. reported fungal diversity in the soil of roots of *Oxytropis glacialis* and reported OTUs associated with swainsonine producers.

Arbuscular mycorrhizal fungi (AMF) can affect floral traits, fruit yield, resource allocation, and seed germination of cherry tomato. In this volume, Wang L. et al. showed that nutrient levels can interact with AMF treatments to affect plant responses. It has been highlighted that tall fescue (*Lolium arundinaceum*) is affected by both *Epichloë* endophytes and AMF. *Epichloë* endophytes can aid tall fescue under conditions of saline-alkali stress. The beneficial effect of endophytes under these conditions was improved by one AMF, *Claroideoglomus etunicatum*, but was hindered by a second AMF, *Funneliformis mosseae*. Liu H. et al. thereby highlight the interactions of key symbionts of particular plants.

Cassava (*Manihot esculenta*) is an important staple and cash crop in tropical and subtropical regions, where its tolerance of harsh conditions (drought, in particular) is valued. According to Ha et al., the microbial community of cassava is not adequately studied. Using 14 cassava genotypes, bacterial 16S rDNA sequencing revealed that the richness and diversity of bacteria in the rhizosphere were higher than those in the tuber endosphere. Cassava genotype did affect rhizosphere bacterial communities but not endophytic tuber communities. The bacterial phyla of tubers and rhizosphere were Bacillota (formerly Firmicutes) and Actinomycetota (formerly Actinobacteria), respectively. This study should pave the way for further exploration of the cassava microbiome.

Two sugarcane varieties with differing drought tolerance were the basis for an interesting study by Liu Q. et al. of the function of rhizosphere bacteria in response to water stress. One variety was tolerant; the other was sensitive. Rhizosphere bacterial diversity was reduced by drought unequally in the two varieties: more in the sensitive than in the tolerant variety. Drought-resistant bacteria persist in the rhizosphere of the tolerant variety but do not in that of the sensitive one. This interesting paper should prompt confirmatory studies in additional plant systems.

Positive effects on plant growth and development are associated in particular with these bacterial genera: *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*, *Erwinia*, *Gluconobacter*, *Klebsiella*, *Serratia*, *Pantoea*, *Herbaspirillum*, *Rahnella*, *Pseudomonas*, and *Xanthomonas* (Compant et al., 2011; Kushwaha et al., 2020; Hazarika et al.). The latter authors tackled the culturable, endophytic community of bacteria in leaves and roots of tea (*Camellia sinensis*) in northeastern India. They then focused on *in vitro* and *in planta* traits for plant growth promotion. A total of 106 isolates were identified for further development as bioinoculants that would greatly aid sustainable tea production.

Yuan et al. investigated the expression of *Bradyrhizobium diazoefficiens* genes in nodule samples from five developmental stages of soybean (i.e., branching, flowering, fruiting, pod maturation, and harvest). Their findings hint at further improvements in engineering efficiency of nitrogen fixation.

Chu et al. found that six bacterial phyla characterized the microbiome of roots of six rice cultivars grown in cadmium-contaminated paddy fields in China: Pseudomonadota (formerly Proteobacteria), Bacillota (Firmicutes), Actinomycetota (Actinobacteria), Acidobacteriota (Acidobacteria), Bacteroidota (Bacteroidetes), and Spirochaetota (Spirochaetes). Their most interesting finding is that these endophytes form "a more highly interconnected network and exhibit higher operational taxonomic unit (OTU) numbers, diversities, and abundance" during the reproductive phase of their rice host than during the vegetative stage. Some of the root endophytes

also displayed resistance to cadmium, which points to possible applications.

Xinjiang is an arid region of China in which cotton is grown. Shi et al. compared "the rhizosphere and endosphere microbiomes of healthy and diseased cotton from north and south of the Tianshan Mountains using the methods of PCR-based high-throughput sequencing and real-time quantitative PCR." Interestingly, diseased plants hosted a greater abundance of both rhizosphere and endosphere bacteria and fungi.

Persistent application of chemical fertilizers to sugarcane crops can lead to pollution, degradation of the soil biota, and yield losses in the long term (Robinson et al., 2011). In an ambitious project reported in this volume (Singh et al.), endophytic root bacteria were screened for their potential as biofertilizers. The following two isolates were most promising: *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1. Both strains readily colonized the leading Chinese sugarcane.

A popular, perennial vegetable in China (i.e., Chinese chives, or *Allium tuberosum*) was the focus of a study by Sun et al. of the plant's endophytic bacteria. Bacteria with high relative abundance in an ecological compartment acted as key nodes.

Microbial symbionts of green algae also make an impressive appearance in this volume (Wang H. et al.). Among epiphytic bacteria, a new species, *Hyunsoonleella* sp. HU1-3 (family Flavobacteriaceae), was identified which significantly promoted the growth of its green algal host, *Ulva fasciata*.

Root-knot nematodes are the target of a study (Kassam et al.) employing Indian isolates of two nematophagous fungi: *Arthrobotrys thaumasia* and *Tolypocladium cylindrosporum*. These fungi show potential as biocontrol agents of the plant-parasitic nematodes that are responsible for significant crop damage globally.

From the sugarcane rhizosphere, 58 actinobacteria were isolated and evaluated for their antagonistic potential in a study by Wang Z. et al.. Smut resistance was the focus, and *Streptomyces griseorubiginosus* showed considerable potential as it possessed the metabolic tools to alleviate biotic stress factors like smut fungi.

Wild licorice (*Glycyrrhiza uralensis*) has been an important traditional medicine in China. With wild populations dwindling, best practices for cultivation are now being researched (Yue et al.). Drought is a significant abiotic stress factor for licorice. In prior research, *Bacillus amyloliquefaciens* FZB42 was shown to confer stress tolerance to *Arabidopsis*. Here, the authors demonstrated that this strain could alleviate drought stress; it also increased yields of total flavonoids, liquiritin, and glycyrrhizic acid which are the medicinal products of *G. uralensis*.

In conclusion, after review of the 19 articles of Vol. II, one thing is clear: the "exciting possibilities" of the plant-microbe interactome, to which we alluded in Vol. I, are now bearing fruit. We can expect many more exciting discoveries in the upcoming years.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The editors of this special issue greatly appreciate the contributions of authors and reviewers.

REFERENCES

Bar-On, Y. M., Phillips, R., and Milo, R. (2018). The biomass distribution on Earth. *Proc. Natl. Acad. Sci. U. S. A.* 115, 6506–6511. doi: 10.1073/pnas.1711842115

Braun, K., Romero, J., Liddell, C., and Creamer, R. (2003). Production of swainsonine by fungal endophytes of locoweed. *Mycol. Res.* 107(Pt 8), 980–988. doi: 10.1017/s095375620300813x

Compant, C., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microbial Ecol.* 62, 188–197. doi: 10.1007/s00248-011-9883-y

Depledge, J., Saldivia, M., and Peñasco, C. (2022). Glass half full or glass half empty?: the 2021 glasgow climate conference. *Clim. Policy* 22, 147–157. doi: 10.1080/14693062.2022.2038482

Kushwaha, P., Kashyap, P. L., Bhardwaj, A. K., Pandiyan, K., Srivastava, A. K., and Tiwari, R. K. (2020). Bacterial endophyte mediated plant tolerance to salinity: growth responses and mechanisms of action. *World J. Microbiol. Biotechnol.* 36, 26. doi: 10.1007/s11274-020-2804-9

Müller, H., Berg, C., Landa, B. B., Auerbach, A., Moissl-Eichinger, C., and Berg, G. (2015). Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. *Front. Microbiol.* 6, 138. doi: 10.3389/fmicb.2015.00138

Newman, L. A., and Reynolds, C. M. (2005). Bacteria and phytoremediation: new uses for endophytic bacteria in plants. *Trends Biotechnol.* 23, 6–8. doi: 10.1016/j.tibtech.2004.11.010

Robinson, N., Brackin, R., Vinall, K., Soper, F., Holst, J., Gamage, H., et al. (2011). Nitrate paradigm does not hold up for sugarcane. *PLoS ONE* 6, e19045. doi: 10.1371/journal.pone.0019045

Simard, S. (2022). *The big idea: can forests teach us to live better? | Science and nature books | The Guardian*. Available online at: https://www.theguardian.com/books/2022/mar/21/the-big-idea-can-forests-teach-us-to-live-better-trees-model-suzanne-simard (accessed April 08, 2022).

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ORIGINAL RESEARCH
published: 30 September 2021
doi: 10.3389/fmicb.2021.729022

Diversity of the Bacterial Microbiome Associated With the Endosphere and Rhizosphere of Different Cassava (*Manihot esculenta* Crantz) Genotypes

Jingwen Ha[1†], Yu Gao[1†], Rui Zhang[1], Ke Li[1], Yijie Zhang[1], Xiaolei Niu[1], Xin Chen[2], Kai Luo[1] and Yinhua Chen[1*]*

[1] Hainan Key Laboratory for the Sustainable Utilization of Tropical Bioresources, Hainan University, Haikou, China, [2] Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Kauser Abdulla Malik, Forman Christian College, Pakistan
Derek Lundberg, Max Planck Institute for Developmental Biology, Germany

***Correspondence:**
Kai Luo
luok@hainanu.edu.cn
Yinhua Chen
yhchen@hainanu.edu.cn

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *22 June 2021*
Accepted: *01 September 2021*
Published: *30 September 2021*

Citation:
Ha J, Gao Y, Zhang R, Li K, Zhang Y, Niu X, Chen X, Luo K and Chen Y (2021) Diversity of the Bacterial Microbiome Associated With the Endosphere and Rhizosphere of Different Cassava (Manihot esculenta Crantz) Genotypes. Front. Microbiol. 12:729022. doi: 10.3389/fmicb.2021.729022

Root-associated microbial communities play important roles in plant growth and development. However, little attention has been paid to the microbial community structures associated with cassava, which is a staple food for approximately 800 million people worldwide. Here, we studied the diversity and structure of tuber endosphere and rhizosphere bacterial communities in fourteen cassava genotypes: SC5, SC8, SC9, SC205, KU50, R72, XL1, FX01, SC16, 4612, 587, 045, S0061, and 1110. The results of bacterial 16S rDNA sequencing showed that the richness and diversity of bacteria in the rhizosphere were higher than those in the tuber endosphere across the 14 cassava genotypes. After sequencing, 21 phyla and 310 genera were identified in the tuberous roots, and 36 phyla and 906 genera were identified in the rhizosphere soils. The dominant phylum across all tuber samples was *Firmicutes,* and the dominant phyla across all rhizosphere samples were *Actinobacteria*, *Proteobacteria*, and *Acidobacteria*. The numbers of core bacterial taxa within the tuber endospheres and the rhizospheres of all cassava genotypes were 11 and 236, respectively. Principal coordinate analysis and hierarchical cluster analysis demonstrated significant differences in the compositions of rhizosphere soil microbiota associated with the different cassava genotypes. Furthermore, we investigated the metabolic changes in tuber roots of three genotypes, KU50, SC205, and SC9. The result showed that the abundances of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* in tuber samples were positively correlated with organic acids and lipids and negatively correlated with vitamins and cofactors. These results strongly indicate that there are clear differences in the structure and diversity of the bacterial communities associated with different cassava genotypes.

Keywords: bacterial diversity, cassava, endosphere, rhizosphere, 16S rDNA, metabolites

INTRODUCTION

Plants host diverse and abundant microbial communities that can be considered the "second genomes" of plants. Microbial communities that exist in close association with plants can be categorized into three groups: endophytic, epiphytic, and closely associated (Tringe et al., 2005). Plants and their associated microbes interact with each other and form assemblages of genotypes that are often referred to as "holobionts" (Vandenkoornhuyse et al., 2015; Hassani et al., 2018). Plants attract and select for beneficial microbiomes by releasing signal molecules and providing carbon metabolites as root exudates to endosphere and rhizosphere bacteria (Ryu et al., 2004; Guo et al., 2016; Lopes et al., 2016). Plants can influence net ecosystem changes through deposition of secondary metabolites into the rhizosphere that attract or inhibit the growth of specific microorganisms. This rhizodeposition was made up of small-molecular weight metabolites, amino acids, secreted enzymes, mucilage, and cell lysates (Grayston et al., 1998; Paterson and Sim, 2000). Soil microbes utilize this abundant carbon source, thereby implying that selective secretion of specific compounds may encourage beneficial symbiotic and protective relationships whereas secretion of other compounds inhibit pathogenic associations (Hoffland et al., 1992; Holden et al., 1999). A concrete example is the secretion of isoflavones by soybean roots, which attract a mutualist (*Bradyrhizobium japonicum*) and a pathogen (*Phytopthora sojae*) (Morris et al., 1998). In turn, plants benefit from these relationships, as the microbes change key nutrients into more usable forms (Long, 1989; Bolan, 1991; Zhang et al., 2009). The symbioses between plants and the associated microbes play important roles in the development, health and environmental adaptability of the plant hosts (Spor et al., 2011; Berendsen et al., 2012; Yuan et al., 2018). Previous studies on *Arabidopsis thaliana* (Durán et al., 2018), grapevine (Rolli et al., 2015), and citrus (Zhang et al., 2017) have demonstrated that the bacterial community plays an essential role in plant growth through a variety of mechanisms, including increasing nutrient acquisition, promoting plant hormone production, and protecting plants against pathogen attacks (Ritpitakphong et al., 2016; Álvarez-Pérez et al., 2017; Hassani et al., 2018). Many plant-associated microbes can induce systemic resistance in plants (Liu et al., 2019). For example, stem inoculation with the bacterial strains *Bacillus amyloliquefaciens* (GB03) and *Microbacterium imperiale* (MAIIF2a) mitigates *Fusarium* root rot in cassava (Freitas et al., 2019). *Bacillus cereus* AR156 is a plant growth-promoting rhizobacterium (PGPR) that induces resistance against a broad spectrum of pathogens in *A. thaliana* (Niu et al., 2011). Moreover, harnessing the plant microbiome to maximize crop production is increasingly considered a viable and sustainable approach for the future of agriculture (Geddes et al., 2015; Qiu et al., 2019).

Endophytic microbes and rhizosphere exophytic microbes are affected both by their host plants and by environmental stimuli. In some plants, the diversity and composition of endophytic communities are highly variable between cultivars (Liotti et al., 2018; López et al., 2018). For example, two *Rosa* cultivars with different powdery mildew susceptibilities were determined to share only 34.2% of operational taxonomic units (OTUs), and the resistant cultivar had significantly lower fungal diversity than the susceptible cultivar in the early stage of development (Zhao et al., 2018b). Some studies have shown that the species has a stronger influence on bacterial community composition by growing *Populus*, *Quercus*, and *Pinus* in three soils originating from different field sites (Bonito et al., 2014). The host plant species is the most important factor that determine the leaf endophytic bacterial communities collected from 5 species of plants (*Asclepias viridis*, *Ambrosia psilostachya*, *Sorghastrum nutans*, *Panicum virgatum*, and *Ruellia humilis*) (Ding et al., 2013). Moreover, the taxonomic composition of the extraordinarily diverse communities of microorganisms associated with plants is determined partly by the plant genotype (Korkama et al., 2006; Peiffer et al., 2013; van der Heijden and Schlaeppi, 2015). Genotype effects on the fungal and bacterial microbiomes have been detected in *Triticum aestivum* (Simonin et al., 2020), rice (*Oryza sativa*) (Edwards et al., 2015), maize (*Zea mays*) (Walters et al., 2018), and potato (*Solanum tuberosum*) (van Overbeek and van Elsas, 2008; Inceoğlu et al., 2010). The influence of plant genotypes on belowground microbiota can be attributed to differences in plant growth performance, as well as in the varying amounts of nutrients provided to soil through litter and root exudates (Korkama et al., 2006; van der Heijden and Schlaeppi, 2015; Hugoni et al., 2018) and through their symbionts (Smith and Read, 2008; Gorka et al., 2019). Rhizosphere-associated microbes obtain essential nutrients from plants through host root exudates, and crucial nutrients can be converted to more usable forms, including sugars, organic acids, amino acids, and peptides, by microbes before being assimilated by plants (Ryu et al., 2004). Therefore, through the release of a broad variety of secondary metabolites and root exudates, plants have the capacity to drive and shape plant-associated microbial communities (Raaijmakers et al., 2009). The plant genotype, by determining the community structure of its microbial partners, can be expected to exert cascading effects on ecosystem functions related to nutrient cycling. Overall, plant genetic control of the microbial community is of considerable interest for crop plant breeding and for exploring the possibility of designing a "healthy" microbiome (Morella et al., 2020).

Cassava (*Manihot esculenta* Crantz) is a member of the *Euphorbiaceae* family, has strong environmental adaptability and is tolerant of barrenness and drought (Luo, 2005). Owing to its starch-enriched tuberous root, cassava is an important cash crop in tropical and subtropical areas, and it can also be converted into a large number of products; for example, it is a major resource used in the production of starch, biofuel, and animal feed (Utsumi et al., 2012; Okogbenin et al., 2013). Previous studies have shown that the agronomical characteristics of cassava are significantly influenced by different PGPR strains (Suja et al., 2015). Microbial inoculation significantly improved the mineral nutrient uptake, yield, harvest index, and repression of root rot infection in cassava compared with those in uninoculated controls (Hridya et al., 2013).

The application of beneficial bacteria plays an important role in increasing plant growth and protecting against pathogen infection in cassava (Freitas et al., 2019). With the development of next-generation sequencing technologies, culture-independent methods have been employed to determine the profiles of the cassava-associated microbial communities by 16S rDNA sequencing. Li et al. (2020) showed that cassava cultivars recruited various endophytic microbial taxa from tuberous roots to affect the ability of root rot resistance. Similarly, structure of microbiomes of cassava genotypes were analyzed, and revealed their potential roles in cassava bacterial blight resistance (Zhang et al., 2021). Such previous studies have been made on cassava-associated bacteria overall, and few have focused on the comprehensive investigation of endophytic and rhizospheric bacteria of cassava genotypes. Besides, we currently have a poor understanding of how tuber metabolites influence microbial community structure.

In this study, fourteen cassava genotypes with significant differences in genetic background were used to investigate the relationship between cassava-associated bacteria and genetic differences among cassava genotypes by 16S rDNA gene tag sequencing analysis. Moreover, we examined the effects of cassava tuber metabolites collected from three typical cassava genotypes on the different bacterial communities from the tuberous roots and rhizosphere soil. Our results will provide new insight into the linkages between cassava-associated bacteria and cassava genotypes.

MATERIALS AND METHODS

Study Sites and Sample Collection

A total of fourteen cassava genotypes (SC5, SC8, SC9, SC205, KU50, R72, XL1, FX01, SC16, 4612, 587, 045, S0061, and 1110) were provided by Prof. Wenquan Wang, and were grown in the same field in Chengmai county, Hainan Province, China (19°85′ N, 110°08′ E, elevation 83 m a.s.l.) (**Supplementary Table 1**). The average annual temperature at the study location is 23.8°C, the annual precipitation is 1786.1 mm and the average annual sunshine hours are 2,059 h. The soil type at the site is a red loam.

Two samples (tuberous roots and rhizosphere soil) of the fourteen genotypes were taken with three biological replicates in March 2019. The detail of endosphere and rhizosphere microbes sampling was as follows. The whole tuberous roots were taken out and the bulk soil was removed by careful shaking. Soil still adhering to the tubers was collected with sterile tweezers and defined as the rhizosphere soil. In order to remove the majority of rhizosphere-associated microbes and enrich for endophytic microbes, the tubers were washed with water and sterilized, first with 75% alcohol and then with a sodium hypochlorite solution containing 1% active chlorine. Then, the tubers were washed with sterilized water, and cleaned using sterilized filter paper, and placed into sterilized bags. All the samples were stored at −80°C in liquid nitrogen until DNA extraction (Dong et al., 2018). The tuberous roots of three cassava genotypes, KU50, SC205, and SC9, were collected for metabonomic analysis, and three biological replicate samples were taken for each cassava genotype.

DNA Extraction and Illumina MiSeq Sequencing

The tuber samples were ground into powder by the liquid nitrogen grinding method for the extraction of the endophytic flora. Genomic DNA was extracted from the freeze-dried tuber powder (50 mg) and freeze-dried soil samples (0.20 g) with E.Z.N.A.^{TM} Mag-Bind Soil DNA kits (Omega, United States), following the manufacturer's instructions. We measured the concentration of the DNA using a Qubit 2.0 (life, United States) to ensure that adequate amounts of high-quality genomic DNA had been extracted. The 16S rDNA V3–V4 amplicon was amplified using KAPA HiFi Hot Start Ready Mix (2×) (TaKaRa Bio Inc., Japan) and individual barcoded primers with gene-specific regions of those primers corresponding to 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Dethlefsen and Relman, 2011). The polymerase chain reaction amplification conditions were as follows: the reaction mixtures in each tube contained 2 μL of target DNA (10 ng/μL), 15 μL of 2 × KAPA HiFi Hot Start Ready Mix, 1 μL of amplicon PCR forward primer (10 μM), 1 μL of amplicon PCR reverse primer (10 μM), and 11 μL of sterile distilled water, with a total volume of 30 μL. The plate was sealed and polymerase chain reaction (PCR) performed in a thermal instrument (Applied Biosystems 9700, United States) using the following program: the thermal cycling conditions for the primary PCRs consisted of 3 min at 93°C, followed by 5 cycles of 30 s at 94°C, 20 s at 45°C, and 30 s at 65°C, followed by 20 cycles of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C, and a final extension for 5 min at 72°C. The PCR products were checked using electrophoresis in 1% (w/v) agarose gels in TBE buffer (Tris, boric acid, EDTA) stained with ethidium bromide (EB) and visualized under UV light.

After PCR amplification, quantification of the bacterial 16S rDNA was performed using Qubit3.0 DNA detection kits. Next, the samples were loaded onto an Illumina MiSeq high-throughput sequencing platform for paired-end sequencing (Shao et al., 2017) and sequenced by Sangon BioTech (Shanghai, China). The raw Illumina MiSeq sequences were processed and analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package (version 1.8.0) (Caporaso et al., 2010). The paired-end reads were merged into longer contigs and quality filtered to remove contigs with lengths < 200 nt, average quality scores of < 20, and contigs containing > 3 nitrogenous bases by PANDAseq. The quality-filtered sequences were subsequently clustered in OTUs at 97% similarity and annotated using the Ribosomal Database Project (RDP) and Silva bacterial databases to determine the phylogeny and relative abundances of the OTUs (Cole et al., 2007). The unclassified OTUs and the reads identified as chimeras through UCHIME (Edgar et al., 2011) were removed from downstream analysis. The raw data were submitted to the NCBI Sequence Read Archive (Accession No. PRJNA750582).

Extraction of Tuber Metabolites

Twenty-five milligrams (± 2%) of each tuberous sample was accurately weighed out and ground. The sample to

be tested was then extracted, and gas chromatography-mass spectrometry (GC-MS) detection was performed. Briefly, GC was performed on an HP-5 MS capillary column (5% phenyl/95% methylpolysiloxane 30 m × 250 μm i.d., 0.25 μm film thickness, Agilent J and W Scientific, Folsom, CA, United States) to separate the derivatives at a constant flow rate of 1 mL/min helium. One microliter of sample was injected in split mode in a 20:1 split ratio by the autosampler. The injection temperature was 280°C, the interface was set to 150°C, and the ion source was adjusted to 230°C. The temperature-increase program was as follows: initial temperature of 60°C for 2 min, 10°C/min increase rate to 300°C and holding at 300°C for 5 min. MS was performed with the full-scan method within a range from 35 to 750 (m/z) (Sangster et al., 2006; Want et al., 2013).

The obtained raw data were converted into the netCDF format (xcms input file format) through Agilent MSD ChemStation (Smith et al., 2006). R (v3.1.3) was used to obtain data matrices, including the mass to charge ratio (m/z), retention time, and peak area (intensity). Metabolite annotations were performed with the AMDIS program. The databases used for annotation were the National Institute of Standards and Technology (NIST) commercial database and the Wiley Registry metabolome database. Among them, the metabolite alkane retention index was used for further qualitative substance analysis according to the retention index provided by the Golm Metabolome Database (GMD),[1] and most of the substances were further confirmed by standard products.

Bioinformatics and Statistical Analysis

R software (version 4.0.2) was used for bioinformatics analysis, and certain plots were generated using the "ggplot2" package. The "vegan" package was used to calculate the number of microorganisms and abundance based on the 16S OTU table. For any sample, we used total sum scaling to calculate the relative abundance and expressed the relative abundance as percentages. The richness and diversity statistics including the richness [the abundance-based coverage estimator (ACE)] and the Shannon diversity index were also calculated using mothur (Schloss et al., 2009). The modified pipeline is described on the mothur website. A *t*-test or two-way analysis of variance (ANOVA) with Duncan's multiple range test was performed for multiple comparisons to determine the significant differences in the total number and α-diversity index of bacteria in the tuberous roots and rhizosphere soil, and Excel 2019 software was used to visualize the differences. All statistical tests performed in this study were considered significant at $P < 0.05$ with SPSS version 20.0 software. Differences were considered significant at $P < 0.05$. The effects of cassava genotypes on the core and unique microbial OTUs in each tuber and the soil environment were analyzed according to the methods provided by Shade and Handelsman (2012) and Zhao et al. (2018a), respectively. The OTUs that consistently appeared in the three biological replicates of all plant genotypes were considered the core microbiome, while the OTUs that were present in all three biological replicates of only one plant genotype were considered unique microbiomes. The significant differences in the microbiome of a given cassava genotype among treatments were tested using one-way ANOVA and the least significant difference (LSD) test ($P < 0.05$). These results were visualized using Venn diagrams. Principal coordinates analyses (PCoAs) based on Bray-Curtis distance were used to evaluate the differences among the microbial communities of the different cassava genotypes in the tuberous roots and rhizosphere soil. Hierarchical clustering analysis (HCA) was performed based on the β-diversity distance matrix, and then the unweighted pair group method with arithmetic mean (UPGMA) algorithm was used to build a cluster tree. Linear discriminant analysis (LDA) and effect size (LEfSe) analyses were performed using the LEfSe tool (Segata et al., 2011). Differences in rhizosphere bacterial abundance were analyzed by LEfSe. The LEfSe analysis used the Kruskal-Wallis rank sum test to detect significantly different abundances and generated LDA scores to estimate the effect size (threshold: ≥ 2).

The relative content (percentage) of each metabolite produced by the three cassava genotypes and their biological roles were determined and then visualized with a stacked column chart to compare the compositions and structures of the tuber metabolites. Unsupervised principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA) based on the "MetaboAnalyst" package in R were used to compare the compositions and structures of tuber metabolites among the different cassava genotypes and to identify significant differences in tuber metabolites among genotypes. The correlations between metabolites and bacterial phyla were estimated using Mantel tests (type = Spearman) in the "vegan" package. In addition, Pearson correlation analyses were performed with the "corrplot" package and used to reveal correlations between the abundance of the microbial flora and the composition of tuber metabolites.

RESULTS

General Characteristics of 16S rDNA Based on Sequencing Data

In this study, we obtained 3,392,789 and 3,648,420 raw reads from the MiSeq sequencing analysis of the two sampling sites (each sampling site consisted of 14 cassava cultivars × 3 biological replicates), with an average of 80,781 and 86,867 reads per sample. After quality filtering, a total of 3,225,194 and 3,418,691 reads were obtained from the two sampling sites, with an average of 76,790 and 81,397 reads obtained in each sample (**Supplementary Table 1**). The reads were clustered into a total of 3,927 and 239,156 OTUs according to 97% sequence similarity. The taxonomic assignment of the OTUs resulted in the identification of 21 phyla and 310 genera in tuberous roots and 36 phyla and 906 genera in rhizosphere soil (**Supplementary Table 2**).

Microbial Taxonomic Analysis at the Phylum Level

The relative abundances of the 10 most abundant phyla (>1% of relative abundance in at least one sample) are shown in

[1] http://gmd.mpimp-golm.mpg.de/

Figure 1A and **Supplementary Table 2**. *Firmicutes* was the dominant phylum (>10% relative abundance) across all tuber samples, accounting for 46.9–89.3% of the total high-quality sequences. *Proteobacteria* and *Actinobacteria* were the next most abundant phyla (>1% relative abundance) in all tuber samples, accounting for 5.6–49.4% and 1.0–14.2% of the total high-quality sequences, respectively. Interestingly, the abundances of *Actinobacteria* in SC8 and *Acidobacteria* in 587 were extremely high compared with those in the other tuber samples. However, among the rhizosphere samples, *Actinobacteria*, *Proteobacteria* and *Acidobacteria* were the dominant phyla (> 10% relative abundance) across all rhizosphere samples, accounting for 17.4–29.5%, 15.2–28.5%, and 11.0–21.8% of the total high-quality sequences, respectively. *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* were the next most abundant phyla (> 1% relative abundance) in all rhizosphere samples, accounting for 4.9–26.2%, 4.5–14.4%, 2.7–6.5%, and 1.5–6.5% of the total high-quality sequences, respectively. Among the rhizosphere samples, the abundances of *Firmicutes* and *Acidobacteria* were higher in SC5, SC8, SC9, SC205, KU50, XL1, and FX01 than in the rhizospheres of the other genotypes (**Figure 1A**). The identities and relative abundances of bacterial phyla between tuberous roots and rhizosphere soil were obviously different. Based on the Venn diagram analysis, 21 phyla in the rhizosphere soil were found to be common to all tuberous samples, and 15 phyla were exclusive to the rhizosphere samples (**Figure 1B**).

Comparison of Bacterial Community Structures at the Genus Level

Based on the heatmap analysis of the relative abundances of the 50 most abundant classified genera, there were clearly significantly different bacterial community structures between the tuberous roots and rhizosphere soil of the fourteen cassava genotypes analyzed (**Figure 2**). The 50 most abundant genera belonged to 13 phyla (**Supplementary Table 3**).

Lactococcus and *Bacillus* were the dominant genera (>10% relative abundance) in all tuber samples, accounting for 20.0–41.3% and 18.5–32.7% of the total high-quality sequences, respectively. Interestingly, the abundance of *Pseudomonas* in 045 was much higher than those in the other tuber samples. *Oceanobacillus* and *Carnobacterium* were the next most abundant genera (> 1% relative abundance) in all tuber samples, accounting for 4.1–6.9% and 2.3–5.3% of the total high-quality sequences, respectively (**Figure 2A**). There were 11 core genera in the tuberous roots, accounting for only 3.5% of the total tuber bacterial community (**Figure 3A**). The core bacterial genera of the fourteen cassava genotypes included *Lactococcus*, *Bacillus*, *Oceanobacillus*, *Acinetobacter*, *Carnobacterium*, *Sphingomonas*, *Streptococcus*, *Exiguobacterium*, *Leuconostoc*, *Enterococcus*, and *Phenylobacterium*. Among them, 6 genera had significant differences in abundance among genotypes ($P < 0.05$), namely, *Lactococcus*, *Bacillus*, *Oceanobacillus*, *Acinetobacter*, *Streptococcus*, and *Exiguobacterium* (**Supplementary Table 4**).

The distributions of the genera differed greatly across the different rhizosphere samples. A total of eight genera (*Gp1*, *Gaiella*, *Bacillus*, *Lactococcus*, *Gp3*, *Ktedonobacter*, *Rhodoplanes*, and *Spirillospora*) were highly abundant (>1% relative abundance) in all rhizosphere samples (**Figure 2B**). The core bacterial genera in the rhizosphere soils remained similar among the different genotypes of cassava. There were 236 core bacterial genera, accounting for 26.0% of the total rhizosphere bacterial community (**Figure 3B**). The relative abundances of most of the core bacterial genera showed significant differences among genotypes ($P < 0.05$) (**Supplementary Table 4**).

Effects of Host Genetics Based on α-Diversity Analysis

To compare the α-diversity of samples with different sequence counts, we refined the data (i.e., we randomly picked an equal number of sequences across samples) using QIIME. The rarefaction curves showed the richness of the observed OTUs (**Supplementary Figure 1**) and indicated that the sequencing depth was sufficient to fully capture the diversity present. Microbial abundance and α-diversity were estimated using the population of bacteria, the bacterial richness (ACE) and the bacterial Shannon index, and had a statistical analysis performed with genotype and sampling site as explanatory variables. The highest richness was detected in the rhizosphere soil samples, which had significantly higher OTU and bacterial numbers and ACE and Shannon index values than the tuber samples. Among the tuber samples, SC8 had the highest number of OTUs ($n \geq 306$ OTUs), and 4612 had the lowest number of OTUs ($n = 49$ OTUs). Among the rhizosphere soil samples, SC16 had the highest number of OTUs ($n \geq 6{,}858$ OTUs), and SC205 had the lowest number of OTUs ($n \geq 4{,}992$ OTUs). Further, the differences in the total number and microbial alpha-diversity of bacteria were tested with the *t*-test or two-way analysis of variance (ANOVA) with Duncan's multiple range test and were considered different at $P < 0.05$. Our results showed that there were no significant differences in the number of bacterial OTUs among the fourteen genotypes ($P > 0.05$) (**Supplementary Table 5**). Similarly, among the tuber samples, no significant differences in microbial abundance or α-diversity were observed among the fourteen genotypes ($P > 0.05$). Overall, SC8 had the highest number of bacteria and α-diversity (**Figure 4** and **Supplementary Table 5**). However, the comparison of the microbial abundance and α-diversity metrics of the rhizosphere soils revealed disparities in the bacterial number, ACE, and Shannon indices among the fourteen genotypes ($P = 0.000032$, 0.005132, and $8.5974E^{-22}$, respectively). Furthermore, SC16, 587, 4612, and FX01 showed significantly higher bacterial diversity than the other genotypes (**Figure 4** and **Supplementary Table 5**).

Effects of Host Genetics Based on β-Diversity Analysis

A β-diversity analysis based on PCoA (**Figures 5A,B**) and HCA (**Figure 5C**) was performed to compare the bacterial compositions of the different samples. PCoA plots based on Bray-Curtis distances showed that bacterial communities in the tuberous roots were not clearly separated, and 40.47% (25.38 and 15.09%) of the overall variation could be explained. In contrast, the bacterial communities in the rhizosphere soil

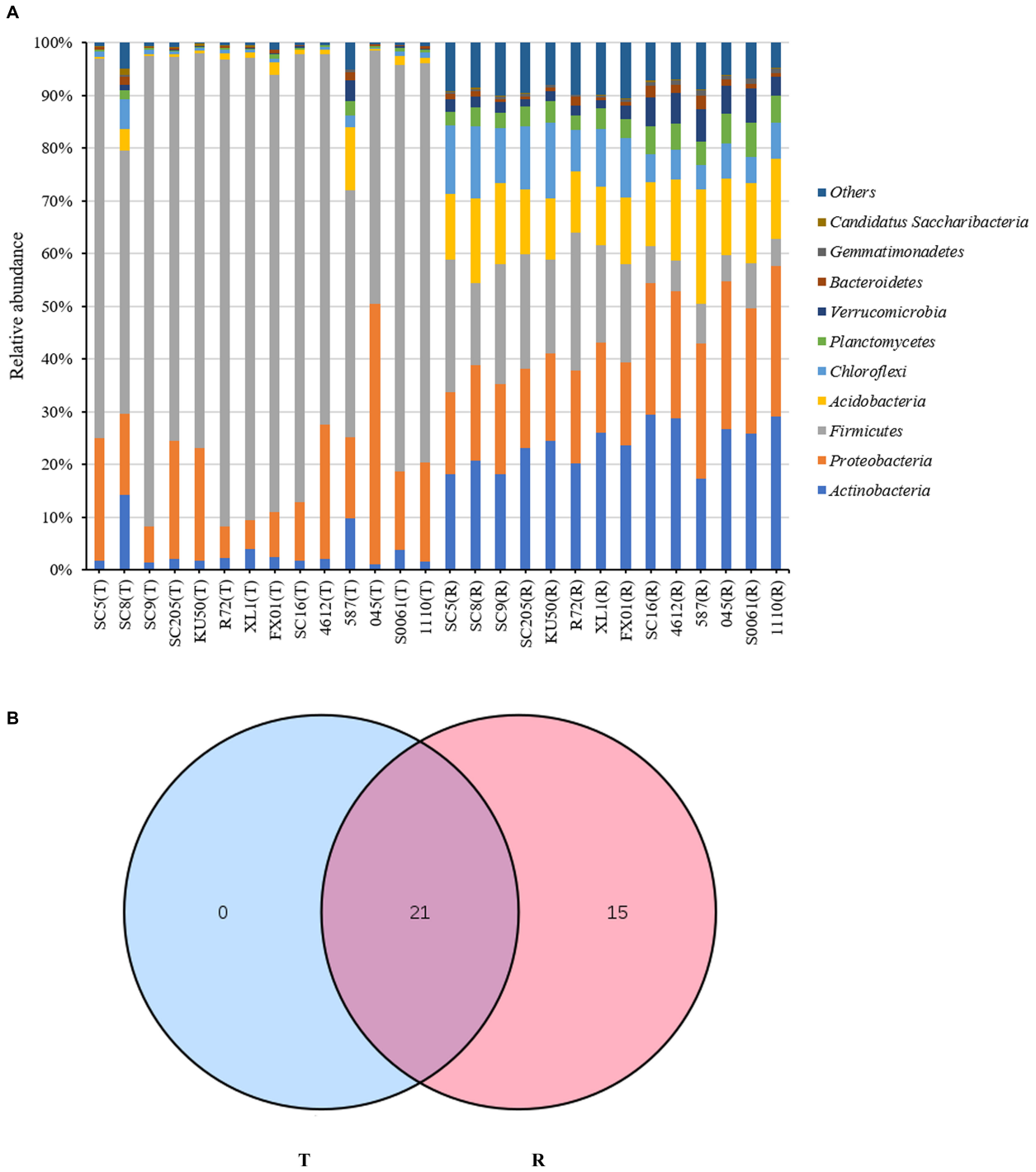


FIGURE 1 | (A) Histogram of the relative abundances at the phyla level of the TOP 10 bacterial communities. **(B)** Number of bacterial phyla in tuberous roots and rhizosphere soil of different cassava genotypes. T stands for tuberous roots; R stands for rhizosphere.

were clearly separated based on the cassava genotypes, which explained 52.68% (42.44 and 10.24%) of the overall variation (**Figures 5A,B**). Similar results were also obtained from the HCA tree. A cluster tree of all rhizosphere soil samples was constructed using HCA (**Figure 5C**). The bacterial communities in the rhizosphere soils of the different genotypes were clustered, and all branches were clustered based on the cassava genotypes. Thus, these results indicate that there is a correlation between the bacterial community in the rhizosphere of cassava and the genetic background of the cassava genotype.

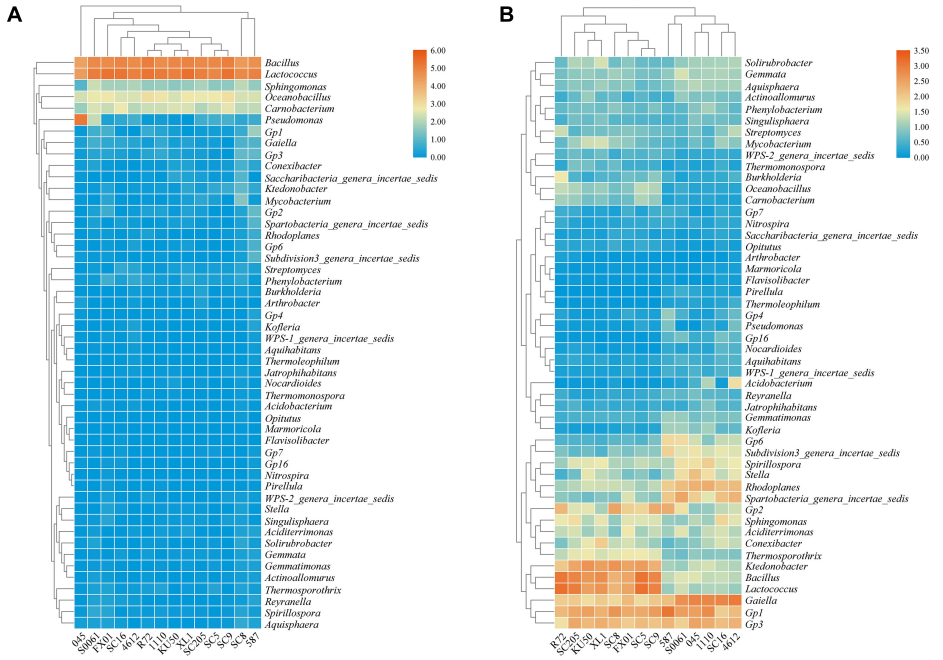


FIGURE 2 | Heatmap of the relative abundances at the genus level of the TOP 50 bacterial communities in **(A)** the tuberous roots and **(B)** the rhizosphere soil of different cassava genotypes.

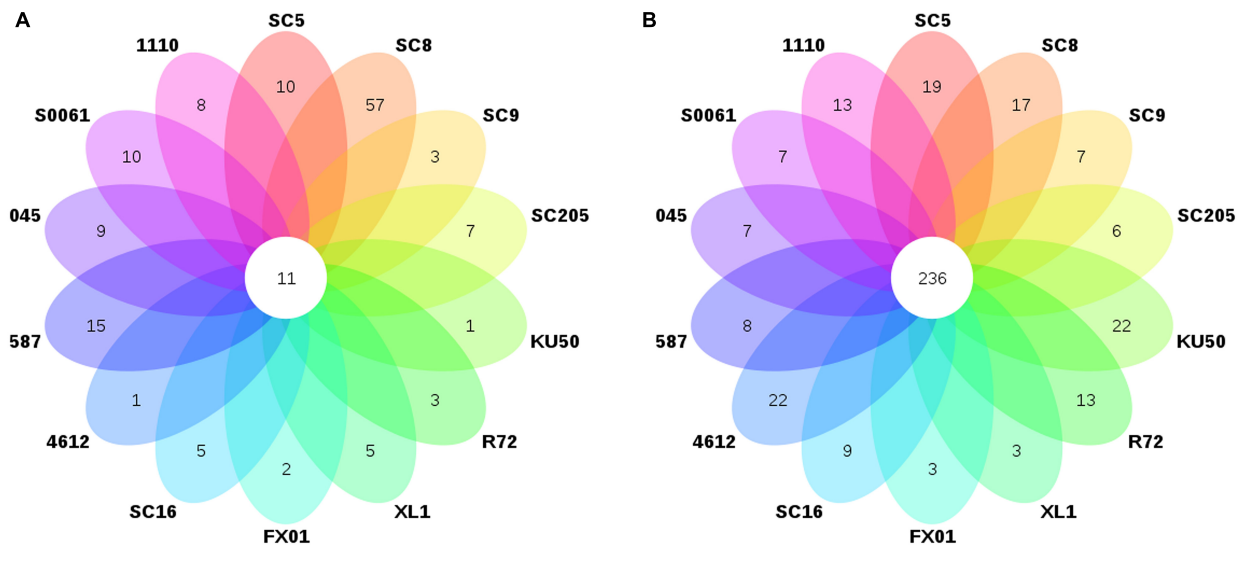


FIGURE 3 | Number of bacterial genera in **(A)** the tuberous roots and **(B)** the rhizosphere soil of different cassava genotypes.

Bacterial Groups With Significant Differences Among Genotypes

In addition to characterizing their α- and β-diversities, another primary purpose of comparing the microbial communities was to identify specialized bacterial groups in the rhizosphere soils of each genotype. LEfSe can be used to analyze bacterial community data at any taxonomic level and to provide biological class explanations to establish statistical significance, biological consistency, and effect-size estimation of predicted biomarkers (Segata et al., 2011). We performed a statistical analysis of rhizosphere soil bacterial communities of the different cassava genotypes at the genus level, and a total of 323 distinct bacterial groups were identified using the default logarithmic LDA value of 2 (**Supplementary Figure 2**). The SC5 microbiome was characterized by the presence of *Bacillus* [LAD(log10) > 4.0]; SC8 was characterized by the presence of *Ktedonobacter* and

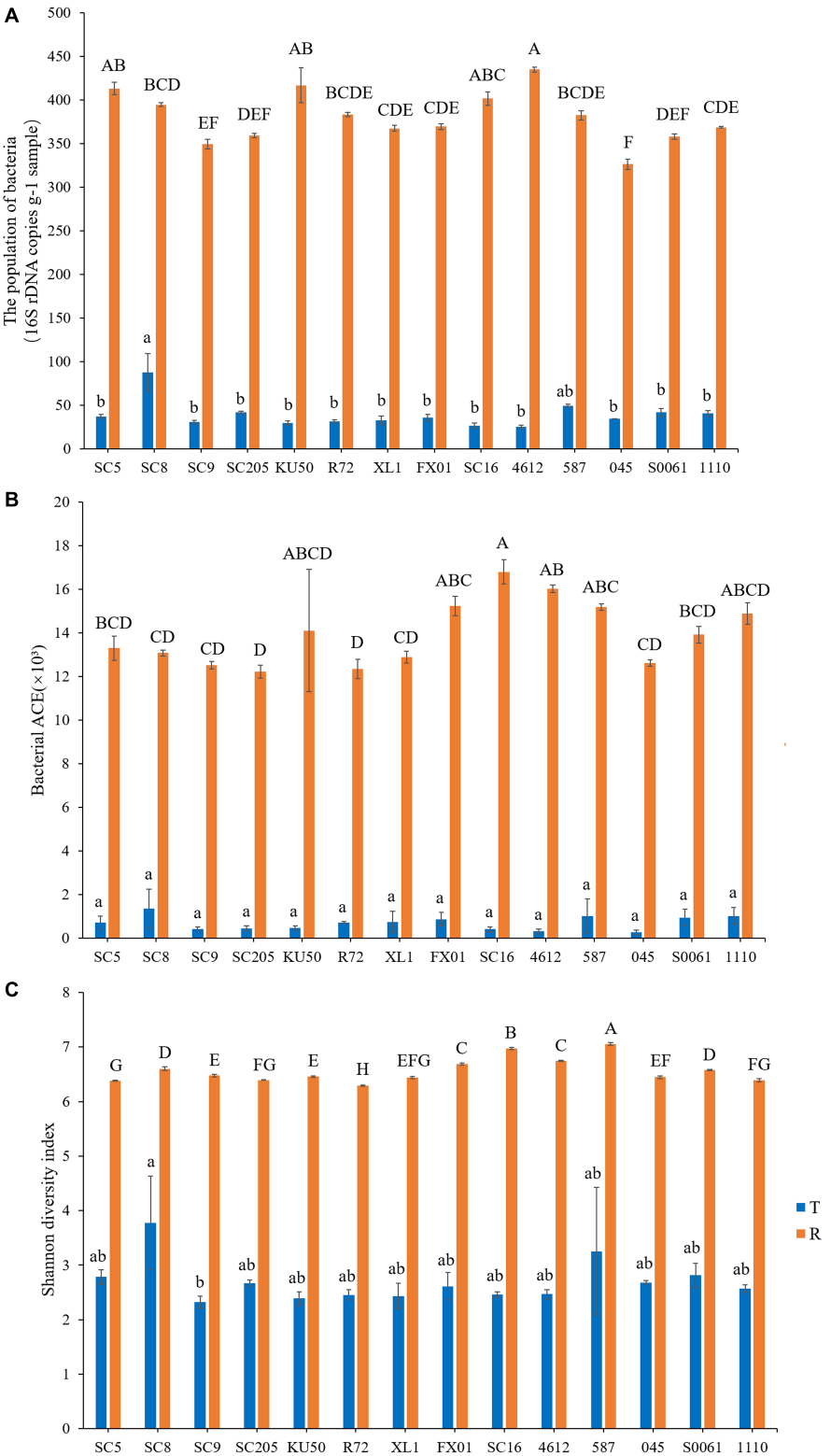


FIGURE 4 | Microbial population **(A)**, richness (abundance-based coverage estimator, ACE) **(B)**, and Shannon diversity **(C)** in the tuberous roots and rhizosphere soil of different cassava genotypes. Error bars represent standard deviations (SDs). Different lowercase letters and capital letters represent significant differences ($P < 0.05$) within tuberous roots and rhizosphere soil according to Duncan test, respectively. T stands for tuberous roots; R stands for rhizosphere.

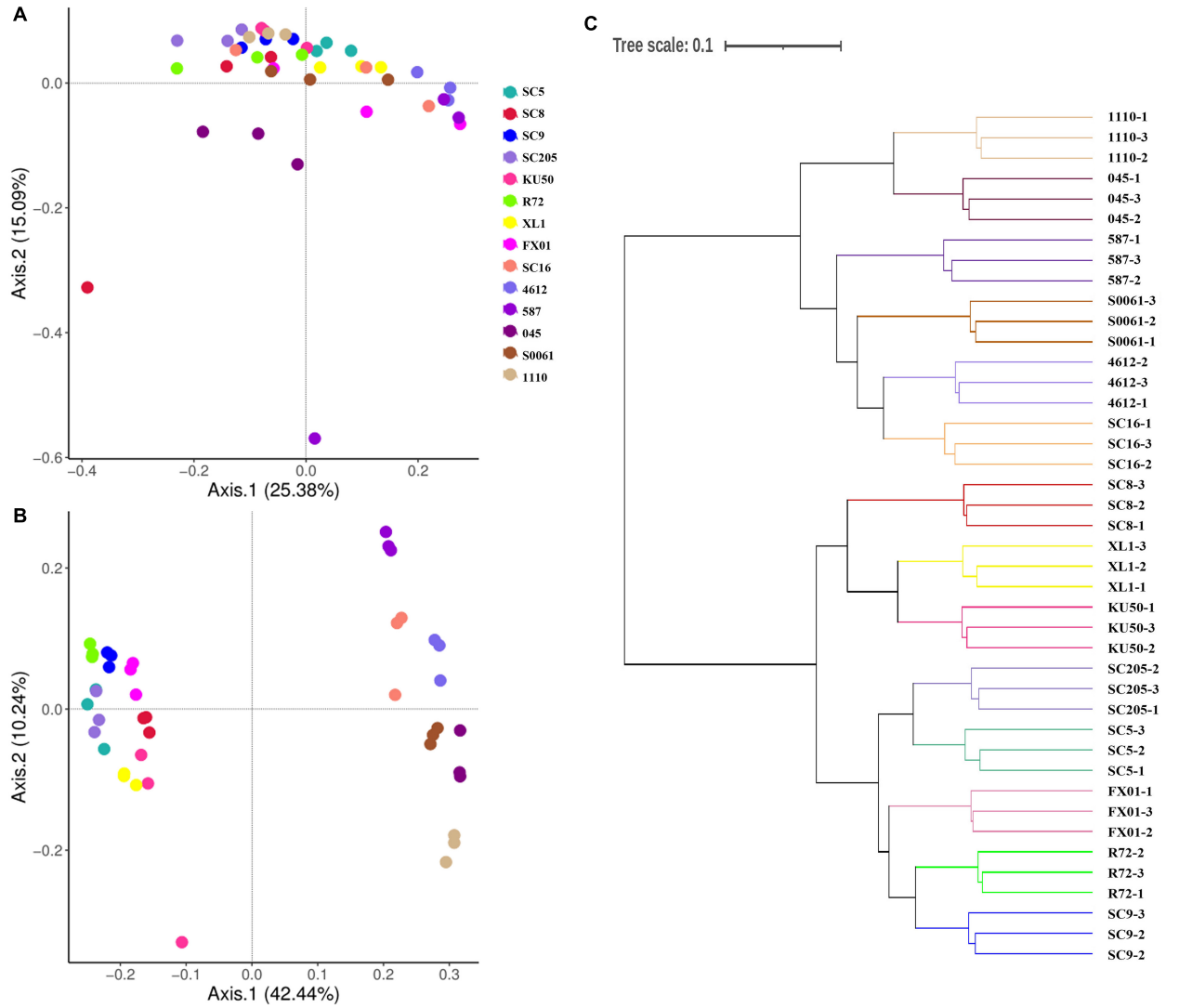


FIGURE 5 | Principal coordinates analyses (PCoA) were performed based on **(A)** tuber and **(B)** rhizosphere soil bacterial OTU distributions using Bray-Curtis distance. **(C)** Hierarchical cluster analysis (HCA) was used to form a cluster tree of rhizosphere soil bacterial communities of different cassava genotypes.

Gp2 [LAD(log10) > 4.0]; R72 was characterized by the presence of *Lactococcus* and *Burkholderia* [LAD(log10) > 4.0]; FX01 was characterized by the presence of *Thermosporothrix* and *Aciditerrimonas* [LAD(log10) > 4.0]; SC16 was characterized by the presence of *Sphingomonas* [LAD(log10) > 4.0]; 4612 was characterized by the presence of *Gaiella* and *Acidobacterium* [LAD(log10) > 4.0]; 587 was characterized by the presence of *Gp1*, *Subdivision3_genera_incertae_sedis* and *Gp6* [LAD(log10) > 4.0]; 045 was characterized by the presence of *Stella* and *Gp3* [LAD(log10) > 4.0]; S0061 was characterized by the presence of *Spartobacteria_genera_incertae_sedis* [LAD(log10) > 4.0]; and 1,110 was characterized by the presence of *Rhodoplanes* and *Spirillospora* [LAD(log10) > 4.0]. Interestingly, no bacterial genera from the rhizosphere of SC9, SC205, or KU50 had an LAD (log10) greater than 4.0.

Influence of Tuber Metabolites on Microbial Communities

The α- and β-diversities of the tuber microbial communities were not significantly different among cassava genotypes, while the bacterial communities in the rhizosphere soils of KU50, SC205, and SC9 clustered into different groups. No bacterial genera had an LAD (log10) of greater than 4.0 for these three cassava genotypes, so they were selected for metabolome analysis.

A total of 78 compounds were detected in the tuber metabolites recovered from the tuberous roots of the three different cassava genotypes. The types of chemicals were the same across the three cassava genotypes. The identified compounds were categorized into nucleic acids, lipids, vitamins, cofactors, organic acids, peptides, and carbohydrates (**Supplementary Figure 3A**). Among them, the content of carbohydrates in the

tuberous roots of SC9 was higher than that in the tuberous roots of the other genotypes; the content of peptides in the tuberous roots of KU50 was higher than that in the tuberous roots of the other genotypes; and the content of organic acids in the tuberous roots of SC205 was higher than that in the tuberous roots of the other genotypes. However, distinct differences in the abundances of certain compounds were detected (**Supplementary Figure 3B**), and the 19 most abundant metabolites in each of the three cassava genotypes were significantly different ($P < 0.05$) (**Supplementary Table 6**). PCA ordination showed that the tuber metabolite distributions of the three cassava genotypes were significantly separated from each other, indicating that the metabolite compositions and structures of the three cassava genotypes were quite different (**Supplementary Figure 3C**); the first two principal components of the PCA explained 73% (49.8 and 23.2%) of the total variation in the metabolites. To identify the metabolites that were notably different among the three cassava genotypes, OPLS-DA was performed on the metabolites of the three cassava genotypes. The analysis revealed that the metabolites with substantial differences among the three genotypes of cassava included sugars (5), sugar acids (4), sugar alcohols (2), organic acids (12), amino acids (3), amides/amines (4), and others (3) (**Supplementary Figure 3D**).

A correlation analysis between bacterial phyla and metabolites indicated that higher relative abundances of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were positively correlated with organic acids and lipids produced by the tuberous roots of the three cassava genotypes and negatively correlated with vitamins and cofactors (**Figure 6A**). Among the rhizosphere soils of the three cassava genotypes, higher relative abundances of most bacterial phyla were positively correlated with peptides, vitamins and cofactors and negatively correlated with carbohydrates, organic acids, lipids, and nucleic acids (**Figure 6B**). Further examination of the core genera, as defined by Bowen et al. (2017), indicated that tuber metabolites from the rhizosphere soil increased the relative abundances of 10 core bacterial genera, including *Lactococcus* and *Bacillus,* compared with those in tuberous roots. Interestingly, the identities and relative abundances of the core microbiome genera in the rhizosphere soil were both significantly different from those in the tuberous roots (**Supplementary Table 4**).

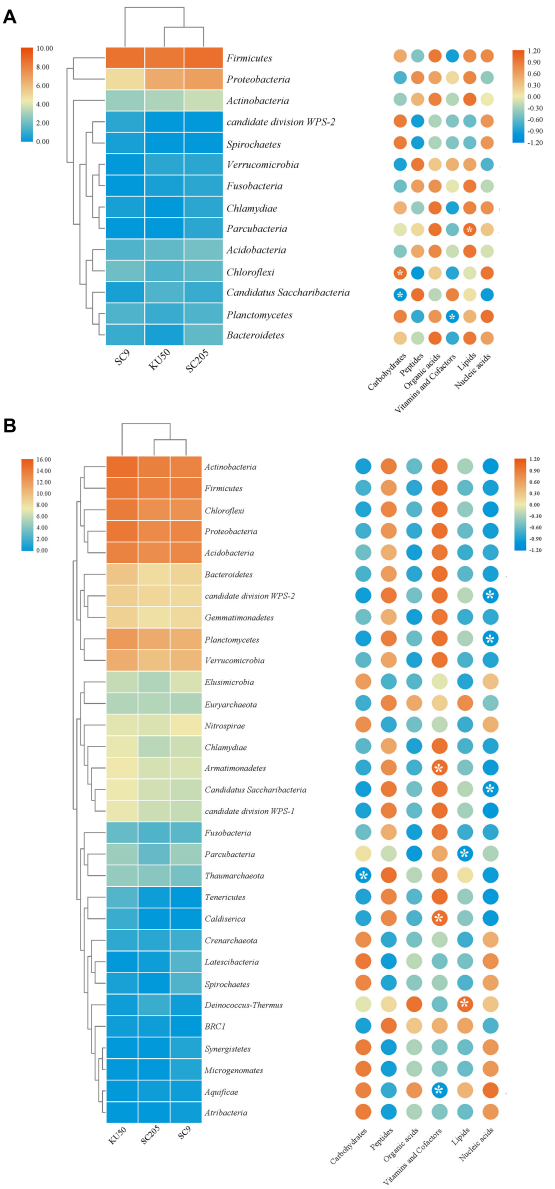


FIGURE 6 | Relative abundance of bacterial phyla detected in **(A)** tuber and **(B)** rhizosphere soil of three cassava genotypes, and correlation analysis of bacterial community compositions and metabolite components. *Indicates significant differences ($P < 0.05$).

DISCUSSION

Plants and microbes interact in order to obtain nutrients to improve their growth and stress resistance, especially through root-microbe interactions (Edwards et al., 2015; Wu et al., 2020). Microbial communities have been shown to be impacted by the microhabitat (Jin et al., 2017), soil type (Bonito et al., 2014), and host genotype (Ofek-Lalzar et al., 2014; Zhang et al., 2019). At present, the microbial diversity of soils, roots, leaves, and aerial organs has been extensively investigated by high-throughput sequencing (Buée et al., 2009; Abdelfattah et al., 2016a,b, 2017; Liu et al., 2018; Yuan et al., 2018). To extend our knowledge of bacterial diversity as it relates to cassava genotypes, we used culture-independent high-throughput sequencing technology to investigate the diversity and community structure of bacteria present in the tuber endospheres and rhizospheres of fourteen cassava genotypes (SC5, SC8, SC9, SC205, KU50, R72, XL1, FX01, SC16, 4612, 587, 045, S0061, and 1110). Our results showed that the diversity of bacterial communities in the tuber endosphere and rhizosphere depends on the plant genotype and the tuber metabolites.

Previous reports have suggested that microbial density is generally higher in the rhizosphere than in the root and

that bacterial diversity and richness gradually decrease from soils to epiphytes to endophytes (Bulgarelli et al., 2012, 2015; Lundberg et al., 2012; Edwards et al., 2015; Hacquard et al., 2015). In this study, the diversity and richness of bacteria in the rhizospheres of all cassava genotypes were higher than those in the tuber endospheres. Moreover, the cassava genotype did not significantly influence the endophytic bacterial community structure (**Figure 4**). Similar results have also been reported in previous studies: there were no significant differences in richness or diversity among the endophytes of different cassava genotypes (Li et al., 2020). Studies on *A. thaliana* have consistently suggested that root endosphere bacterial communities are strongly influenced by soil type and soil properties but that host genotype has a limited effect on the root microbiome (Bulgarelli et al., 2012; Lundberg et al., 2012; Thiergart et al., 2020). However, we detected a significant effect of cassava genotypes on the rhizosphere bacterial community structure (**Figure 5**), which is consistent with other previous findings (Miethling et al., 2000; Marschner et al., 2004; Garbeva et al., 2008; Bonito et al., 2014; Burns et al., 2015; Leff et al., 2018). For example, a significant effect of plant genotype on rhizosphere microbial communities was observed by comparing the rhizospheres of different experimental crops grown in soils of the same type (Liu et al., 2020). Some previous studies of the tree phyllosphere and maize rhizosphere separately showed that host genetics played an important role in shaping the bacterial microbiome (Laforest-Lapointe et al., 2016; Walters et al., 2018). Our results provide comprehensive empirical evidence for the selection of the microbial community by cassava and a theoretical framework for the coevolution between cassava and microbes; in this framework, cassava plants use exudates to recruit, filter, and enrich certain microbial taxa that have specific functions (Müller et al., 2016; Martin et al., 2017; Sasse et al., 2018), and competition among microbes for these resources drives their rapid evolutionary radiation and consequent divergence to reduce competition (Foster et al., 2017).

Generally, genetic based-interactions among genotypes are complex and have been recently gaining attention (Rasche et al., 2006; Xu et al., 2009; Aira et al., 2010; Ýnceoğlu et al., 2012; Cheng et al., 2020), and even minor genotype differences as between genetically modified and parental lines are believed to affect the microbial colonization of plant, particularly in vegetatively propagated crop. The seed stem-associated bacterial communities, independently of the genotypes and the soil type, is also a possible factor determining the specificity of the bacterial community in the tuber root system compartments. Nevertheless, the influence of genotype in our study is very evident.

In all cassava tuber samples, the dominant bacterial phyla were *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (>1% of high-quality sequences) (**Figure 1A**). It has been previously reported that *Proteobacteria*, *Firmicutes*, and *Actinobacteria* are the dominant bacterial phyla in cassava (Li et al., 2020). Similar results based on both culture-dependent and culture-independent approaches have previously been reported for endophytes of other plants (Khan Chowdhury et al., 2017; Yang et al., 2017). In ginseng, *Proteobacteria* was found to have the highest abundance, followed by *Firmicutes* and *Actinobacteria* (Khan Chowdhury et al., 2017). Similarly, in peony, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* have been reported to be the dominant bacterial phyla (Yang et al., 2017). In all cassava rhizosphere samples, the dominant bacterial phyla were *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* (>1% of high-quality sequences) (**Figure 1A**); these findings are in accordance with Sarr et al. (2017), who reported that the same soil bacterial communities were associated with cassava cultivation in Cameroon. *Actinobacteria* have also been shown to be enriched in exophytes of other plants, such as *Pinus pinaster* and maize-wheat (*Triticum aestivum*)/barley (*Hordeum vulgare*) rotation systems (Pérez-Izquierdo et al., 2019; Xiong et al., 2021); these bacteria are used as biocontrol agents to control soil- and seed-borne plant diseases (Priyadharsini and Dhanasekaran, 2015). These results suggest that rhizosphere microorganisms could play an important role in cassava cropping in tropical regions that experience various recurrent plant diseases.

The β-diversity analyses showed that bacterial communities in the rhizosphere soil varied across the different plant genotypes. PCoA indicated that the bacterial communities in the tuberous roots were not clearly separated by cassava genotype but that those in the rhizosphere soil were clearly separated by cassava genotype. These results were also supported by heatmap analyses at the genus level. Moreover, HCA demonstrated that the rhizosphere bacterial communities of the fourteen cassava genotypes were clustered based on the cassava genotypes (**Figure 5C**); these findings support the view that the cassava genotype influences the bacterial rhizosphere community. Similarly, Schlemper et al. (2017) reported that the community structures of the rhizosphere microbiome were significantly different among seven different sorghum cultivars. However, further studies are needed to confirm this hypothesis and to confirm the effects of genetic diversity on the compositions of root-associated bacterial communities.

We noted a few genera that were consistently enriched in the cassava tuberous roots, including *Lactococcus*, *Bacillus*, *Oceanobacillus*, and *Carnobacterium* (**Figure 2A**). *Lactococcus* and *Bacillus* improve plant resistance to diseases such as bacterial blight and root rot in cassava and wilt disease in cucumber (Xu et al., 2014; Freitas et al., 2019; Zhang et al., 2021). In addition, *Pseudomonas* was also a dominant genus in the tuber endosphere of 045. *Pseudomonas* species have been used to alleviate heavy metal toxicity and the negative effects of saline sodic field growth on wheat (Hassan et al., 2017). Eight genera (*Gp1*, *Gaiella*, *Bacillus*, *Lactococcus*, *Gp3*, *Ktedonobacter*, *Rhodoplanes*, and *Spirillospora*) were highly abundant in all rhizosphere samples (**Figure 2B**). This result is in accordance with Bao et al. (2019), who reported that the dominant bacteria in a paddy soil included *Bacillus*, *Acidobacteria/Gp1*, *Acidobacteria/Gp3*, and *Ktedonobacter*. Wu et al. (2021) found that the enrichment of beneficial bacteria, mainly *Gaiella*,

contributed to the ability of ramie to tolerate poor soils. Our results showed that the cultivation of different cassava genotypes recruited different unique and core microbes to the cassava tubers and rhizospheres and that the microbes were significantly different in identity and relative abundance (**Figure 3**). This result is mainly attributed to the process by which cassava plants recruit different microbes, i.e., the release of a wide variety of exudates from tubers (Garbeva et al., 2004; Raaijmakers et al., 2009). LEfSe analysis identified specialized bacterial groups exclusively in the rhizosphere soil (**Supplementary Figure 2**), which suggests that these groups may play critical roles in maintaining the structure and function of rhizospheric soil bacterial communities. For example, *Burkholderia*, found in the R72 rhizosphere, was more abundant in a pineapple-banana crop rotation soil than in a banana monoculture soil, has the capacity to suppress Fusarium wilt of banana (Wang et al., 2015). Other reports who have demonstrated that bacteria from the *Burkholderia* genera possess a potential biocontrol ability through the production of varying compounds that inhibit plant pathogens (Mendes et al., 2011; Raaijmakers and Mazzola, 2012; Tenorio-Salgado et al., 2013). *Spartobacteria_genera*, found in the S0061 rhizosphere, was stimulated potentially to suppress the Fusarium wilt disease by sustainable biofertilizer application (Shen et al., 2015). Furthermore, we also found that most core microbes in the tubers and rhizosphere had plant growth-promoting potential. These core genera are known to produce various antibiotics, including bacillibactin and lipopeptides (produced by *Bacillus*) (Li et al., 2014; Liu et al., 2017), 2, 4-diac-etylphlor-oglucinol and phenazines (produced by *Pseudomonas*) (Mazurier et al., 2009; Hu et al., 2017), fusaricidin (produced by *Paenibacillus*) (Finch et al., 2018; Li and Chen, 2019), and thiopeptide and ectoine (produced by *Streptomyces*) (Cha et al., 2016). Overall, identifying these core and unique microbiomes is important for understanding the responsive microbial components associated with different plant genotypes.

In this study, the bacterial communities in the rhizosphere soils conditioned by the tuber metabolomes collected from three typical cassava genotypes, KU50, SC205, and SC9, were significantly different from each other taxonomically; in contrast, the bacterial communities in the tuberous roots were not significantly different. The correlation analysis between bacterial phyla and the produced exudates revealed that these differences could be linked to the exudation of certain tuber metabolites (**Figure 6**). These results thus support the notion that specific compounds within changing plant exudate profiles may drive soil microbial dynamics (Badri et al., 2013). Although the potential to mobilize soil nutrients is clearly already present in the soil microbiome, tuber exudates increase the functional potential of soil microbial communities. In addition, exudate concentrations can play a major role in shaping the abundances of microbial functional genes, which may be beneficial to plants (Badri et al., 2013). Moreover, the contents of measured metabolites in the three genotypes of cassava and their biological roles were significantly different; the metabolites included sugars, sugar acids, sugar alcohols, organic acids, amino acids, amides/amines, and others (**Supplementary Figure 3**). These differences could reflect a level of microbial community functional redundancy that was stimulated by the metabolomes. In a study of the rhizosphere microbiome of *A. thaliana*, Chaparro et al. (2013) suggested that plants exude sugars that are used by a wide variety of microorganisms as well as more specific exudates, such as phenolic compounds, that may be intended to attract more specific microbes. Amino acids, as specific chemoattractants for microorganisms, promote the chemotaxis of soil microbes to the rhizosphere (Barbour et al., 1991). Organic acids play a crucial role in nutrient acquisition (P, Fe, and Mn) by plants growing in nutrient-poor soils (Dakora and Phillips, 2002). Our result is in accordance with the finding that specific exudates of different *Sorghum bicolor* genotypes may influence the rhizosphere microbial community composition (Funnell-Harris et al., 2008). The available mineral nutrients in soils are not sufficient to meet the requirements of plants for optimal growth; thus, plants have evolved systems to recruit symbiotic microbial partners that increase the availability of nutrients (Tinker, 1984; Landeweert et al., 2001; Gyaneshwar et al., 2002; Adesemoye and Kloepper, 2009). Many studies of root exudate-mediated microbial defenses have developed robust models of coevolution between plants and soil microbes; for example, in the "cry for help" and "legacy effects" models (Weller et al., 2002; Yuan et al., 2018), plants select for microbial communities that help to suppress plant pathogens. The exudates released by different genotypes of the same plant species can vary, which affects the microbial community composition of the rhizosphere (Micallef et al., 2009; Inceoğlu et al., 2010).

CONCLUSION

The different cassava genotypes did not affect the richness or diversity of the endophytic bacterial community, but they affected the richness and diversity of the exophytic bacterial community. Furthermore, the cassava genotype shaped the endophytic and exophytic community structures and affected the relative abundances of core bacterial genera. The bacterial community structures varied between the tuber endosphere and the rhizosphere across cassava genotypes. The more dominant bacterial phyla associated with tubers and the rhizosphere were *Firmicutes* and *Actinobacteria*, respectively. Moreover, the majority of genera were associated with the tuberous roots and rhizosphere soils of specific cassava genotypes; this may have been due to the exudation of certain metabolites from the cassava tubers. These results suggest that plant genotypes affect the community composition of endophytic bacteria and may affect the community composition of exophytic bacteria through the exudation of metabolites.

DATA AVAILABILITY STATEMENT

The raw sequences were deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA750582.

AUTHOR CONTRIBUTIONS

YC and KL conceived and designed the experiments. JH, YG, RZ, YZ, XN, and XC performed the experiments and data analyses. JH drafted the manuscript. JH, YG, YC, and KL revised the manuscript. All authors have read and approved the submitted version.

FUNDING

This research was funded by grants from the Key R&D Program of Hainan Province (ZDYF2019063), the National Key Research and Development Program of China (2018YFD1000500), and the China Agriculture Research System (CARS-11-HNCYH).

ACKNOWLEDGMENTS

We are grateful to the Dr. Hong Li from Hainan University for technical editing of the manuscript and special thanks to Dr. Xiaofei Zhang from Alliance Bioversity-CIAT for suggestion to manuscript revision. We also appreciate the reviewers and editors for the patience to the work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.729022/full#supplementary-material

REFERENCES

Abdelfattah, A., Cacciola, S. O., Mosca, S., Zappia, R., and Schena, L. (2017). Analysis of the fungal diversity in citrus leaves with greasy spot disease symptoms. *Microb. Ecol.* 73, 739–749. doi: 10.1007/s00248-016-0874-x

Abdelfattah, A., Wisniewski, M., Droby, S., and Schena, L. (2016a). Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. *Hortic. Resengland.* 3:16047. doi: 10.1038/hortres.2016.47

Abdelfattah, A., Wisniewski, M., Li Destri Nicosia, M. G., Cacciola, S. O., and Schena, L. (2016b). Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. *PLoS One* 11:e0160470. doi: 10.1371/journal.pone.0160470

Adesemoye, A. O., and Kloepper, J. W. (2009). Plant-microbes interactions in enhanced fertilizer-use efficiency. *Appl. Microbiol. Biot.* 85, 1–12. doi: 10.1007/s00253-009-2196-0

Aira, M., Gómez-Brandón, M., Lazcano, C., Bååth, E., and Domínguez, J. (2010). Plant genotype strongly modifies the structure and growth of maize rhizosphere microbial communities. *Soil Biol. Biochem.* 42, 2276–2281. doi: 10.1016/j.soilbio.2010.08.029

Álvarez-Pérez, J. M., González-García, S., Cobos, R., Olego, M. Á, Ibañez, A., Díez-Galán, A., et al. (2017). Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. *Appl. Environ. Microb.* 83, e1564–e1517. doi: 10.1128/AEM.01564-17

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *Biol. Chem.* 288, 4502–4512. doi: 10.1074/jbc.M112.433300

Bao, Q. L., Wang, F. H., Bao, W. K., and Huang, Y. Z. (2019). Effects of rice straw addition on methanogenic archaea and bacteria in two paddy soils. *Environ. Sci.* 40, 4202–4212. doi: 10.13227/j.hjkx.201901195

Barbour, W. M., Hattermann, D. R., and Stacey, G. (1991). Chemotaxis of Bradyrhizobium japonicum to soybean exudates. *Appl. Environ. Microb.* 57, 2635–2639. doi: 10.1128/aem.57.9.2635-2639.1991

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Bolan, N. S. (1991). A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant Soil* 134, 189–207. doi: 10.1007/BF0001 2037

Bonito, G., Reynolds, H., Robeson, M. S. II, Nelson, J., Hodkinson, B. P., Tuskan, G., et al. (2014). Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. *Mol. Ecol.* 23, 3356–3370. doi: 10.1111/mec.12821

Bowen, J. L., Kearns, P. J., Byrnes, J., Wigginton, S., Allen, W. J., Greenwood, M., et al. (2017). Lineage overwhelms environmental conditions in determining rhizosphere bacterial community structure in a cosmopolitan invasive plant. *Nat. Commun.* 8:433. doi: 10.1038/s41467-017-00626-0

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R. H., Uroz, S., et al. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytol.* 184, 449–456. doi: 10.1111/j.1469-8137.2009.03003.x

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Dröge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren, van Themaat, E., Ahmadinejad, N., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Burns, J. H., Anacker, B. L., Strauss, S. Y., and Burke, D. J. (2015). Soil microbial community variation correlates most strongly with plant species identity, followed by soil chemistry, spatial location and plant genus. *Aob Plants* 7:lv030. doi: 10.1093/aobpla/plv030

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cha, J. Y., Han, S., Hong, H. J., Cho, H., Kim, D., Kwon, Y., et al. (2016). Microbial and biochemical basis of a Fusarium wilt-suppressive soil. *Isme J.* 10, 119–129. doi: 10.1038/ismej.2015.95

Chaparro, J. M., Badri, D. V., Bakker, M. G., Sugiyama, A., Manter, D. K., and Vivanco, J. M. (2013). Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PLoS One* 8:e55731. doi: 10.1371/journal.pone.0055731

Cheng, Z., Lei, S., Li, Y., Huang, W., Ma, R., Xiong, J., et al. (2020). Revealing the Variation and Stability of Bacterial Communities in Tomato Rhizosphere Microbiota. *Microorganisms* 8:170. doi: 10.3390/microorganisms8020170

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., et al. (2007). The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res.* 35, D169–D172. doi: 10.1093/nar/gkl889

Dakora, F. D., and Phillips, D. A. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Spr. Net.* 245, 201–213. doi: 10.1007/978-94-017-1570-6_23

Dethlefsen, L., and Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *P. Natl. Acad. Sci. USA* 1(Suppl. 1), 4554–4561. doi: 10.1073/pnas.1000087107

Ding, T., Palmer, M. W., and Melcher, U. (2013). Community terminal restriction fragment length polymorphisms reveal insights into the diversity and dynamics of leaf endophytic bacteria. *BMC Microbiol.* 13:1. doi: 10.1186/1471-2180-13-1

Dong, M., Yang, Z., Cheng, G., Peng, L., Xu, Q., and Xu, J. (2018). Diversity of the Bacterial Microbiome in the Roots of Four Saccharum Species: *S. spontaneum*, *S. robustum*, *S. barberi*, and *S. officinarum*. *Front. Microbiol.* 9:267. doi: 10.3389/fmicb.2018.00267

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973.e–983.e. doi: 10.1016/j.cell.2018.10.020

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinform.* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *P. Natl. Acad. Sci. USA* 112, E911–E920. doi: 10.1073/pnas.1414592112

Finch, E. A., Caruso, T., and Engl, C. (2018). Effects of *Paenibacillus polymyxa* inoculation on below-ground nematode communities and plant growth. *Soil. Biol. Biochem.* 121, 1–7. doi: 10.1016/j.soilbio.2018.01.026

Foster, K. R., Schluter, J., Coyte, K. Z., and Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature* 548, 43–51. doi: 10.1038/nature23292

Freitas, M. A., Medeiros, F. H., Melo, I. S., Pereira, P. F., Peñaflor, M. F. G., Bento, J. M., et al. (2019). Stem inoculation with bacterial strains *Bacillus amyloliquefaciens* (GB03) and *Microbacterium imperiale* (MAIIF2a) mitigates *Fusarium* root rot in cassava. *Phytoparasitica* 47, 135–142. doi: 10.1007/s12600-018-0706-2

Funnell-Harris, D. L., Pedersen, J. F., and Marx, D. B. (2008). Effect of sorghum seedlings, and previous crop, on soil fluorescent *Pseudomonas* spp. *Plant Soil* 311, 173–187. doi: 10.1007/s11104-008-9669-2

Garbeva, P., Van Elsas, J. D., and Van Veen, J. A. (2008). Rhizosphere microbial community and its response to plant species and soil history. *Plant Soil* 302, 19–32. doi: 10.1007/s11104-007-9432-0

Garbeva, P., van Veen, J. A., and van Elsas, J. D. (2004). Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. *Annu. Rev. Phytopathol.* 42, 243–270. doi: 10.1146/annurev.phyto.42.012604.135455

Geddes, B. A., Ryu, M. H., Mus, F., Garcia Costas, A., Peters, J. W., Voigt, C. A., et al. (2015). Use of plant colonizing bacteria as chassis for transfer of N2-fixation to cereals. *Curr. Opin. Biotech.* 32, 216–222. doi: 10.1016/j.copbio.2015.01.004

Gorka, S., Dietrich, M., Mayerhofer, W., Gabriel, R., Wiesenbauer, J., Martin, V., et al. (2019). Rapid transfer of plant photosynthates to soil bacteria via ectomycorrhizal hyphae and its interaction with nitrogen availability. *Front. Microbiol.* 10:168. doi: 10.3389/fmicb.2019.00168

Grayston, S. J., Wang, S., Campbell, C. D., and Edwards, A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* 30, 369–378. doi: 10.1016/S0038-0717(97)00124-7

Guo, L., Zheng, S., Cao, C., and Li, C. (2016). Tillage practices and straw-returning methods affect topsoil bacterial community and organic C under a rice-wheat cropping system in central China. *Sci. Rep-UK* 6:33155. doi: 10.1038/srep33155

Gyaneshwar, P., Kumar, G. N., Parekh, L., and Poole, P. (2002). Role of soil microorganisms in improving P nutrition of plants. *Plant Soil* 245, 83–93. doi: 10.1007/978-94-017-1570-6_15

Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., et al. (2015). Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* 17, 603–616. doi: 10.1016/j.chom.2015.04.009

Hassan, T. U., Bano, A., and Naz, I. (2017). Alleviation of heavy metals toxicity by the application of plant growth promoting rhizobacteria and effects on wheat grown in saline sodic field. *Int. J. Phytoremediat.* 19, 522–529. doi: 10.1080/15226514.2016.1267696

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58. doi: 10.1186/s40168-018-0445-0

Hoffland, E., van den Boogaard, R. I. K. I., Nelemans, J. A. A. P., and Findenegg, G. (1992). Biosynthesis and root exudation of citric and malic acids in phosphate-starved rape plants. *New Phytol.* 122, 675–680. doi: 10.1111/j.1469-8137.1992.tb00096.x

Holden, M. T., Ram Chhabra, S., de Nys, R., Stead, P., Bainton, N. J., Hill, P. J., et al. (1999). Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* 33, 1254–1266. doi: 10.1046/j.1365-2958.1999.01577.x

Hridya, A. C., Byju, G., and Misra, R. S. (2013). Effect of biocontrol agents and biofertilizers on root rot, yield, harvest index and nutrient uptake of cassava (*Manihot esculanta* Crantz). *Arch. Agron. Soil Sci.* 59, 1215–1227. doi: 10.1080/03650340.2012.702896

Hu, J., Wei, Z., Weidner, S., Friman, V. P., Xu, Y. C., Shen, Q. R., et al. (2017). Probiotic *Pseudomonas* communities enhance plant growth and nutrient assimilation via diversity-mediated ecosystem functioning. *Soil Biol. Biochem.* 113, 122–129. doi: 10.1016/j.soilbio.2017.05.029

Hugoni, M., Luis, P., Guyonnet, J., and Haichar, F. (2018). Plant host habitat and root exudates shape fungal diversity. *Mycorrhiza* 28, 451–463. doi: 10.1007/s00572-018-0857-5

Inceoğlu, O., Salles, J. F., van Overbeek, L., and van Elsas, J. D. (2010). Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields. *Appl. Environ. Microb.* 76, 3675–3684. doi: 10.1128/AEM.00040-10

Ýnceoğlu, Ö, Falcão Salles, J., and van Elsas, J. D. (2012). Soil and cultivar type shape the bacterial community in the potato rhizosphere. *Microb. Ecol.* 63, 460–470. doi: 10.1007/s00248-011-9930-8

Jin, T., Wang, Y., Huang, Y., Xu, J., Zhang, P., Wang, N., et al. (2017). Taxonomic structure and functional association of foxtail millet root microbiome. *Gigascience* 6, 1–12. doi: 10.1093/gigascience/gix089

Khan Chowdhury, M., Jeon, J., Ok Rim, S., Park, Y. H., Kyu Lee, S., and Bae, H. (2017). Composition, diversity and bioactivity of culturable bacterial endophytes in mountain-cultivated ginseng in Korea. *Sci Rep-Uk* 7:10098. doi: 10.1038/s41598-017-10280-7

Korkama, T., Pakkanen, A., and Pennanen, T. (2006). Ectomycorrhizal community structure varies among Norway spruce (*Picea abies*) clones. *New Phytol.* 171, 815–824. doi: 10.1111/j.1469-8137.2006.01786.x

Laforest-Lapointe, I., Messier, C., and Kembel, S. W. (2016). Host species identity, site and time drive temperate tree phyllosphere bacterial community structure. *Microbiome* 4:27. doi: 10.1186/s40168-016-0174-1

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T. W., and van Breemen, N. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol. Evol.* 16, 248–254. doi: 10.1016/s0169-5347(01)02122-x

Leff, J. W., Bardgett, R. D., Wilkinson, A., Jackson, B. G., Pritchard, W. J., De Long, J. R., et al. (2018). Predicting the structure of soil communities from plant community taxonomy, phylogeny, and traits. *Isme J.* 12, 1794–1805. doi: 10.1038/s41396-018-0089-x

Li, B., Li, Q., Xu, Z., Zhang, N., Shen, Q., and Zhang, R. (2014). Responses of beneficial *Bacillus amyloliquefaciens* SQR9 to different soilborne fungal pathogens through the alteration of antifungal compounds production. *Front. Microbiol.* 5:636. doi: 10.3389/fmicb.2014.00636

Li, H., Yan, C., Tang, Y., Ma, X., Chen, Y., Chen, S., et al. (2020). Endophytic bacterial and fungal microbiota in different cultivars of cassava (*Manihot esculenta* Crantz). *J. Microbiol.* 58, 614–623. doi: 10.1007/s12275-020-9565-x

Li, Y., and Chen, S. (2019). Fusaricidin produced by *Paenibacillus polymyxa* WLY78 induces systemic resistance against Fusarium wilt of cucumber. *Int. J. Mol. Sci.* 20:5240. doi: 10.3390/ijms20205240

Liotti, R. G., da Silva, Figueiredo, M. I., da Silva, G. F., de Mendonça, E., and Soares, M. A. (2018). Diversity of cultivable bacterial endophytes in *Paullinia cupana* and their potential for plant growth promotion and phytopathogen control. *Microbiol. Res.* 207, 8–18. doi: 10.1016/j.micres.2017.10.011

Liu, J., Abdelfattah, A., Norelli, J., Burchard, E., Schena, L., Droby, S., et al. (2018). Apple endophytic microbiota of different rootstock/scion combinations suggests a genotype-specific influence. *Microbiome* 6:18. doi: 10.1186/s40168-018-0403-x

Liu, L., Huang, X., Zhang, J., Cai, Z., and Chang, Y. (2020). Deciphering the combined effect and relative importance of soil and plant traits on the development of rhizosphere microbial communities. *Soil Biol. Biochem.* 148:107909. doi: 10.1016/j.soilbio.2020.107909

Liu, N., Luo, X., Tian, Y., Lai, D., Zhang, L., Lin, F., et al. (2019). The stereoisomeric *Bacillus subtilis* HN09 metabolite 3,4-dihydroxy-3-methyl-2-pentanone induces disease resistance in *Arabidopsis* via different signalling pathways. *BMC Plant Biol.* 19:384. doi: 10.1186/s12870-019-1985-6

Liu, Y., Chen, L., Wu, G., Feng, H., Zhang, G., Shen, Q., et al. (2017). Identification of root-secreted compounds involved in the communication between cucumber, the beneficial *Bacillus amyloliquefaciens*, and the soil-borne pathogen *Fusarium oxysporum*. *Mol. Plant Microbe In Mpmi* 30, 53–62. doi: 10.1094/MPMI-07-16-0131-R

Long, S. R. (1989). Rhizobium-legume nodulation: life together in the underground. *Cell* 56, 203–214. doi: 10.1016/0092-8674(89)90893-3

Lopes, L. D., Pereira, E., Silva, M., and Andreote, F. D. (2016). Bacterial abilities and adaptation toward the rhizosphere colonization. *Front. Microbiol.* 7:1341. doi: 10.3389/fmicb.2016.01341

López, S. M. Y., Pastorino, G. N., Franco, M. E. E., Medina, R., Lucentini, C. G., Saparrat, M. C. N., et al. (2018). Microbial endophytes that live within the seeds of two tomato hybrids cultivated in Argentina. *Agron. J.* 8:136. doi: 10.3390/agronomy8080136

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Luo, T. (2005). *SSR identification and classification of cassava germplasms*. master's thesis, China, IL: University of Guangxi.

Marschner, P., Crowley, D., and Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* 261, 199–208. doi: 10.1023/B:PLSO.0000035569.80747.c5

Martin, F. M., Uroz, S., and Barker, D. G. (2017). Ancestral alliances: plant mutualistic symbioses with fungi and bacteria. *Science* 356:eaad4501. doi: 10.1126/science.aad4501

Mazurier, S., Corberand, T., Lemanceau, P., and Raaijmakers, J. M. (2009). Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to Fusarium wilt. *Isme J.* 3, 977–991. doi: 10.1038/ismej.2009.33

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Micallef, S. A., Channer, S., Shiaris, M. P., and Colón-Carmona, A. (2009). Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. *Plant Signal Behav.* 4, 777–780. doi: 10.1093/jxb/erp053

Miethling, R., Wieland, G., Backhaus, H., and Tebbe, C. C. (2000). Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. *Microb. Ecol.* 40, 43–56. doi: 10.1007/s002480000021

Morella, N. M., Weng, F. C., Joubert, P. M., Metcalf, C., Lindow, S., and Koskella, B. (2020). Successive passaging of a plant-associated microbiome reveals robust habitat and host genotype-dependent selection. *P. Natl. Acad. Sci. USA* 117, 1148–1159. doi: 10.1073/pnas.1908600116

Morris, P. F., Bone, E., and Tyler, B. M. (1998). Chemotropic and contact responses of *phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiol.* 117, 1171–1178. doi: 10.1104/pp.117.4.1171

Müller, D. B., Vogel, C., Bai, Y., and Vorholt, J. A. (2016). The plant microbiota: systems-level insights and perspectives. *Annu. Rev. Genet.* 50, 211–234. doi: 10.1146/annurev-genet-120215-034952

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., et al. (2011). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe In Mpmi* 24, 533–542. doi: 10.1094/MPMI-09-10-0213

Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S. J., Hadar, Y., and Minz, D. (2014). Niche and host-associated functional signatures of the root surface microbiome. *Nat. Commun.* 5:4950. doi: 10.1038/ncomms5950

Okogbenin, E., Setter, T. L., Ferguson, M., Mutegi, R., Ceballos, H., Olasanmi, B., et al. (2013). Phenotypic approaches to drought in cassava: review. *Front. Physiol.* 4:93. doi: 10.3389/fphys.2013.00093

Paterson, E., and Sim, A. (2000). Effect of nitrogen supply and defoliation on loss of organic compounds from roots of *Festuca rubra*. *J. Exp. Bot.* 51, 1449–1457. doi: 10.1093/jexbot/51.349.1449

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *P. Natl. Acad. Sci. USA* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pérez-Izquierdo, L., Zabal-Aguirre, M., González-Martínez, S. C., Buée, M., Verdú, M., Rincón, A., et al. (2019). Plant intraspecific variation modulates nutrient cycling through its below ground rhizospheric microbiome. *J. Ecol.* 107, 1594–1605. doi: 10.1111/1365-2745.13202

Priyadharsini, P., and Dhanasekaran, D. (2015). Diversity of soil allelopathic Actinobacteria in Tiruchirappalli district, Tamilnadu, India. *J. Saudi Soc. Agr. Sci.* 14, 54–60. doi: 10.1016/j.jssas.2013.07.001

Qiu, Z., Egidi, E., Liu, H., Kaur, S., and Singh, B. K. (2019). New frontiers in agriculture productivity: optimised microbial inoculants and in situ microbiome engineering. *Biotechnol. Adv.* 37:107371. doi: 10.1016/j.biotechadv.2019.03.010

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 50, 403–424. doi: 10.1146/annurev-phyto-081211-172908

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Rasche, F., Hödl, V., Poll, C., Kandeler, E., Gerzabek, M. H., van Elsas, J. D., et al. (2006). Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. *FEMS Microbiol. Ecol.* 56, 219–235. doi: 10.1111/j.1574-6941.2005.00027.x

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Métraux, J. P., and L'Haridon, F. (2016). The microbiome of the leaf surface of *Arabidopsis* protects against a fungal pathogen. *New Phytol.* 210, 1033–1043. doi: 10.1111/nph.13808

Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M. L., et al. (2015). Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. *Environ. Microbiol.* 17, 316–331. doi: 10.1111/1462-2920.12439

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Paré, P. W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* 134, 1017–1026. doi: 10.1104/pp.103.026583

Sangster, T., Major, H., Plumb, R., Wilson, A. J., and Wilson, I. D. (2006). A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *Analyst* 131, 1075–1078. doi: 10.1039/b604498k

Sarr, P. S., Sugiyama, A., Begoude, A. D., Yazaki, K., Araki, S., and Nawata, E. (2017). Molecular assessment of the bacterial community associated with Cassava (*Manihot esculenta* Crantz) cultivation in Cameroon. *Microbiol. Res.* 197, 22–28. doi: 10.1016/j.micres.2016.12.011

Sasse, J., Martinoia, E., and Northen, T. (2018). Feed your friends: do plant exudates shape the root microbiome? *Trends Plant Sci.* 23, 25–41. doi: 10.1016/j.tplants.2017.09.003

Schlemper, T. R., Leite, M., Lucheta, A. R., Shimels, M., Bouwmeester, H. J., van Veen, J. A., et al. (2017). Rhizobacterial community structure differences among sorghum cultivars in different growth stages and soils. *Fems Microbiol. Ecol.* 93:8. doi: 10.1093/femsec/fix096

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Shao, Y., Huo, D., Peng, Q., Pan, Y., Jiang, S., Liu, B., et al. (2017). Lactobacillus plantarum HNU082-derived improvements in the intestinal microbiome prevent the development of *hyperlipidaemia*. *Food Funct.* 8, 4508–4516. doi: 10.1039/c7fo00902j

Shen, Z., Ruan, Y., Wang, B., Zhong, S., Su, L., Li, R., et al. (2015). Effect of biofertilizer for suppressing Fusarium wilt disease of banana as well as enhancing microbial and chemical properties of soil under greenhouse trial. *Appl. Soil Ecol.* 93, 111–119. doi: 10.1016/j.apsoil.2015.04.013

Simonin, M., Dasilva, C., Terzi, V., Ngonkeu, E., Diouf, D., Kane, A., et al. (2020). Influence of plant genotype and soil on the wheat rhizosphere microbiome:

evidences for a core microbiome across eight African and European soils. *Fems Microbiol Ecol* 96, fiaa067. doi: 10.1093/femsec/fiaa067

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006). XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78, 779–787. doi: 10.1021/ac051437y

Smith, S., and Read, D. (eds) (2008). *Mycorrhizal symbiosis*. London, UK: Academic Press.

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi: 10.1038/nrmicro2540

Suja, S. P., Hegde, V., Thangaraj, M. K., and Anjanadevi, I. P. (2015). Screening of rhizobacteria associated with cassava for plant growth promotion and biocontrol potential. *J. Root Crops* 40, 66–73.

Tenorio-Salgado, S., Tinoco, R., Vazquez-Duhalt, R., Caballero-Mellado, J., and Perez-Rueda, E. (2013). Identification of volatile compounds produced by the bacterium Burkholderia tropica that inhibit the growth of fungal pathogens. *Bioengineered* 4, 236–243. doi: 10.4161/bioe.23808

Thiergart, T., Durán, P., Ellis, T., Vannier, N., Garrido-Oter, R., Kemen, E., et al. (2020). Root microbiota assembly and adaptive differentiation among European *Arabidopsis* populations. *Nat. Ecol. Evol.* 4, 122–131. doi: 10.1038/s41559-019-1063-3

Tinker, P. B. (1984). *The role of microorganisms in mediating and facilitating the uptake of plant nutrients from soil in Biológical processes and soil fertility*. Dordrecht: Springer, 77–91. doi: 10.1007/978-94-009-6101-2_7

Tringe, S. G., von Mering, C., Kobayashi, A., Salamov, A. A., Chen, K., Chang, H. W., et al. (2005). Comparative metagenomics of microbial communities. *Science* 308, 554–557. doi: 10.1126/science.1107851

Utsumi, Y., Tanaka, M., Morosawa, T., Kurotani, A., Yoshida, T., Mochida, K., et al. (2012). Transcriptome analysis using a high-density oligomicroarray under drought stress in various genotypes of cassava: an important tropical crop. *DNA Res.* 19, 335–345. doi: 10.1093/dnares/dss016

van der Heijden, M. G., and Schlaeppi, K. (2015). Root surface as a frontier for plant microbiome research. *P. Natl. Acad. Sci. USA* 112, 2299–2300. doi: 10.1073/pnas.1500709112

van Overbeek, L., and van Elsas, J. D. (2008). Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). *Fems Microbiol. Ecol.* 64, 283–296. doi: 10.1111/j.1574-6941.2008.00469.x

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Walters, W. A., Jin, Z., Youngblut, N., Wallace, J. G., Sutter, J., Zhang, W., et al. (2018). Large-scale replicated field study of maize rhizosphere identifies heritable microbes. *P. Natl. Acad. Sci. USA* 115, 7368–7373. doi: 10.1073/pnas.1800918115

Wang, B., Li, R., Ruan, Y., Ou, Y., Zhao, Y., and Shen, Q. (2015). Pineapple–banana rotation reduced the amount of *Fusarium oxysporum* more than maize–banana rotation mainly through modulating fungal communities. *Soil Biol. Biochem.* 86, 77–86. doi: 10.1016/j.soilbio.2015.02.021

Want, E. J., Masson, P., Michopoulos, F., Wilson, I. D., Theodoridis, G., Plumb, R. S., et al. (2013). Global metabolic profiling of animal and human tissues via UPLC-MS. *Nat. Protoc.* 8, 17–32. doi: 10.1038/nprot.2012.135

Weller, D. M., Raaijmakers, J. M., Gardener, B. B., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi: 10.1146/annurev.phyto.40.030402.110010

Wu, L., Li, L., Chen, S., Wang, L., and Lin, X. (2020). Deep eutectic solvent-based ultrasonic-assisted extraction of phenolic compounds from *Moringa oleifera* L. leaves: Optimization, comparison and antioxidant activity. *Sep. Purif. Technol.* 247:117014. doi: 10.1016/j.seppur.2020.117014

Wu, S., Xue, S., Iqbal, Y., Xing, H., and Jie, Y. (2021). Seasonal nutrient cycling and enrichment of nutrient-related soil microbes aid in the adaptation of ramie (*Boehmeria nivea* L.) to nutrient-deficient conditions. *Front. Plant Sci.* 12:644904. doi: 10.3389/fpls.2021.644904

Xiong, C., Zhu, Y. G., Wang, J. T., Singh, B., Han, L. L., Shen, J. P., et al. (2021). Host selection shapes crop microbiome assembly and network complexity. *New Phytol.* 229, 1091–1104. doi: 10.1111/nph.16890

Xu, Y., Wang, G., Jin, J., Liu, J., Zhang, Q., and Liu, X. (2009). Bacterial communities in soybean rhizosphere in response to soil type, soybean genotype, and their growth stage. *Soil Biol. Biochem.* 41, 919–925. doi: 10.1016/j.soilbio.2008.10.027

Xu, Z., Zhang, R., Wang, D., Qiu, M., Feng, H., Zhang, N., et al. (2014). Enhanced control of cucumber wilt disease by *Bacillus amyloliquefaciens* SQR9 by altering the regulation of Its DegU phosphorylation. *Appl. Environ. Microb.* 80, 2941–2950. doi: 10.1128/AEM.03943-13

Yang, R., Liu, P., and Ye, W. (2017). Illumina-based analysis of endophytic bacterial diversity of tree peony (*Paeonia Sect. Moutan*) roots and leaves. *Braz. J. Microbiol.* 48, 695–705. doi: 10.1016/j.bjm.2017.02.009

Yuan, J., Zhao, J., Wen, T., Zhao, M., Li, R., Goossens, P., et al. (2018). Root exudates drive the soil-borne legacy of aboveground pathogen infection. *Microbiome* 6:156. doi: 10.1186/s40168-018-0537-x

Zhang, H., Sun, Y., Xie, X., Kim, M. S., Dowd, S. E., and Paré, P. W. (2009). A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. *Plant* J. 58, 568–577. doi: 10.1111/j.1365-313X.2009.03803.x

Zhang, J., Liu, Y. X., Zhang, N., Hu, B., Jin, T., Xu, H., et al. (2019). NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nat. Biotechnol.* 37, 676–684. doi: 10.1038/s41587-019-0104-4

Zhang, L., Zhang, J., Wei, Y., Hu, W., Liu, G., Zeng, H., et al. (2021). Microbiome-wide association studies reveal correlations between the structure and metabolism of the rhizosphere microbiome and disease resistance in cassava. *Plant Biotechnol. J.* 19, 689–701. doi: 10.1111/pbi.13495

Zhang, Y., Xu, J., Riera, N., Jin, T., Li, J., and Wang, N. (2017). Huanglongbing impairs the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome. *Microbiome* 5:97. doi: 10.1186/s40168-017-0304-4

Zhao, J., Zhou, X., Jiang, A., Fan, J., Lan, T., Zhang, J., et al. (2018a). Distinct impacts of reductive soil disinfestation and chemical soil disinfestation on soil fungal communities and memberships. *Appl. Microbiol. Biot.* 102, 7623–7634. doi: 10.1007/s00253-018-9107-1

Zhao, Y., Xiong, Z., Wu, G., Bai, W., Zhu, Z., Gao, Y., et al. (2018b). Fungal endophytic communities of two wild *Rosa* varieties with different powdery mildew susceptibilities. *Front. Microbiol.* 9:2462. doi: 10.3389/fmicb.2018.02462

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ORIGINAL RESEARCH
published: 30 September 2021
doi: 10.3389/fmicb.2021.738058

Prospecting Endophytic Bacteria Endowed With Plant Growth Promoting Potential Isolated From *Camellia sinensis*

*Shabiha Nudrat Hazarika[1,2], Kangkon Saikia[1], Atlanta Borah[1] and Debajit Thakur[1]**

[1] Microbial Biotechnology Laboratory, Life Sciences Division, Institute of Advanced Study in Science and Technology, Guwahati, India, [2] Department of Molecular Biology and Biotechnology, Cotton University, Guwahati, India

Endophytes are well-acknowledged inoculants to promote plant growth, and extensive research has been done in different plants. However, there is a lacuna about the endophytes associated with tea clones and their benefit to promote plant growth. The present study focuses on isolating and characterizing the beneficial endophytic bacteria (EnB) prevalent in commercially important tea clones cultivated in North Eastern India as plant growth promoters. Diversity of culturable EnB microbiome, *in vitro* traits for plant growth promotion (PGP), and applicability of potent isolates as bioinoculant for *in vivo* PGP abilities have been assessed in the present study. A total of 106 EnB identified as members of phyla Proteobacteria, Firmicutes, and Actinobacteria were related to 22 different genera and six major clusters. Regarding PGP traits, the percentage of isolates positive for the production of indole acetic acid, phosphate solubilization, nitrogen fixation siderophore, ammonia, and 1-aminocyclopropane-1-carboxylic acid deaminase production were 86.8, 28.3, 78.3, 30.2, 95.3, and 87.7, respectively. In total, 34.0, 52.8, and 17.0% of EnB showed notable production of hydrolytic enzymes like cellulase, protease, and amylase, respectively. Additionally, based on the bonitur score, the top two isolates K96 identified as *Stenotrophomonas* sp. and M45 identified as *Pseudomonas* sp. were evaluated for biofilm formation, motility, and *in vivo* plant growth promoting activity. Results suggested strong biofilm formation and motility in K96 and M45 which may attribute to the colonization of the strains in the plants. Further *in vivo* plant growth promotion experiment suggested sturdy efficacy of the K96 and M45 as plant growth promoters in nursery condition in commercial tea clones Tocklai vegetative (TV) TV22 and TV26. Thus, this study emphasizes the opportunity of commercialization of the selected isolates for sustainable development of tea and other crops.

Keywords: endophytic bacteria, *Camellia sinensis*, plant growth promotion, bio-inoculum, 16S rRNA gene, diversity, biofilm

OPEN ACCESS

Edited by:
Gustavo Santoyo, Universidad Michoacana de San Nicolás de Hidalgo, Mexico

Reviewed by:
Pramod Kumar Sahu, Indian Council of Agricultural Research (ICAR), India
Vuyo Mavumengwana, South African Medical Research Council, South Africa

***Correspondence:**
Debajit Thakur
debajitthakur@yahoo.co.uk

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *08 July 2021*
Accepted: *20 August 2021*
Published: *30 September 2021*

Citation:
Hazarika SN, Saikia K, Borah A and Thakur D (2021) Prospecting Endophytic Bacteria Endowed With Plant Growth Promoting Potential Isolated From Camellia sinensis. Front. Microbiol. 12:738058. doi: 10.3389/fmicb.2021.738058

INTRODUCTION

Plants interact with a broad range of diverse bacteria having a beneficial, neutral, or pathogenic impact on their hosts, in both natural and managed ecosystems. The majority of bacterial symbionts known as epiphytes colonize the rhizosphere (Compant et al., 2005; Lugtenberg and Kamilova, 2009). But a subset of bacteria from the rhizosphere are capable to enter and proliferate

within plants as endophytes establishing a mutualistic association with the plants (Rosenblueth and Martínez-romero, 2006; Hallmann and Berg, 2007). Endophytes (bacteria or fungi) are an endosymbiotic group of microorganisms, ubiquitous in nature and well-known to dwell inside the plant endosphere without causing any apparent harmful effect on the host plant (Hallmann et al., 1997). Endophytic bacteria (EnB) have been isolated from almost all plants and plant parts such as roots, stems, leaves, barks, floral organs, and even seeds (Ryan et al., 2008; Compant et al., 2011; Dudeja et al., 2012). EnB studies in rice (Fujita et al., 2017), wheat (Emami et al., 2019), tomato (Khan et al., 2014), potato (Sessitsch et al., 2004), sugarcane (Mendes et al., 2007), and many other crops, medicinal plants, halophytes, and woody trees were mainly focussed, but endophytes from tea plants were rarely considered. To maintain stable symbiosis, endophytes ameliorate the host plant to tolerate abiotic and biotic stresses through phosphate solubilization (Liu et al., 2014; Oteino et al., 2015), nitrogen fixation (Hurek and Reinhold-Hurek, 2003; Zhu et al., 2012), indole-3-acetic acid (IAA) (Patten and Glick, 1996; Hassan, 2017), siderophore (Zhang et al., 2011), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Zhang et al., 2011; Khan et al., 2014) synthesis and augment the resistance of plants to insects, pests, and pathogens by producing bioactive metabolites (Etminani and Harighi, 2018) and polymer hydrolyzing enzymes such as cellulase, amylase, chitinase, and protease (El-Deeb et al., 2012) and promote plant growth and development. Bacterial endophytes that have potentially beneficial effects on plant growth and development has been found in many genera including *Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Burkholderia, Curtobacterium, Erwinia, Gluconobacter, Klebsiella, Serratia, Pantoea, Herbaspirillum, Rahnella, Pseudomonas,* and *Xanthomonas* (Zinniel et al., 2002; Berg et al., 2005; Compant et al., 2011).

Camellia sinensis (L) O. Kuntze (Tea), an economically important crop, is the world's most-consumed non-alcoholic beverage which has been commercially produced by more than 30 countries worldwide (Hayat et al., 2015), with 1283.3 M. Kgs produced by India making it the world's second-largest producer of tea (Sarani, 2020). In India, tea is extensively cultivated in Assam, Darjeeling, Nilgiri, and other North-Eastern (NE) states of India (Mondal et al., 2004). Assam plays a pivotal role in the national economy by producing 623.23 M. Kgs of tea, being the leading producer and exporter of commercial tea (Sarani, 2020). With the favorable climatic and topographic conditions of Meghalaya, suitable tea varieties brought from Assam and Darjeeling were transplanted at tea gardens located at Umsning, Meghalaya, India, in 1978 which were acclaimed by tea makers to be of very high quality (Directorate of Food Processing Meghalaya, n.d.). Tocklai Tea Research Institute (TTRI), Jorhat, Assam, India, has released 33 Tocklai Vegetative (TV) series tea clones, 153 region-specific garden series clones, and 16 biclonal seed stocks to the tea industry (Patel et al., 2019) and classified them based on their yield and quality into a standard clone (greater than average quality and yield), quality clone (high quality with average yield), and yield clone (average quality with high yield) (Das et al., 2012).

Owing to the production of piles of nutraceuticals in tea plants, consumption of tea drinks is related to numerous medicinal and nutritional benefits (Sharangi, 2009) such as antioxidant (Frei and Higdon, 2003), anti-inflammatory (Hsu et al., 2003), anti-carcinogenic (Kris-Etherton et al., 2002; Cooper et al., 2005), anti-allergic (Maeda-Yamamoto et al., 2007), and anti-microbial effect (Hamilton-Miller, 1995; Siddiqui et al., 2016). Although studies on tea phytochemicals and their potent effects have been well characterized, research on endophytic bacteria associated with tea clones is rather sparse.

Compared to the rhizospheric bacterial community (Dutta and Thakur, 2017) and endophytic fungal community (Win et al., 2018), EnB community of tea plants has received less attention. The interaction studies between EnB microflora associated with different tea clones cultivated especially in the NE region of India have not yet been investigated thoroughly. The culturable microbial diversity of EnB associated with tea clones has barely been explored. Also, the ability to utilize the EnB as plant growth promoters has not been well reported. Thus, the current study aims to investigate EnB community prevalent in selected commercially popular tea clones (TV1, TV22, TV9, Teenali 17, and TV25) using molecular, phylogenetic, and functional approaches. To evaluate the plant growth stimulation by plant growth-promoting bacteria (PGPB), phosphate solubilization, production of phytohormone, siderophore and ammonia, biological nitrogen fixation, and stress alleviation by production of ACC deaminase were carried out. To identify and explore the microbial diversity of the endophytic isolates molecular techniques such as 16S rRNA and phylogenetic analysis were used. Further, to determine the efficacy of the potent plant growth-promoting isolates, a nursery experiment was performed using two different yield clones, TV22 and TV26, which are extensively used for commercial cultivation in NE India. Thus, the results endowed the importance of EnB microflora as plant growth promoters that can be used to develop microbial formulations to reduce the use of agrochemicals and for sustainable development of tea plants including the soil.

MATERIALS AND METHODS

Sampling of Plant Material

Random sampling method was used to collect leaf and root samples from selected healthy tea plants of five clones, TV1, TV9, Teenali 17, TV22, and TV25 (**Table 1**), and all clones were selected based on their commercial importance. The samples were collected from two locations: Kopati Tea estate, Assam, India, and MEG tea estate, Meghalaya, India. The leaves and roots were collected aseptically in sterile ziplock bags as composite samples for each tea clone, transported to the laboratory in an ice box, and further processing for isolation was done within 1 month.

Isolation of Endophytic Bacteria From Tea Clones

EnB was isolated by using surface sterilization and serial dilution method as described (Schulz et al., 1993; Sturz et al., 1998) with

TABLE 1 | Collection of Tea clones.

Sl. no.	Collected samples			Tissue of origin	Sampling site and GIS location
	Tea clones	Jat/Population/Parents	Type of clone		
1	TV1	Assam-China (Cinnamara)	Standard	Leaf, root	Kopati tea estate, Darrang, Assam, India (26.5875°N, 92.2507°E); Meg Tea, Tea Development Centre, Umsning, Meghalaya, India (25.741729° N, 91.889094° E)
2	TV9	Burma (Cambod)	Standard	Leaf, root	
3	TV22	Cambod type (Indo-China)	Yield	Leaf, root	
4	Teenali 17	Cambod type	Estate	Leaf, root	
5	TV25	Cambod type: Ayapathar D × Ayapathar A (DA/4)	Yield	Leaf, root	

slight modifications. In brief, plant materials were washed for 3–5 min in running tap water to remove any debris and rinsed in 70% ethanol for 30 s followed by surface sterilization for 5 min using 1.5 and 2% v/v sodium hypochlorite solution for leaves and roots, respectively, followed by washing with sterile distilled water thrice for 5 min. The efficacy of surface sterilization was validated by spreading 100 μL aliquots of distilled water on nutrient agar (NA) plates taken from the last wash of the samples.

The surface-sterilized samples were excised and macerated in 0.9% sodium chloride (NaCl) with a sterile mortar pestle. EnB isolation was done by spread plate method, 100 μL of the aliquots were spread on selective media, namely Nutrient Agar, King's B agar, Pikovskaya's agar, Pseudomonas agar (HiMedia, India) followed by incubation at 28 ± 2°C for 48–72 h. Distinguished colonies based on phenotypic characters (size, elevation, color, form, and margin) were selected and pure culture of the isolates were made to determine the culturable bacterial population. For further studies and long-term preservation pure cultures were maintained at 4°C and −80°C as 20% (w/v) glycerol stocks, respectively.

Molecular Characterization of Endophytic Bacteria

DNA Extraction and 16S rRNA Gene Amplification

Genomic DNA was extracted from isolates according to the protocol in the maker's instructions as mentioned in QIAamp DNA mini kit (Qiagen, Hilden, Germany). The purity and concentration of the eluted DNA were quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific, United States) and stored at 4°C until required for PCR.

In addition, 16S rRNA gene of the isolates was amplified using 1 μL (10 μmol L^{-1}) forward (27F 5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primers (1492R 5′- GGT TAC CTT GTT ACG ACT T-3′), 5 μL (10x) Taq buffer, 2.5 μL (2.5 mmol L^{-1}) dNTPs, 0.5 μL (2.5 U) Taq polymerase in a total volume of 50 μL reaction mixture. PCR cycling conditions were set up in a Thermal cycler (Eppendorf, United States) as follows: 94°C for 5 min for initial denaturation of DNA, followed by 35 cycles of denaturation at 94°C for 30 s, annealing of primer at 55°C for 40 s, extension of DNA strand at 72°C for 1 min and final extension at 72°C for 10 min (Passari et al., 2015). The amplicons were then separated on a 1.2% agarose gel with ethidium bromide, visualized and documented in a BioRad Gel Doc XRS+ (Hercules, CA, United States). The PCR amplicons were then outsourced to 1st Base DNA Sequencing service, Malaysia for Sanger sequencing to determine the nucleotide sequence of DNA.

Sequence Analysis and Phylogenetic Analysis

The 16S rRNA sequences were subjected to de-novo assembly using UGENE 37.0 and contigs generated were queried for homology using NCBI BLASTn and EZ taxon server 2.1 programs. The identified accessions were then submitted to GenBank and accession numbers were collected for each isolate. The multiple sequence alignment was carried out using MAFFT v7.45 (Katoh and Standley, 2013) and corrected by trimAl v1.4 using the gappyout algorithm (Capella-Gutiérrez et al., 2009) followed by a validation step using pmodel test tool of ETE3 toolkit (Huerta-Cepas et al., 2016). The evolutionary relationship between the accessions was analyzed by the maximum likelihood method using RAxML with 1,000 bootstrap steps (Stamatakis, 2014) and a phylogenetic tree was constructed using. Fig Tree 1.4.4 (Rambaut, 2018).

Screening of Plant Growth Promotion Traits *in vitro*

The EnB isolates were screened *in vitro* for identifying the traits required for nutrient acquisition and plant growth, like production of phytohormone, ammonia, and iron chelating agents, fixation of nitrogen from the atmosphere, production of stress alleviating enzymes, and hydrolytic enzymes. For all the PGP assays *Bacillus pseudomycoides* strain SN29 (GenBank accession no. KJ767523) (Dutta et al., 2015) was used as a positive control.

Estimation of Production of Phytohormone Indole-3-Acetic Acid

Gordon and Weber's (1951) method was used to determine IAA production quantitatively and qualitatively with modifications. EnB isolates were inoculated in minimal salt (MS) medium amended with 2 mg mL^{-1} L-Tryptophan and incubated in an orbital shaker (170 rpm) at 28 ± 2°C for 48 h. To obtain bacterial cells free supernatant centrifugation at 10,000 rpm for 15 min was done. To the supernatant Salwoski reagent (0.5 M $FeCl_3$ and 70% perchloric acid) was added in 1:2 ratio (v/v) and incubated in dark

for 25 min at room temperature. After incubation optical density was measured at 530 nm using a multimode reader (Varioskan, Thermo Fisher Scientific). Commercial IAA (Sigma-Aldrich) was used as a standard to quantify IAA production. The experiment was done in triplicates.

Estimation of Phosphate Solubilization

Phosphate solubilization by EnB isolates was screened by spot inoculation on Pikovskaya's agar medium (PKA) followed by incubation at 28 ± 2°C for 48 h. To quantify the potential of isolates to solubilize tricalcium phosphate, they were grown in NBRIP (National Botanical Research Institute's Phosphate Growth) medium at 28°C for 48 h as described by Fiske and Subbarow (1925) and centrifuged at 10,000 rpm for 20 min to obtain supernatant. The amount of phosphate content in the supernatant was determined by adding ammonium molybdate reagent in equal volume and measured by colorimetry at 650 nm. A standard curve of KH_2PO_4 was used to determine the amount of phosphate solubilized which was expressed in mg L^{-1}. The experiment was done in triplicates.

Estimation of Siderophore Production

Quantitative analysis of siderophore production was determined by Chrome Azurol S (CAS) shuttle assay as described (Schwyn and Neilands, 1987; Alexander and Zuberer, 1991). The EnB isolates were grown in MS media at 28 ± 2°C for 48 h and after incubation culture was centrifuged at 10,000 rpm for 10 min and mixed with an equal volume of CAS reagent. The un-inoculated MS medium served as a control. The colorimetric measurements were performed by using a multimode reader at 630 nm. The percentage of siderophore produced (psu) by isolates was calculated by the following formula as described (Payne, 1994).

$$Siderophore\ production\ units\ (psu) = \frac{(Ar - As)\,x100}{Ar}$$

where Ar = absorbance of reference and As = absorbance of the sample at 630 nm

Estimation of Ammonia Production and Nitrogen Fixation

Ammonia production was determined as described (Yuen and Pollard, 1954; Singh et al., 2014). EnB isolates were inoculated into Peptone Water Broth medium and incubated at 28 ± 2°C, 150 rpm for 48 h. The bacteria cells were removed by centrifugation at 8,000 rpm for 5 min. To the supernatant, Nessler's reagent was added in a 2:1 ratio (v/v). Yellow to brown color development indicated ammonia production. Ammonium sulfate (0.1–5.0 μmol mL^{-1}) was used as a standard to quantify the amount of ammonia production, the absorbance of which was measured at 450 nm by a multimode reader and expressed as μmol mL^{-1}. The experiment was done in triplicates.

Nitrogen-fixing abilities of the EnB isolates were determined qualitatively by culturing in Nitrogen-free (NF) medium containing (g L^{-1}) mannitol 20 g, K_2HPO_4 0.2 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, K_2SO_4 0.1 g, $CaCO_3$ 5 g, and agar 20 g and Jensen's agar medium by spot inoculation method as described (Kifle and Laing, 2016). The growth of the isolates was monitored after incubation at 28 ± 2°C for 48 h.

Qualitative Estimation of 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Activity

ACC deaminase activity of the EnB isolates was qualitatively assessed by spot inoculation method in Dworkin and Foster (DF) salt medium (Dworkin and Foster, 1958) amended with 3 mM ACC as sole nitrogen source. The inoculated plates were incubated for 48 h at 28 ± 2°C and monitored. The colonies growing on the plates were recognized as ACC deaminase producers (Glick et al., 2007).

Screening of Hydrolytic Enzyme Production

For the production of carboxymethyl cellulase (CMCase), EnB isolates were spot inoculated on M9-CMC agar plates containing (g L^{-1}), $NaNO_3$ 1 g, K_2HPO_4 1 g, KCl 1 g, $MgSO_4$ 0.5 g, yeast extract 5 g, agar 15 g, and CMC 2 g as substrate and incubated for 48 h at 28 ± 2°C. The incubated plates were flooded with 0.2% congo red solution for 15 min and de-stained with 0.1 M NaCl for 15 min. The formation of clear halo zones around colonies after 3–5 min confirmed cellulose hydrolysis (Meddeb-mouelhi et al., 2014).

The starch degrading ability of isolates to produce amylolytic enzymes was determined by spot inoculation on starch agar plates containing (g L^{-1}) meat extract 3 g, peptic digest of animal tissue 5 g, starch soluble 2 g, and agar 15 g. Lugol solution (5 g iodine crystals and 10 g potassium iodide in 100 mL of distilled water) was flooded in plates for 2 min after incubation at 28 ± 2°C for 48 h. On starch hydrolysis, a clear zone around the colonies indicated amylase production (Iverson and Millis, 1974).

Protease production by EnB isolates was evaluated by spot inoculation on skim milk agar (SMA) medium (Sgroy et al., 2009). The inoculated plates were incubated at 28 ± 2°C for 48 h. Proteolytic activity was confirmed by the formation of clear halo zones around colonies.

Quantification and Microscopic Evaluation of Bacterial Biofilm Development

In vitro biofilm formation was quantified using microtiter plate assay method described (O'Toole and Kolter, 1998) with minor modifications. Overnight grown cultures of EnB were re-suspended in nutrient broth (NB) medium and incubated in a rotary shaker (180 rpm) at 28°C to obtain the final OD_{600} = 0.2 (10^7 CFU mL^{-1}) bacterial cells. Aliquots of 200 μL of EnB cultures were moved to 96 well microtiter plate and incubated at 28 ± 2°C for 48 h in static condition. NB broth without inoculation was taken as control. To remove the planktonic bacteria, the wells were emptied and washed thrice with 1X phosphate-buffered saline (PBS), pH 7.2 followed by staining with 0.1% crystal violet solution for 30 min. Excess bound stains on wells were removed and the wells were washed with

1X PBS and allowed to air dry. The bound crystal violet was then solubilized using 100% ethanol and the intensity of biofilm formation was measured at 570 nm using a multimode plate reader (Gupta et al., 2019).

Light microscopy and confocal microscopy were used to evaluate bacterial biofilm development. Freshly grown overnight bacterial cultures (20 μL) of K96 and M45 were inoculated in sterile glass coverslip (20 mm) and placed in a 60 mm culture plate and incubated at 28 ± 2°C for 24–48 h under static condition. Loosely adhered cells were removed from the surface by washing glass coverslip thrice with 1X PBS and stained with 0.1% crystal violet (CV) for 10 min and 0.1% acridine orange (AO) for 20 min followed by removal of excess stain by washing with 1X PBS and allowed to dry. To examine the extent of microbial attachment or biofilm formation on the glass surface the slides stained with CV were visualized under a compound microscope.

Confocal Laser Scanning Microscope (Clsm) imaging was performed using a Leica Tcs Sp8 confocal laser scanning microscope (Leica, Mannheim, Germany) equipped with a visible light laser, Leica Dmi 6000B inverted light microscope, and an Hc Pl Apo Cs2 63X/1.40 Oil immersion objective. Acridine Orange (Ao) was excited using a 488 nm argon laser line, while the fluorescent emission was detected from 502 to 526 nm. The pinhole aperture size was adjusted to 1.8 Airy units and detector gain was set to 630 for visualization of the biofilm. In the z-plane, 36 fluorescent optical images were collected to span the full depth of the biofilms. Leica Application Suite X software was used for visualization and ImageJ software was used for the analysis of the images.

Cell Surface Hydrophobicity Assay

Microbial Adhesion to Hydrocarbons (MATH) method was used to establish CSH by K96 and M45 EnB isolates as described (Rosenberg, 2006). The percentage of hydrophobicity was described as follows:

$$\textit{Percentage of hydrophobicity}\ (\%) = \frac{(A0 - A1)\,x100}{A0}$$

where A0 is the initial absorbance of bacterial culture at 400 nm and A1 is the final absorbance of the aqueous phase at 400 nm.

Motility Assay

Bacterial motility assays (swimming and swarming) were performed by the protocol described (Gupta et al., 2019) with slight modification. Luria broth (LB) medium with 0.3% agar (w/v) and 0.5% (w/v) agar supplemented with 5 g L^{-1} D-glucose was used for swimming motility and swarming motility assay, respectively. To study the bacterial migration freshly grown bacterial culture (5 μL, OD_{600} = 0.2) of cell density (10^7 cells mL^{-1}) was inoculated on each assay plate and incubated in an inverted position at 28 ± 2°C for 48 h and the swarm diameter for both swimming and swarming motilities was measured at 24 and 48 h of incubation and expressed in mm. The twitching motility assay was done by inoculation of freshly grown bacterial isolates in LB medium with 0.6% agar followed by incubation at 28 ± 2°C for 48 h (Henrichsen, 1983). The bacterial twitching motility was visualized under the microscope (Motic BA410).

Plant Growth Promotion Experiment

Plant Material

For the plant growth promotion experiment in nursery conditions, 3-months-old tea vegetative clones TV22 (Indo-China) and TV26 (Ayapthar D × Ayapathar A) were collected from Bateli Tea estate, Assam. These TV clones are Cambod type yield clones (high yield average quality). The procured tea clones were propagated mainly through vegetative propagation using cuttings for commercial cultivation. The cuttings consist of a 3–4 cm long single leaf with a stem below the node about 2.5 cm and about 0.5 cm of stem above it. Before propagation, the cuttings were first treated with 0.1% solution of zinc sulfate. For callusing or rooting of cuttings they are transferred to nursery beds. After rooting (6–8 weeks), the cuttings were transferred to polythene sleeves where they were grown and maintained in nursery condition. For vegetative propagation of tea in the tea estate, the sleeves were prepared following standard protocol described in Field Management Notes, TTRI, Jorhat. The polythene sleeves prepared are 15 ± 17.7 cm layflat wide, 20 ± 25 cm long, and 150 gauge thick in size. The soil used in the sleeves contains well-drained sandy loam (15–20% clay less than 0.002 mm, 0–50% silt 0.002–0.2 mm, and 50–70% sand greater than 0.2 mm) of good tilth having ideal pH of 4.5.

Preparation of Bacterial Inoculum and Treatment

For the PGP experiment in nursery condition, two potent isolates K96 (*Stenotrophomonas* sp., GenBank Accession number MW905624) and M45 (*Pseudomonas* sp., GenBank Accession number MZ008002) were selected based on *in vitro* PGP abilities. For mass production, the pure bacterial cultures were inoculated in NB (HiMedia, India) and grown for 24 h at 28 ± 2°C at 180 rpm. The cultures were centrifuged at 10,000 rpm for 15 min and the pellets were washed twice with PBS to obtain a final suspension of 10^8 CFU mL^{-1} (Xu et al., 2014). Then, 10 mL of the resulting cell suspension was used to treat the tea clones in the nursery condition by soil drenching method in 15 days interval for 6 months (Dutta and Thakur, 2017; Borah et al., 2019). The nursery experiment was divided into three treatments, namely T1:K96 inoculation, T2 :M45 inoculation, T3 :inoculated with consortia of K96 and M45, and control, without bacterial inoculum, with three replicates each. To retain the soil moisture the saplings were watered once daily until harvesting. Harvesting of the tea saplings was done after 6 months of the last inoculum treatment. Physiological parameters of plants (number of leaves, shoot and root length along with their fresh and dry weight, and chlorophyll content) were recorded and compared to control.

Chlorophyll Estimation

Mature tea leaves were obtained from the treated and untreated tea clones to quantitatively determine the chlorophyll content in the leaf tissues as described by Hiscox and Israelstam (1979). To facilitate the extraction of chlorophyll, 100 mg of fresh leaf tissue was cut into small pieces and immersed in 7 mL DMSO (HiMedia). After extraction at 65°C for 30 min in dark using a

water bath, the supernatant was decanted and absorbance was determined at 645 and 663 nm against DMSO as blank using a multimode reader. Chlorophyll a and b were calculated by the following formula (Arnon, 1949):

$$Chlorophyll\ a\ (mg/g)\ =\ 12.7\ (A663) - 2.69\ (A645)\ x\frac{V}{1000xW}$$

$$Chlorophyll\ b\ (mg/g)\ =\ 22.9\ (A645) - 4.68\ (A663)\ x\frac{V}{1000xW}$$

where A = absorbance at specific wavelengths; V = final volume of chlorophyll extract; W = fresh weight of tissue extracted.

Data Analysis

The Vennture software (Martin et al., 2012) was used to make a Venn diagram to represent all the isolates producing different PGP traits. The plant growth parameters were analyzed statistically by subjecting to two-way Anova (analysis of variance). Statistically significant data was determined at $p \leq 0.05$. The interrelationships among the treatments and vegetative parameters were evaluated using principal component analysis (PCA) in Matlab R (2017a). In addition, to determine the absolute value difference between group means of control and treatment fold change analysis was done. The resulting values are generated in a log2 scale such that the up or down-regulation will be equidistant to the baseline.

RESULTS

Endophytic Bacteria Isolation From Selected Tea Clones

Altogether 106 culturable EnB were isolated from leaves and roots of five different tea clones using different enriched media and identified based on their morphological characteristics. The population density of EnB isolates in the selected tea clones varied from 3.2 to 4.4 $\log_{10}$ colony forming units g^{-1} (CFU g^{-1}) per fresh weight (**Supplementary Figure 1**). In addition, 59.43% EnB (n = 63) isolates were obtained from roots and 40.56% (n = 43) from leaves. The bacterial isolates were confirmed to be endophytes as no bacterial colonies were observed in the control plates. The relative abundance of each EnB isolated was determined at the genus level in both leaf and root samples of the five different tea clones. In leaf samples, as evident from **Figure 1A**, *Alcaligenes* sp. (42.58%) was most abundant in TV1 clone, *Brevibacillus* sp. (100%) in TV9, *Bacillus* sp. (28.83%) and *Alcaligenes* sp. (28.83%) in TV22, *Brevundimonas* sp. (67.14%) in TV25, and *Alcaligenes* sp. (32.05%) in Teenali17. In root samples, as shown in **Figure 1B**, *Pseudomonas* sp. (79.97%) was most abundant in TV1, *Bacillus* sp. (47.38%) in TV9, *Microbacterium* sp. (36.67%) in TV22, *Stenotrophomonas* sp. (62.52%) in TV25, and *Bacillus* sp. (49.98%) in Teenali 17.

The Shannon diversity indices of EnB were more in leaf (H = 2.3) as compared to the root (H = 2.14). Simpson diversity index was 0.125 in leaf and 0.177 in the root. Species richness (S) was found to be 63 in root and 43 in leaf samples. The species evenness value was found to be 0.610 in leaf and 0.516 in the root. The average population size is found to be 2.87 and 3.94 in leaf and root samples, respectively. The population of EnB isolates with their diversity indices is shown in **Table 2**.

Molecular Identification of Endophytic Bacteria Isolates

The present study revealed a diverse population of bacterial isolates within 22 different genera based on 16S rRNA gene sequencing. The sequence results procured from BLAST validated the identity with 91–100% similarity. The 16s rRNA sequence GenBank accession numbers of individual isolates and sample details are listed in **Supplementary Table 1**. The phylogenetic analysis by maximum likelihood revealed 6 major clusters (**Figure 2**). Cluster II was the largest consisting of 42 isolates of different genera belonging to *Bacillus* sp., *Sporosarcina* sp., *Paenibacillus* sp., Lysinibacillus sp., *Planococcus* sp., *Brevibacillus* sp., and *Exiguobacterium* sp. Actinobacteria species viz. *Microbacterium* sp., *Streptomyces* sp., *Curtobacterium* sp., *Janibacter* sp., *Rhodococcus* sp., *and Nocardia* sp. were distributed separately in cluster I which consisted of 11 isolates. *Pseudomonas* sp., *Acinetobacter* sp., and *Serratia* sp. were found to be in cluster III containing 15 isolates. Cluster IV comprised of 21 isolates, where it was mostly dominated by *Alcaligenes* sp. followed by *Achromobacter* sp. and *Advenella* sp. All 10 isolates in cluster V belongs to *Stenotrophomonas* sp. and 7 isolates in cluster VI belong to the genera *Ochrobactrum* sp. and *Brevundimonas* sp. Cluster I, II, and the rest of the clusters are found to be divided into two distinct sub-nodes from the origin, following a separate hierarchy. Taxa within the same genera and similar tissue (viz. leaf and root) can be distinguished separately in the subclusters with minimum evolutionary distance. In most of the cases, a diverse phylogenetic relationship was observed between accessions originating from different tea clones.

Plant Growth-Promoting Characteristics of Bacterial Endophytes

All the 106 isolates when screened for various PGP traits *in vitro* showed at least one trait as shown in **Figure 3A** and **Supplementary Table 2**. On the combined evaluation of all the six PGP traits, seven isolates (K63, K71, K96, M2, M29, M37, and M45) revealed the capacity to produce all the traits (Phosphate solubilization, IAA, Ammonia and Siderophore production, Nitrogen fixation and ACC deaminase activity) which is shown in the Venn diagram in **Figure 3B**.

Indole-3-Acetic Acid Production and Phosphate Solubilization

The ability to biosynthesize IAA was observed in 92 isolates, 41 isolates from leaf, and 51 from the root. The majority of isolates to produce IAA belong to the genera *Bacillus* sp. (26 isolates) followed by *Alcaligenes* sp. (16 isolates), *Pseudomonas* sp. (11 isolates), and *Stenotrophomonas* sp. (10 isolates). Quantitatively IAA production in presence of L-Tryptophan ranges from 3.00 ± 1.19 to 129.84 ± 4.72 $\mu g\ mL^{-1}$. Twelve (11.32%) produced more than 40 $\mu g\ mL^{-1}$. Isolate *Ochrobactrum* sp. (K39)

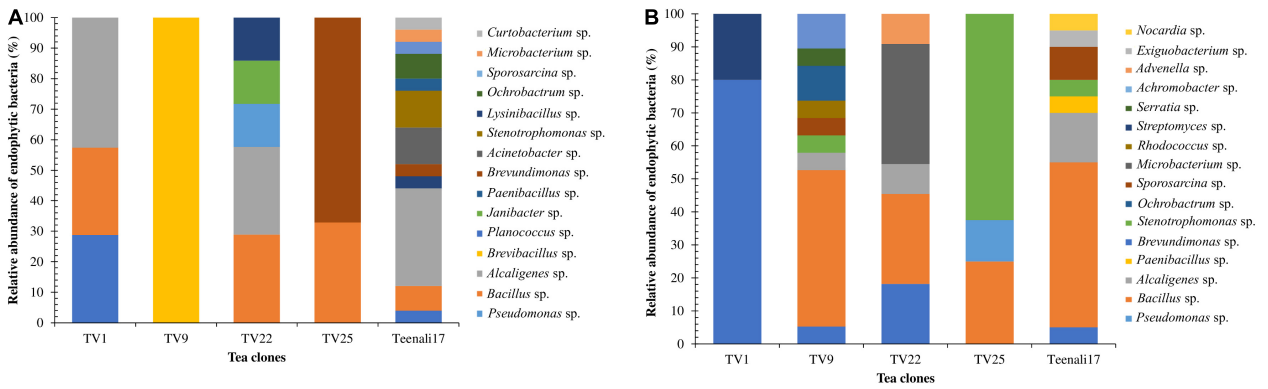


FIGURE 1 | Relative abundance (in percentage) of endophytic bacterial genera procured from **(A)** leaf and **(B)** roots of five different tea clones (TV1, TV9, TV22, TV25, and Teenali 17).

showed the highest production of IAA of 129.84 ± 4.72 μg mL^{-1} followed by *Stenotrophomonas* sp. (K96) 123.55 ± 2.50 μg mL^{-1}.

In addition, 28.30% of all the isolates (n = 30), among which 12 from leaf and 18 from roots, were identified to solubilize inorganic phosphate based on the development of clear zones around colonies in Pikovskaya's agar medium. Among the isolates *Bacillus* sp. (10 isolates) followed by *Pseudomonas* sp. (6 isolates) showed maximum phosphate solubilizing activity. The efficiency of phosphate solubilization varies from 2.79 ± 0.67 to 81.42 ± 5.73 mg L^{-1}. The highest phosphate solubilizing ability was shown by *Bacillus* sp. (K98) which solubilized 81.42 ± 5.73 mg L^{-1} calcium phosphate available in the medium followed by *Serratia* sp. (K61) 73.24 ± 0.38 mg L^{-1} and *Stenotrophomonas* sp. (K96) 71.62 ± 2.2 mg L^{-1}.

TABLE 2 | Population of endophytic bacterial isolates with diversity indices.

Isolate name	Leaf	Root
Acinetobacter sp.	3	0
Stenotrophomonas sp.	3	7
Brevundimonas sp.	3	1
Lysinibacillus sp.	1	0
Pseudomonas sp.	3	8
Ochrobactrum sp.	2	1
Alcaligenes sp.	13	5
Sporosarcina sp.	1	3
Paenibacillus sp.	2	1
Bacillus sp.	7	24
Rhodococcus sp.	0	1
Exiguobacterium sp.	0	1
Serratia sp.	0	1
Achromobacter sp.	0	2
Planococcus sp.	1	0
Janibacter sp.	1	0
Brevibacillus sp.	1	0
Curtobacterium sp.	1	0
Advenella sp.	0	1
Streptomyces sp.	0	2
Nocardia sp.	0	1
Microbacterium sp.	1	4
Taxa	15	16
Total number of organisms	43	63
Shannon diversity index_H	2.3	2.14
Simpson diversity index_SDI	0.125	0.177
Species evenness	0.62	0.516
Berger-Parker Dominance Index	0.302	0.381
Margalef Richness Index	3.72	3.62
Menhinick Index	2.29	2.02

Ammonia Production and Nitrogen Fixation

The majority of isolates (101) produced ammonia, accounting for 41 isolates from leaf and 60 isolates from roots. The majority of ammonia producers belong to the genera *Bacillus* sp. (29 isolates) followed by *Alcaligenes* sp. (17 isolates) and *Pseudomonas* sp. (11 isolates). The amount of ammonia produced falls in the range 0.61 ± 0.26–4.92 ± 0.19 μmol mL^{-1} with 39 isolates (36.79%) having the capacity to produce ammonia more than 3.0 μg mL^{-1}. *Sporosarcina* sp. (M15) produced the highest amount of ammonia of 4.92 ± 0.19 μmol mL^{-1} in the medium.

Also, 78.30% of the total isolates were capable of growing on NF media and Jensen's agar media, where 28 were from leaf and 55 from roots. Maximum nitrogen fixation was observed in *Bacillus* sp. (31 isolates) subsequently by *Pseudomonas* sp. (11 isolates), *Stenotrophomonas* sp. (10 isolates), and *Alcaligenes* sp. (10 isolates). NF media and Jensen's agar media are defined medium without nitrogen used for detection of nitrogen-fixing bacteria. The growth of EnB on medium indicates that these bacteria can fix atmospheric nitrogen.

Siderophore Production and 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Activity

Siderophore production in liquid media was detected in 32 (30.18%) isolates among which 12 isolates were from leaf and 20 from roots, where maximum production was shown by genera *Bacillus* sp. (9 isolates). Quantitative estimation of siderophore production showed that isolate *Brevundimonas* sp. (K4) and

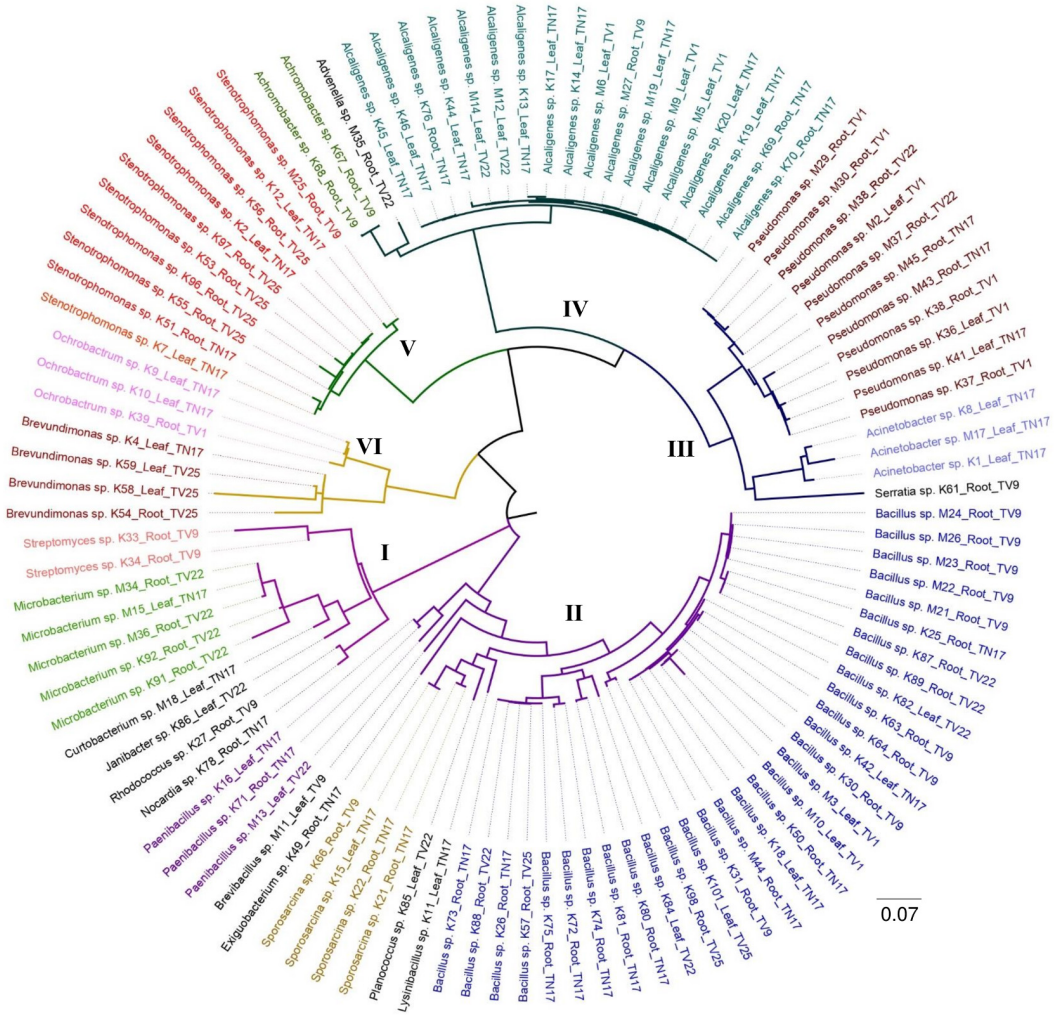


FIGURE 2 | The maximum likelihood phylogenetic tree of endophytic bacterial isolates obtained from five different tea clones based on 16S rRNA gene sequences; the scale bar represents the distance from the origin to the most distant taxa. Clades that are distinctly clustered are represented by six different groups.

Bacillus sp. (K87) produced the highest quantities of siderophore units (SU) of 83.22% followed by 81.2% SU by *Pseudomonas* sp. (M45) and 79.06% SU by *Stenotrophomonas* sp. (K96). A total of 17 isolates produced significantly higher quantities of siderophores above 40% siderophore units.

Out of 93 EnB isolates, 39 from leaf and 54 from roots utilized ACC as the sole nitrogen source and exhibit ACC deaminase activity. Genera *Bacillus* sp. (29 isolates) showed maximum ACC deaminase activity followed by *Alcaligenes* sp. (15 isolates) and *Stenotrophomonas* sp. (10 isolates). The isolates showing similar diameters as the control plate was considered to be weak or negative isolates and the isolates showing greater diameter than control plates were considered to be positive.

Hydrolytic Enzyme Production Assays

Qualitative analysis of 106 EnB isolates for hydrolysis of extracellular enzymes such as cellulase, protease, and amylase was performed. A total of 56 (52.83%) isolates hydrolyzed proteins by the formation of clear zones around colonies in SMA medium. For amylase screening, 20 (18.87%) isolates hydrolyzed starch by the production of amylase in starch agar medium by the formation of clear zones around bacterial isolates and cellulase enzyme production by 37 (34.90%) isolates were confirmed by the development of clear halo zones in the vicinity of colonies in M9-CMC medium (**Figure 3A**, **Table 3**, and **Supplementary Table 2**).

Concluding Evaluation of the *in vitro* Plant Growth Promotion Traits

A bonitur scale was generated similar to that described by El-Sayed et al. (2014) to select the best EnB isolates having high plant growth-promoting potential. In this scale PGP traits examined are scored. The maximum possible score in the traits examined here is 13 points: 3 points each for IAA production and phosphate solubilization; 1 point each for nitrogen fixation, ammonia, siderophore, and ACC deaminase production, and production

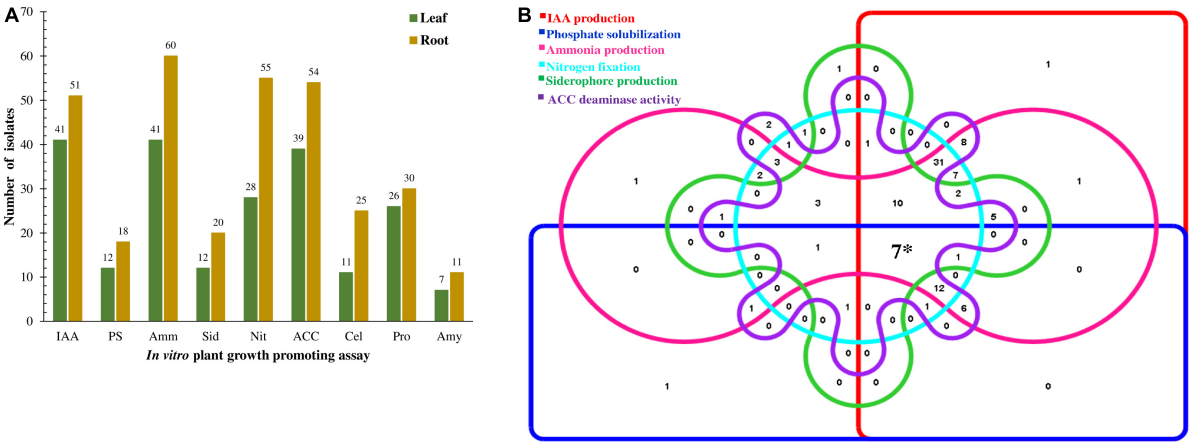


FIGURE 3 | Representation of plant growth-promoting traits showed by endophytic bacteria isolated from the tea clones. **(A)** Quantitative distribution of bacterial isolates according to PGP traits and isolation source and **(B)** Venn diagram illustrating the number of isolates showing PGP traits. The 106 isolates were allocated to 6 different PGP traits. (*7 showed PGP traits for all the attributes).

TABLE 3 | Endophytic bacterial isolates with their PGP traits and general ranking for their ability to function as plant growth promoters based on bonitur scale.

Isolate code	Organism name	Accession no.	Plant growth promoting traits						Hydrolytic enzymes			Total assessment points (13)	Rank
			IAA[a]	PS[b]	Amm[c]	Sid[d]	Nit[e]	ACC[f]	Cel[g]	Pro[h]	Amy[i]		
K96	*Stenotrophomonas* sp.	MW905624	3	3	1	1	1	1	1	1	1	13	1st
M45	*Pseudomonas* sp.	MZ008002	2	2	1	1	1	1	1	1	1	11	2nd
K55	*Stenotrophomonas* sp.	MW898448	2	1	1	0	1	1	1	1	1	9	3rd
K61	*Serratia* sp.	MN493913.1	1	3	1	0	1	1	1	1	0	9	4th
K98	*Bacillus* sp.	MN577376.1	0	3	1	1	1	1	0	1	1	9	5th
K9	*Ochrobactrum* sp.	MN493877.1	1	0	1	1	1	1	1	1	1	8	6th
K51	*Stenotrophomonas* sp.	MN493906.1	1	0	1	1	1	1	1	1	1	8	7th
K71	*Paenibacillus* sp.	MN493921.1	1	2	1	1	1	1	0	1	0	8	8th
M2	*Pseudomonas* sp.	MW905608	1	1	1	1	1	1	1	1	0	8	9th
M3	*Bacillus sp.*	MN577378.1	1	3	1	0	1	1	0	1	0	8	10th

IAA[a], Indole Acetic Acid production (1 ≤ 50, 2 = 50–100, 3 ≥ 100 μg mL^{-1}); PS[b], Phosphate solubilization (1 ≤ 30, 2 = 30–60, 3 ≥ 60 μg mL^{-1}); Amm[c], Ammonia production (μmol mL^{-1}) (1 = positive); Sid[d], Siderophore production (%) (1 = positive); Nit[e], Nitrogen fixation (1 = positive); ACC[f], ACC deaminase activity (1 = positive); Cel[g], Cellulase activity (1 = positive); Pro[h], Protease activity (1 = positive); Amy[i], Amylase activity (1 = positive). 0 indicates no activity or growth.

of hydrolytic enzymes was given as the assessment values. The assessment revealed that isolate *Stenotrophomonas* sp. (K96) was the foremost isolate exhibiting the highest score of 13 points followed by *Pseudomonas* sp. (M45) exhibiting 11 points. Based on the bonitur scale the top 10 EnB isolates with the ranking for their ability to function as plant growth promoters are shown in **Table 3** and the ranking of all the isolates are shown in **Supplementary Table 2**.

Biofilm Formation Assay

Bacterial isolate M45 showed strong biofilm formation and isolate K96 showed moderate biofilm formation at 48 h, i.e., during the stationary growth phase. The same was observed when biofilm was developed on a glass slide and observed under a compound microscope at 24 and 48 h after staining with crystal violet (**Figures 4A–D**). The attachment of bacterial cells increased significantly after 12 h (initial period of lag phase) as assessed using crystal violet (**Figure 4E**).

CLSM microscopy observations revealed the thickness of the biofilms in the z-plane as 9 μm for K96 and 10 μm M45, respectively. The biofilm growth revealed that the cells of K96 and M45 attached to the glass surface and colonized the whole surface (**Figure 5** and **Supplementary Figure 4**).

Cell Surface Hydrophobicity Assay

The bacterial CSH plays a crucial role in bacterial aggregation which sequentially promotes bacterial dispersion and survival in soil and aid in microbial adhesion to plant surfaces during biofilm formation. In this study, 38.29% CSH was shown by K96 and 27.77% CSH by M45.

Motility Assay

The bacterial motility was studied over time for bacterial isolates M45 and K96. Both the isolates were seen to exhibit twitching, swimming, and swarming motility. Isolates M45 and K96 isolates produced a flat, widely

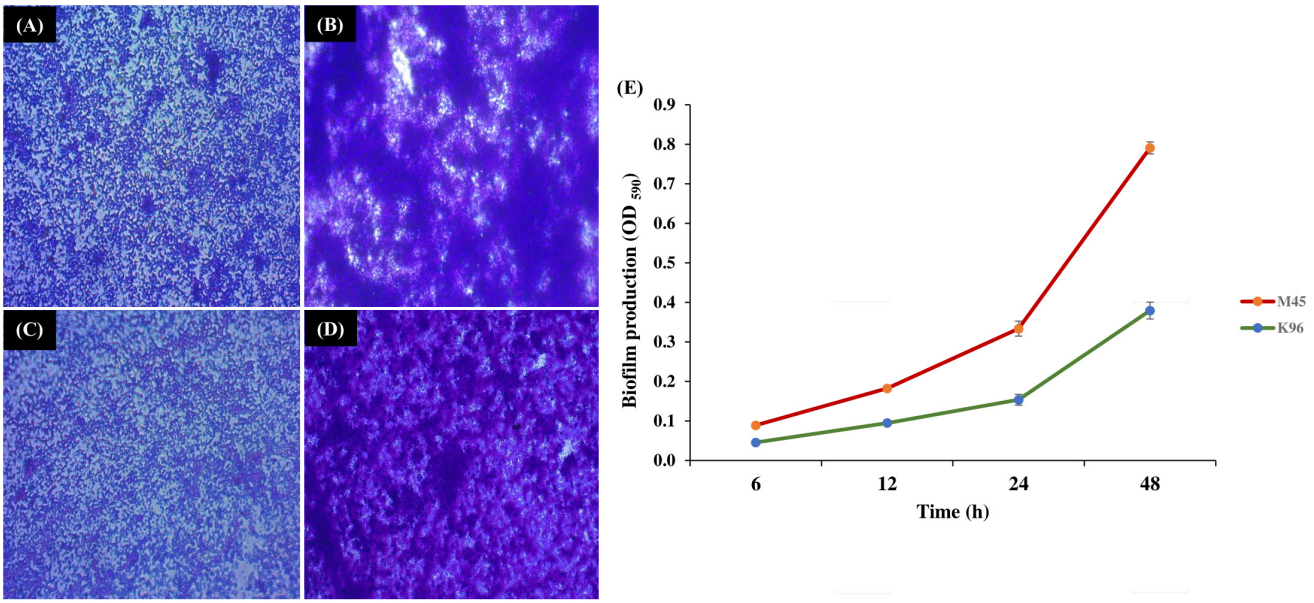


FIGURE 4 | Biofilm formation by potent bacterial isolates. Light microscopy images of biofilm formation by **(A,B)** M45 (*Pseudomonas* sp.), and **(C,D)** K96 (*Stenotrophomonas* sp.) at 24 and 48 h, respectively. The biofilms were stained by crystal violet; **(E)** Quantification of biofilm production for 48 h by M45 and K96 after elution of crystal violet stain by ethanol.

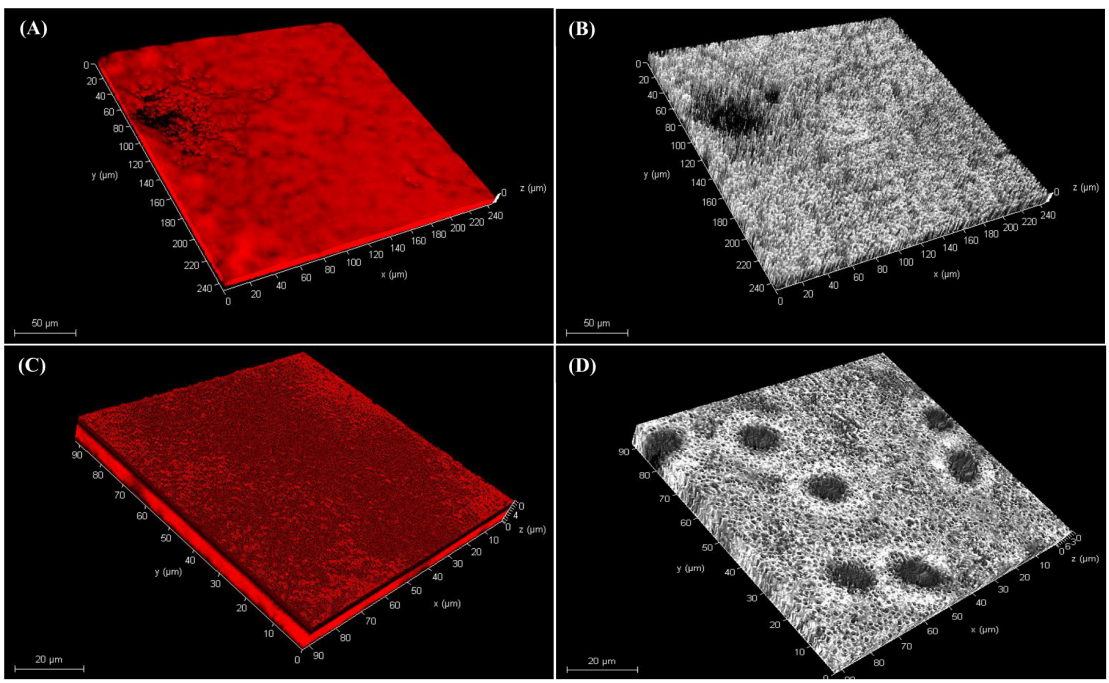


FIGURE 5 | Confocal laser scanning microscopy of biofilm. Three-dimensional (3D) views of **(A,B)** M45 and **(C,D)** K96 biofilms grown for 48 h. Bacteria were stained with acridine orange and observed by CLSM.

spread, irregularly shaped colony at 100X magnification in media-specific for twitching motility (**Figures 6A,B**). The isolates were seen to exhibit swarming motility with diameter 53 mm ± 1.2 and 45 mm ± 0.25 for K96 and M45, respectively, within a time period of 48 h (**Figures 6C–E**). Swimming assays revealed that M45 and K96 isolates are motile presenting concentric halos on 0.3% agar plates at 48 h (**Figures 6F–H**).

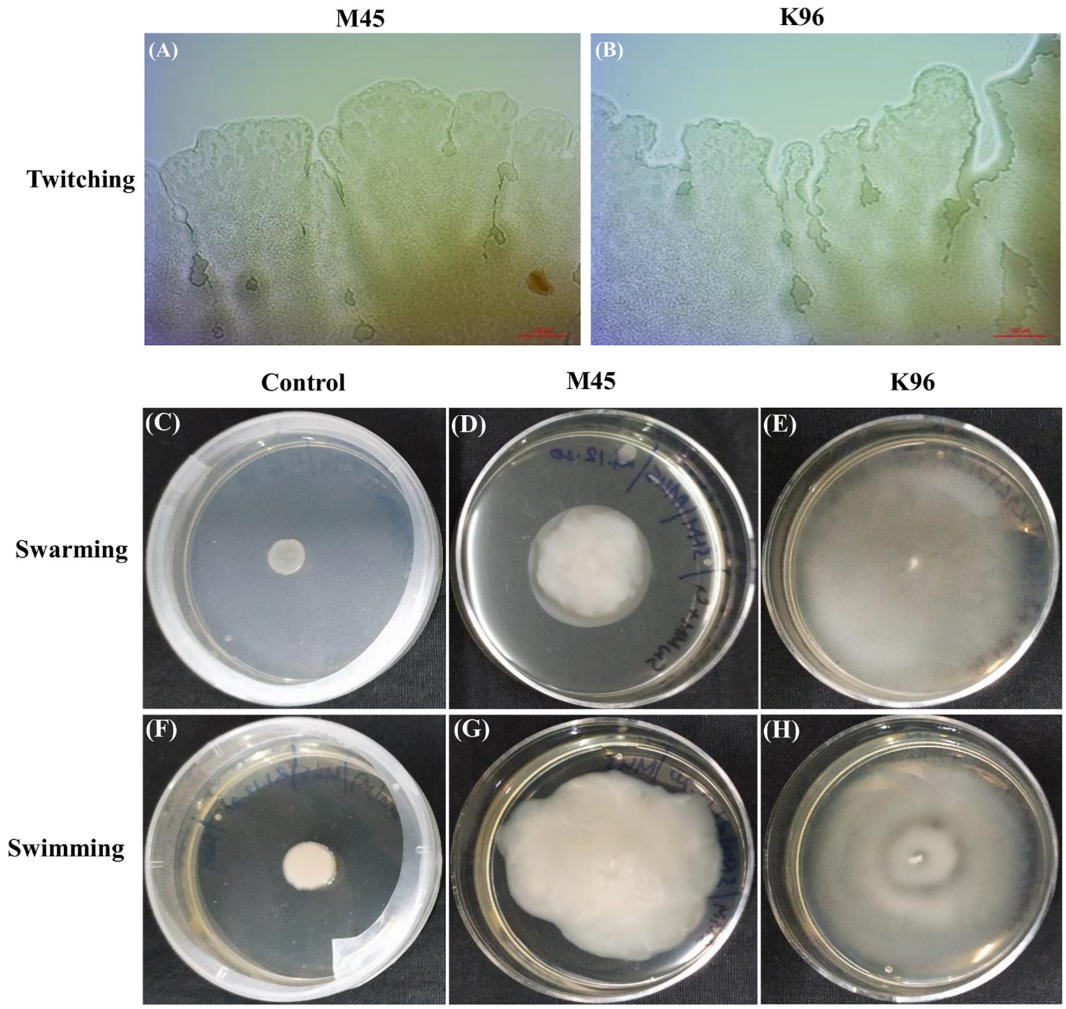


FIGURE 6 | Motility assays. **(A,B)** Light microscopy of colony edges of M45 and K96 in twitching motility. Both M45 and K96 isolates produced a flat, widely spread, irregularly shaped colony at 100X magnification. **(C–E)** Swarming motility [NA plate with 5 g glucose/L and 0.5% (w/v) agar] and **(F–H)** Swimming motility [LB with 0.3% (w/v)] of Control, M45 and K96 incubated at 28°C for 48 h.

Plant Growth Promotion Assay *in vivo* Using Selected Isolates

Based on bonitur score, isolates with the top two scores namely K96 and M45 were selected to observe their effective contribution to promote plant growth in the treated plant group compared to the control group *in vivo* experiments in nursery conditions (**Supplementary Figure 2**). All the treated plant groups showed a significant ($p < 0.05$) increase in different plant growth-promoting vegetative parameters in comparison to the control plant group (**Figure 7**) on inoculation with the potent EnB isolates. PCA resulted in two major principal components (PC) with a variance of 70.96% (PC1) and 15.21% (PC2) on reduction of the dimension of the various PGP parameters (**Figure 8**). The analysis revealed a major distinction between treatment and control groups throughout the PC1. Among the treatment groups viz. T1 and T3 the interrelationship among the vegetative parameters in clone TV26 and TV22 were reflected by PC2 and PC1, respectively, where a minor distinction was observed in the case of the T1 group for both the clones. Likewise, PC1 represented a higher degree of correlation of parameters dry weight root and shoot (DWR and DWS), fresh weight shoot and root (FWS and FWR), and chlorophyll b (Chlb) toward clone TV22 in T1 and T2 group. Likewise, parameters chlorophyll a (Chla), root length (RL), number of leaves (NL), and shoot length (SL) were correlated mostly toward clone TV26 in both the treatment groups.

The level of growth in vegetative parameters of treated plants compared to untreated plants was measured by fold change analysis. T3 showed considerable increase in the number of fold change compared to T1 and T2 in TV22 tea clone with an increase in shoot length by 4.99-fold, root length by 3.93-fold, fresh shoot weight by 2.96-fold, dry shoot weight by 2.38-fold, fresh root weight by 2.51 fold, and dry root weight by 1.64-fold. However, T1 showed a maximum increase in the number of leaves of TV22 clone by 3.59-fold. In the case of clone

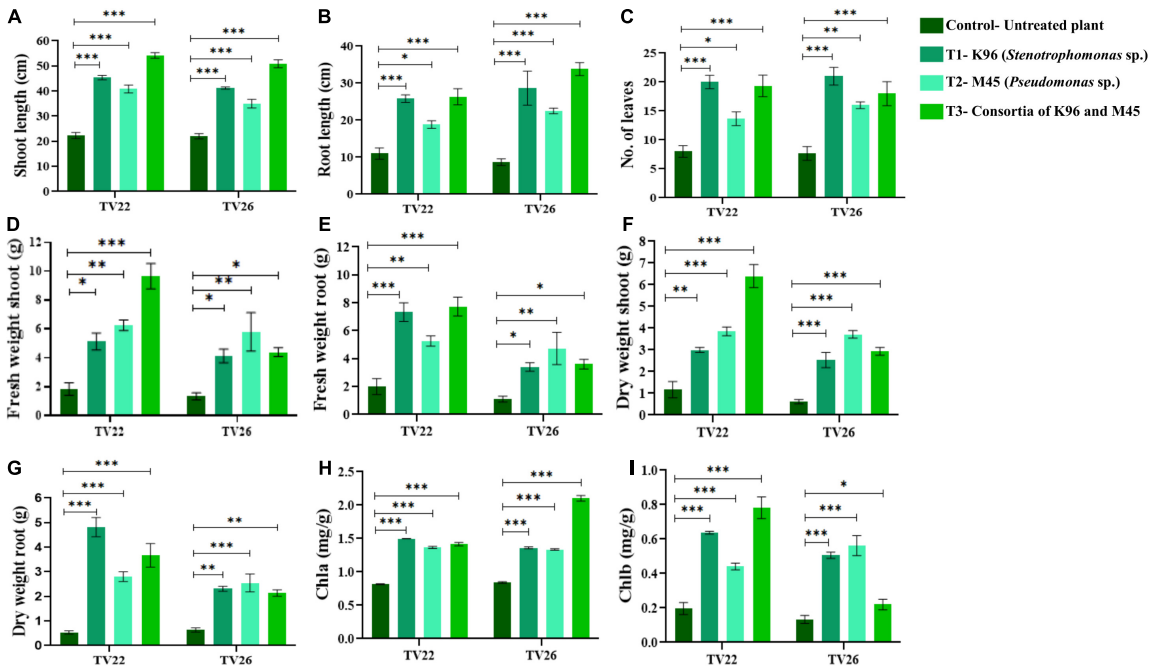


FIGURE 7 | Evaluation of PGP experiment to show the effect of treatments of endophytic bacterial inoculum in two tea clones TV22 and TV26 for **(A)** shoot length; **(B)** root length; **(C)** number of leaves **(D)** fresh weight shoot; **(E)** fresh weight root; **(F)** dry weight shoot; **(G)** dry weight root; **(H)** chlorophyll a; and **(I)** chlorophyll b. Error bar represents the standard error of means of replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

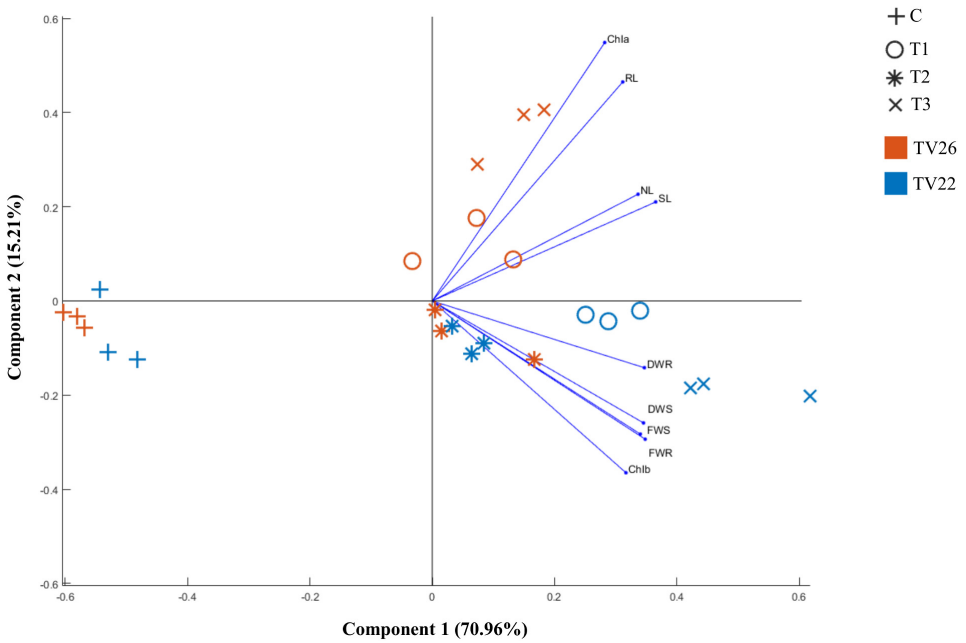


FIGURE 8 | Principle component analysis biplot based on correlation matrices of the PGP dataset of growth parameters and treatment groups of tea clones TV22 and TV26. C- Control, Untreated plant; T1-Treatment 1, K96 (*Stenotrophomonas* sp.); T2- Treatment 2, M45 (*Pseudomonas* sp.); T3- Treatment 3, Consortia of K96 and M45.

TV26, the degree of growth promotion was more significant when treated with T2 that showed a 2.16 fold increase for fresh shoot weight, 1.63-fold for dry weight shoot, 1.86-fold for fresh root weight, and 0.94 fold for dry root weight. On the other hand, T3 showed maximum shoot length and root length with an increase of 4.85 and 4.65, respectively, and T1

showed a maximum increase in the number of leaves by 3.73 fold (**Supplementary Figures 3A–F**).

DISCUSSION

Over the past few decades to prevent food shortage worldwide and escalate crop yield, extensive amounts of chemical fertilizers, pesticides, and herbicides have been used that resulted in environmental hazards and is one of the major areas of concern in tea and other agricultural production (Sharma and Singhvi, 2017; Xie et al., 2019). It has become important to seek a substitute to take over these chemicals. The beneficial bacteria that inhabit inside plants can improve plant growth under natural and challenging conditions (Glick, 2012; Santoyo et al., 2016). They are capable of improving plant nutrient uptake, amending soil health, and priming plant defense to develop quality clones of tea with higher yield potential and confer sustainability in agriculture (Jain and Kumar, 2017). Consequently, understanding the EnB community associated with tea to delineate their function in PGP has become important. To endeavor beneficial effects on plant growth, PGPB has to colonize the endosphere after colonization in the rhizosphere. Many hypotheses including motility, attachment, plant-polymer degradation, production of growth phytohormones, phosphate solubilization, siderophore production, and evasion of plant defenses have been described to be involved in growth promotion by PGPB (Glick, 2014; Liu et al., 2017).

Camellia sinensis known for its therapeutic aid in several diseases is being widely consumed worldwide. Tea plants harbor a broad range of beneficial EnB which can be used as bioinoculants in promoting safe and sustainable agriculture. With the possibility to isolate diverse culturable EnB, leaf, and roots of five different tea clones were selected as variation in the endophytic community depends on the plant. Most t of the EnB isolates was recovered from root tissues as compared to leaf tissues which are unanimous with several studies that indicated the dominance of EnB in root tissues (Ma et al., 2013; Kandel et al., 2017).

PCR amplification followed by 16S rRNA gene sequencing and phylogenetic analysis determined the molecular identity and genetic diversity of these isolates. Similarity search using NCBI GenBank and EZ Taxon identified all the isolates at genus level showing 91–100% identity with reference sequences. Also, 16S rRNA gene sequencing is regarded as a robust technique for the identification of bacteria at genus and species level which differentiates between closely related bacterial species (Johnson et al., 2019). EnB were grouped into three phyla which consisted of 22 different genera that were grouped into six clusters based on phylogenetic analysis. Proteobacteria (n = 53, 50%) was most dominant which is represented by *Acinetobacter* sp., *Stenotrophomonas* sp., *Brevundimonas* sp., *Pseudomonas* sp., *Ochrobactrum* sp., *Alcaligenes* sp., *Serratia* sp., *Achromobacter* sp., and *Advenella* sp. followed by Firmicutes (n = 42, 39.62%) including *Lysinibacillus* sp., *Sporosarcina* sp., *Paenibacillus* sp., *Bacillus* sp., *Exiguobacterium* sp., *Planococcus* sp., and *Brevibacillus* sp. and Actinobacteria (n = 11, 10.37%) which is represented by *Rhodococcus* sp., *Janibacter* sp., *Curtobacterium* sp., *Streptomyces* sp., *Nocardia* sp., and *Microbacterium* sp. which is similar to the findings as reported by Verma et al. (2015). *Bacillus* was found to be the dominant genus (n = 31, 29.24%) followed by *Alcaligenes* (n = 18, 17.0%), *Pseudomonas* (n = 11,10.37%), and *Stenotrophomonas* (n = 10, 9.43%). *Bacillus* sp. was reported previously as dominant genera as an endophyte in different crops. In Pisarska and Pietr, (2015) and Passari et al. (2016) *Bacillus* was reported as the dominant genus among the EnB isolated from maize and *Clerodendrum colebrookianum* Walp which agrees with our findings.

For the judicious selection of potential indigenous PGP strains, the isolates were evaluated for a range of PGP traits by direct (include phosphate solubilization, production of IAA, ACC deaminase, and nitrogen fixation) and indirect (include siderophore, ammonia, and extracellular enzyme production) methods. All the isolates showed multifarious PGP traits which may be beneficial for the growth of the tea plant.

Phosphorus is an essential nutrient for plant growth which is required for numerous key plant functions inclusive of photosynthesis, nutrient uptake, and energy transfer, and so on. But it is unavailable to plants because most of the phosphorus exists in the soil in an insoluble form as organic phosphate or as mineral salts emerging as a major limiting factor in agricultural systems (Miller et al., 2010; Oteino et al., 2015). Thus, to determine the ability of EnB isolates to solubilize tri-calcium phosphate present in PKA and NBRIP medium quantitative and qualitative analysis was performed. A significant decrease in pH of culture medium due to production of organic acids confirmed phosphate solubilization by 30 of the isolates. As reported (Oteino et al., 2015) the acidity and concentration of soluble phosphate in the medium are inversely proportional. The phosphate solubilization values in this study fall in the range 2.79 ± 0.67- 81.42 ± 5.73 mg L^{-1} which is in agreement with the typical findings in PSB range from 10 to 800 mg L^{-1} as reported (Rodríguez and Fraga, 1999; Passari et al., 2016).

Auxin IAA production by beneficial bacteria is very essential for plant growth-promoting microorganisms as it is one of the most important phytohormones regulating growth and several developmental processes in plants (Egamberdieva et al., 2017). In the present study, noticeably more IAA was produced by isolates in presence of precursor L-tryptophan than previously reported by Hassan (2017) ranging from 4.1 to 23.4 μg mL^{-1} in *Teucrium polium* L. Similarly, Woźniak et al. (2019) reported IAA production by bacteria isolated from wheat, maize, rye, broad bean, burdock, and horsetail in the range 0.13–22.51 μg mL^{-1}. Since IAA production is governed by multiple pathways, the amount of IAA produced may vary among isolates (Moliszewska and Nabrdalik, 2020). Studies revealed that most of the IAA producing EnB belong to phyla Proteobacteria, Firmicutes, and Actinobacteria (Nabrdalik et al., 2018; Hazarika and Thakur, 2020) which is in agreement with this study.

Production of ammonia by EnB potentially serves as a source for fixation of atmospheric nitrogen which stimulates

plant growth and contributes to plant defense. Almost all the isolates produced ammonia among which *Bacillus* sp. produced a higher amount of ammonia which is similar with the report (Brígido et al., 2019; Kang et al., 2020) that can escalate plant biomass by the accumulation of nitrogen.

Siderophore production by plant-associated bacteria assists in plant growth by scavenging iron from the environment because of the low iron bioavailability (Ahmed and Holmström, 2014). The EnB isolates under iron limiting conditions produced a high amount of siderophore in this study. Siderophore production by *Brevundimonas* sp. has rarely been reported earlier which has been confirmed in our study, although it has been isolated from various sources such as rhizospheric soil of sugarcane, potato, and maize (Montañez et al., 2012; Kumar and Gera, 2014; Naqqash et al., 2020). Previous studies reported *Bacillus* sp. (Ferreira et al., 2019), *Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp., and *Ochrobactrum* sp. (Yu et al., 2018; Ferreira et al., 2019) as producers of siderophore which is similar with our study.

Drought is one of the most common stresses induced in tea plants (Lin, 2011). Thus, one of the vital roles of beneficial bacteria associated with tea plants is to alleviate stress and promote growth. One such mechanism exploited by EnB is lowering plant ACC levels, a precursor of ethylene by the production of ACC deaminase (Sessitsch and Nowak, 2008). In the present study, 87.73% of isolates produced ACC deaminase which was much higher than reported by Borah et al. (2019). Production of other extracellular enzymes like cellulase, protease, and amylase plays a significant role in the decay of organic matter and plant growth promotion. In our study, *Bacillus* sp. exhibited the highest number of exoenzyme activities among tested enzymes, which is in accordance with the results reported (El-Deeb et al., 2012). Among other isolates *Pseudomonas* sp., *Stenotrophomonas* sp., *Ochrobactrum* sp., and *Alcaligenes* sp. produced all the enzymes tested. Among isolates, *Serratia* sp. and *Acinetobacter* sp. had cellulase and protease activity; *Streptomyces* sp. and *Brevundimonas* sp. displayed cellulase and amylase activity. Bacterial strains *Brevibacillus* sp., *Lysinibacillus* sp., and *Microbacterium* sp. showed only cellulase activity; *Achromobacter* sp. showed only amylase activity; and *Planococcus* sp., *Janibacter* sp., and *Paenibacillus* sp. produced only protease. This result is in accordance with the study of El-Deeb et al. (2012); Liu et al. (2016), and Passari et al. (2016). Many plant beneficial bacteria produces extracellular enzymes through indirect mechanism for PGP (Gupta et al., 2015). The production of hydrolytic enzymes by EnB emerges as important for colonization of plant roots and movement of bacteria into the interior of plants rendering help in plant growth (Al-Mallah et al., 1987; Quadt-Hallmann et al., 1997; Choubane et al., 2016).

As described by Chen et al. (2013) the ability of bacteria to form a stable biofilm for attachment and persistence on the plant root has been proven to be highly advantageous for plant growth promotion. Colonization of bacteria is crucial for plant-PGPB interactions to aid in plant growth. Thus, for biofilm formation and competitive colonization in the plant-root system bacterial motility plays an important role (Harshey, 2003; Danhorn and Fuqua, 2007). Motility and biofilm formation exhibited by the tea-associated bacteria M45 and K96 denotes their potentiality as PGPB and may colonize and establish on plant root system with ease, aiding in plant growth promotion.

Several reports have shown endophytic bacteria can boost plant biomass in different plants rendering multifarious advantages to host plants (Dutta and Thakur, 2017; Finkel et al., 2017). The PGP experiment was performed in an established natural condition using non-sterile soil. The soil microflora associated with tea is diverse and complex which portrays a substantial challenge in tea cultivation. To assess the efficacy of the indigenous bacterial inoculum and to constitute a compatible interaction with the host the treated tea plants were compared with untreated tea plants in the same condition. Multifarious experiments on PGP traits and bonitur scale assessment ameliorated the selection of potent isolates which augmented all growth parameters tested in the nursery experiment. The most promising effect on the tea plants tested were displayed by consortia of the isolates than the individual inoculum. The multivariate PCA analysis showed accord with the treatment and vegetative growth parameters. The efficiency of the selected isolates was further confirmed as the treated plants deviated significantly from the untreated plants. This was validated by two-way ANOVA analysis. Therefore, the bacteria inoculums utilized in this nursery experiment were assumed to show synergistic PGP effect, colonization, and competence with the other native microbial population in the treated tea clones.

In vivo, the growth of the tea plants is not directly related to the growth-promoting traits such as IAA production, phosphate solubilization, ACC deaminase production, and so on. The specificity of the bacteria's interaction with the plants influenced the growth-promotion. The efficiency of the PGP strains was possibly dependent on the genotype of the host plant or the bacterial genotype. Thus, the genetic factors of both partners appear to influence plant colonization and growth promotion by the EnB (Afzal et al., 2019).

CONCLUSION

Endophytic bacteria residing within the host plant are potential natural bioresources aiding in plant growth promotion. The exploration of endophytes indigenous to tea plants is limited as compared to endophytes from various agricultural systems. This current study helped in establishing the benefits of endophytes associated with tea clones endowed with plant growth-promoting traits, motility, and biofilm-forming ability and suggesting its applicability in tea gardens and other agricultural crops as biofertilizer or as an alternative for chemical fertilizers and pesticides, thereby promoting sustainability. However, further research is needed to establish the colonization of the endophytes within the host and study the correlation with the native soil microflora.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

SH conceptualized, planned, designed, and conducted the laboratory and field experiments, acquired and analyzed the data, interpreted the results, and wrote and reviewed the original draft. KS contributed to data analysis. AB helped in an experimental setup. DT conceptualized and supervised the research work and reviewed and edited the original draft. All authors have read and approved the final manuscript.

FUNDING

This work was partially supported by DST, Government of India in-house R&D core budget funded project for the year 2021–2022.

ACKNOWLEDGMENTS

We thank the Director, Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam, India, for providing facilities for this work; Kopati Tea estate, Assam, India, and Meg Tea estate, Umsning, Meghalaya, for approving and assisting in sample collection; Bioinformatics Facility (BIF), IASST for providing facilities and support for data analysis; and Central Instrumentation Facility (CIF), IASST for providing infrastructure facilities to carry out part of the work. We would like to thank Somarani Dash, IASST for great help in imaging using CLSM. We would like to thank the Department of Science and Technology (DST), Government of India for the fellowship to SH.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.738058/full#supplementary-material

REFERENCES

Afzal, I., Shinwari, Z. K., Sikandar, S., and Shahzad, S. (2019). Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. *Microbiol. Res.* 221, 36–49.

Ahmed, E., and Holmström, S. J. M. (2014). Siderophores in environmental research : roles and applications. *Microb. Biotechnol.* 7, 196–208. doi: 10.1111/1751-7915.12117

Alexander, D. B., and Zuberer, D. A. (1991). Use of chrome Azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol. Fertil. Soils* 12, 39–45. doi: 10.1007/BF00369386

Al-Mallah, M. K., Davey, M. R., and Cocking, E. C. (1987). Enzymatic treatment of clover root hairs removes a barrier to rhizobium-host specificity. *Nat. Biotechnol.* 5, 1319–1322. doi: 10.1038/nbt1287-1319

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. polyphenoloxidase in beta vulgaris. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1

Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A., and Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microb. Ecol.* 51, 215–229. doi: 10.1016/j.femsec.2004.08.006

Borah, A., Das, R., Mazumdar, R., and Thakur, D. (2019). Culturable endophytic bacteria of *Camellia* species endowed with plant growth promoting characteristics. *J. Appl. Microbiol.* 127, 825–844. doi: 10.1111/jam.14356

Brígido, C., Singh, S., Menéndez, E., Tavares, M. J., Glick, B. R., Félix, M. D. R., et al. (2019). Diversity and functionality of culturable endophytic bacterial communities in chickpea plants. *Plants* 8, 1–21. doi: 10.3390/plants8020042

Capella-Gutiérrez, S., Silla-Martínez, J. M., and Gabaldón, T. (2009). TrimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25, 1972–1973. doi: 10.1093/bioinformatics/btp348

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., et al. (2013). Biocontrol of tomato wilt disease by bacillus subtilis isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ. Microbiol.* 15, 848–864. doi: 10.1111/j.1462-2920.2012.02860.x

Choubane, S., Cheba, B. A., and Benourrad, A. (2016). Screening and phenotypic diversity of amylase producing rhizospheric bacteria from some North African plants. *Proc. Technol.* 22, 1197–1204. doi: 10.1016/j.protcy.2016.01.168

Compant, C., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb. Ecol.* 62, 188–197. doi: 10.1007/s00248-011-9883-y

Compant, S., Duffy, B., Nowak, J., Clement, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases : principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Cooper, R., Morré, D. J., and Morré, D. M. (2005). Medicinal benefits of green tea: part ii. review of anticancer properties. *J. Altern. Complement. Med.* 11, 639–652. doi: 10.1089/acm.2005.11.639

Danhorn, T., and Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* 61, 401–422. doi: 10.1146/annurev.micro.61.080706.093316

Das, S. C., Das, S., and Hazarika, M. (2012). "Breeding of the tea plant (*Camellia sinensis*) in India," in *Global Tea Breeding*, eds L. Chen, Z. Apostolides, and Z. M. Chen (Hangzhou: Zhejiang University Press), 69–124. doi: 10.1007/978-3-642-31878-8_3

Directorate of Food Processing Meghalaya (n.d.). *Meg Tea*. Available online at: dofpmeghalaya.org/meg-tea/ (accessed June 26, 2021).

Dudeja, S. S., Giri, R., Saini, R., Suneja-Madan, P., and Kothe, E. (2012). Interaction of endophytic microbes with legumes. *J. Basic Microbiol.* 52, 248–260. doi: 10.1002/jobm.201100063

Dutta, J., Handique, P. J., and Thakur, D. (2015). Assessment of culturable tea rhizobacteria isolated from tea estates of Assam, India for growth promotion in commercial tea cultivars. *Front. Microbiol.* 6:1252. doi: 10.3389/fmicb.2015.01252

Dutta, J., and Thakur, D. (2017). Evaluation of multifarious plant growth promoting traits, antagonistic potential and phylogenetic affiliation of rhizobacteria associated with commercial tea plants grown in Darjeeling, India. *PLoS One* 12:e0182302. doi: 10.1371/journal.pone.0182302

Dworkin, M., and Foster, J. W. (1958). Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75, 592–603. doi: 10.1128/jb.75.5.592-603.1958

Egamberdieva, D., Stephan, J., Wirth, S. J., Alqarawi, A. A., Abd-Allah, E. F., and Hashem, A. (2017). Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Front. Microbiol.* 8:2104. doi: 10.3389/fmicb.2017.02104

El-Deeb, B., Bazaid, S., Gherbawy, Y., and Elhariry, H. (2012). Characterization of endophytic bacteria associated with rose plant (*Rosa damascena trigintipeta*) during flowering stage and their plant growth promoting traits. *J. Plant Interact.* 7, 248–253. doi: 10.1080/17429145.2011.637161

El-Sayed, W. S., Akhkha, A., El-Naggar, M. Y., and Elbadry, M. (2014). In vitro antagonistic activity, plant growth promoting traits and phylogenetic affiliation

of rhizobacteria associated with wild plants grown in arid soil. *Front. Microbiol.* 5:651. doi: 10.3389/fmicb.2014.00651

Emami, S., Alikhani, H. A., Pourbabaei, A. A., Etesami, H., Sarmadian, F., and Motessharezadeh, B. (2019). Effect of rhizospheric and endophytic bacteria with multiple plant growth promoting traits on wheat growth. *Environ. Sci. Pollut. Res.* 26, 19804–19813. doi: 10.1007/s11356-019-05284-x

Etminani, F., and Harighi, B. (2018). Isolation and identification of endophytic bacteria with plant growth promoting activity and biocontrol potential from wild pistachio trees. *Plant Pathol. J.* 34, 208–217. doi: 10.5423/PPJ.OA.07.2017.0158

Ferreira, C. M. H., Vilas-Boas, Â., Sousa, C. A., Soares, H. M. V. M., and Soares, E. V. (2019). Comparison of five bacterial strains producing siderophores with ability to chelate iron under alkaline conditions. *AMB Express* 9:78. doi: 10.1186/s13568-019-0796-3

Finkel, O. M., Castrillo, G., Paredes, S. H., González, I. S., and Dangl, J. (2017). Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* 38, 155–163. doi: 10.1016/j.pbi.2017.04.018

Fiske, C. H., and Subbarow, Y. J. (1925). The colorimetric determination of phosphorus. *Biol. Chem.* 66, 375–400. doi: 10.1007/s13398-014-0173-7.2

Frei, B., and Higdon, J. V. (2003). Proceedings of the third international scientific symposium on tea and human health : role of flavonoids in the diet antioxidant activity of tea polyphenols in vivo : evidence from animal studies. *J. Nutr.* 133, 3275S–3284S.

Fujita, M., Kusajima, M., Okumura, Y., Nakajima, M., Minamisawa, K., and Hideo Nakashita, H. (2017). Effects of colonization of a bacterial endophyte, *Azospirillum* sp. B510, on disease resistance in tomato. *Biosci. Biotechnol. Biochem.* 81, 1657–1662. doi: 10.1080/09168451.2017.1329621

Glick, B. R. (2012). Plant growth-promoting bacteria : mechanisms and applications. *Scientifica* 2012:963401. doi: 10.6064/2012/963401

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Glick, B. R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., and McConkey, B. (2007). Promotion of plant growth by bacterial ACC deaminase. *Crit. Rev. Plant Sci.* 26, 227–242. doi: 10.1080/07352680701572966

Gordon, S. A., and Weber, R. P. (1951). Colorimetric estimation of indoleacetic Acid. *Plant Physiol.* 26, 192–195. doi: 10.1104/pp.26.1.192

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., and Singh, V. (2015). Microbial and biochemical technology plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J. Microb. Biochem. Technol.* 7, 96–102. doi: 10.4172/1948-5948.1000188

Gupta, K., Singh, S. P., Manhar, A. K., Saikia, D., Namsa, N. D., Konwar, B. K., et al. (2019). Inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm and virulence by active fraction of *Syzygium cumini* (L.) skeels leaf extract: in-vitro and in silico studies. *Indian J. Microbiol.* 59, 13–21. doi: 10.1007/s12088-018-0770-9

Hallmann, J., and Berg, G. (2007). "Spectrum and population dynamics of bacterial root endophytes," in *Microbial Root Endophytes*, eds B. J. E. Schulz, C. J. C. Boyle, and T. N. Sieber (Heidelberg: Springer), 15–31.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43, 895–914. doi: 10.1139/m97-131

Hamilton-Miller, J. M. (1995). Antimicrobial properties of tea. *Antimicrob. Agents Ch.* 39, 2375–2377. doi: 10.1128/aac.39.11.2375

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu. Rev. Microbiol.* 57, 249–273. doi: 10.1146/annurev.micro.57.030502.091014

Hassan, S. E. (2017). Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium* L. *J. Adv. Res.* 8, 687–695. doi: 10.1016/j.jare.2017.09.001

Hayat, K., Iqbal, H., Malik, U., Bilal, U., and Mushtaq, S. (2015). Tea and its consumption: benefits and risks. *Crit. Rev. Food Sci. Nutr.* 55, 939–954. doi: 10.1080/10408398.2012.678949

Hazarika, S. N., and Thakur, D. (2020). "Actinobacteria," in *Beneficial Microbes in Agro-Ecology: Bacteria and Fungi*, eds N. Amaresan, M. Senthil Kumar, K. Annapurna, K. Kumar, and A. Sankaranarayanan (Cambridge, MA: Academic Press), 443–476.

Henrichsen, J. (1983). Twitching motility. *Annu. Rev. Microbiol.* 37, 81–93. doi: 10.1146/annurev.mi.37.100183.000501

Hiscox, J. D., and Israelstam, G. F. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.* 57, 1332–1334. doi: 10.1139/b79-163

Hsu, S., Bollag, W. B., Lewis, J., Huang, Q., Singh, B., Sharawy, M., et al. (2003). Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J. Pharmacol. Exp. Ther.* 306, 29–34. doi: 10.1124/jpet.103.049734

Huerta-Cepas, J., Serra, F., and Bork, P. (2016). ETE 3: reconstruction, analysis, and visualization of phylogenomic data. *Mol. Biol. Evol.* 33, 1635–1638. doi: 10.1093/molbev/msw046

Hurek, T., and Reinhold-Hurek, B. (2003). *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. *J. Biotechnol.* 106, 169–178. doi: 10.1016/j.jbiotec.2003.07.010

Iverson, W. G., and Millis, N. F. (1974). a method for the detection of starch hydrolysis by bacteria. *J. App. Bacteriol.* 37, 443–446. doi: 10.1111/j.1365-2672.1974.tb00460.x

Jain, P., and Kumar, P. R. (2017). "Potential role of endophytes in sustainable agriculture-recent developments and future prospects," in *Endophytes: Biology and Biotechnology. Sustainable Development and Biodiversity*, ed. D. K. Maheshwari (Cham: Springer), 15.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., et al. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat. Commun.* 10:5029. doi: 10.1038/s41467-019-13036-1

Kandel, S., Joubert, P., and Doty, S. (2017). Bacterial endophyte colonization and distribution within plants. *Microorganisms* 5:77. doi: 10.3390/microorganisms5040077

Kang, S. M., Bilal, S., Shahzad, R., Kim, Y. N., Park, C. W., Lee, K. E., et al. (2020). Effect of ammonia and indole-3-acetic acid producing endophytic *Klebsiella pneumoniae* YNA12 as a bio-herbicide for weed inhibition: special reference with evening primroses. *Plants* 9:761. doi: 10.3390/plants9060761

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Khan, A. L., Waqas, M., Kang, S. M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., et al. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. *J. Microbiol.* 52, 689–695. doi: 10.1007/s12275-014-4002-7

Kifle, M. H., and Laing, M. D. (2016). Isolation and screening of bacteria for their diazotrophic potential and their influence on growth promotion of maize seedlings in greenhouses. *Front. Plant Sci.* 6:1225. doi: 10.3389/fpls.2015.01225

Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., et al. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* 113, 71–88. doi: 10.1016/s0002-9343(01)00995-0

Kumar, V., and Gera, R. (2014). Isolation of a multi-trait plant growth promoting *Brevundimonas* sp. and its effect on the growth of Bt-Cotton. *3 Biotech.* 4, 97–101. doi: 10.1007/s13205-013-0126-4

Lin, B. B. (2011). Resilience in agriculture through crop diversification: adaptive management for environmental change. *BioScience* 61, 183–193. doi: 10.1525/bio.2011.61.3.4

Liu, F. P., Liu, H. Q., Zhou, H. L., Dong, Z. G., Bai, X. H., Bai, P., et al. (2014). Isolation and characterization of phosphate-solubilizing bacteria from betel nut (*Areca catechu*) and their effects on plant growth and phosphorus mobilization in tropical soils. *Biol. Fertil. Soils* 50, 927–937. doi: 10.1007/s00374-014-0913-z

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M. J., et al. (2017). Inner plant values : diversity, colonization and benefits from endophytic bacteria. *Front. Microbiol.* 8:2552. doi: 10.3389/fmicb.2017.02552

Liu, Y. H., Guo, J. W., Salam, N., Li, L., Zhang, Y. G., Han, J., et al. (2016). Culturable endophytic bacteria associated with medicinal plant *Ferula songorica*: molecular phylogeny, distribution and screening for industrially important traits. *3 Biotech.* 6:209. doi: 10.1007/s13205-016-0522-7

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Ma, B., Lv, X., Warren, A., and Gong, J. (2013). Shifts in diversity and community structure of endophytic bacteria and archaea across root, stem and leaf tissues in the common reed, *Phragmites australis*, along a salinity gradient in a marine tidal wetland of northern China. *Anton. Leeuw Int J Gen. Mol. Microbiol.* 104, 759–768. doi: 10.1007/s10482-013-9984-3

Maeda-Yamamoto, M., Ema, K., and Shibuichi, I. (2007). In vitro and in vivo anti-allergic effects of "benifuuki" green tea containing O-methylated catechin and ginger extract enhancement. *Cytotechnology* 55, 135–142. doi: 10.1007/s10616-007-9112-1

Martin, B., Chadwick, W., Yi, T., Park, S.-S., Lu, D., Ni, B., et al. (2012). VENNTURE–a Novel Venn Diagram investigational tool for multiple pharmacological dataset analysis. *PLoS One* 7:e36911. doi: 10.1371/annotation/27f1021c-b6f2-4b90-98bc-fcacd2679185

Meddeb-mouelhi, F., Kelly, J., and Beauregard, M. (2014). Enzyme and microbial technology a comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. *Enzyme Microb. Technol.* 66, 16–19. doi: 10.1016/j.enzmictec.2014.07.004

Mendes, R., Pizzirani-kleiner, A. A., Araujo, W. L., and Raaijmakers, J. M. (2007). Diversity of cultivated endophytic bacteria from sugarcane : genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. *Appl. Environ. Microbiol.* 73, 7259–7267. doi: 10.1128/AEM.01222-07

Miller, S. H., Browne, P., Prigent-Combaret, C., Combes-Meynet, E., Morrissey, J. P., and O'Gara, F. (2010). Biochemical and genomic comparison of inorganic phosphate solubilization in *Pseudomonas* species. *Environ. Microbiol. Rep.* 2, 403–411. doi: 10.1111/j.1758-2229.2009.00105.x

Moliszewska, E. B., and Nabrdalik, M. (2020). "Application and biological impact of endophytic bacteria as IAA producers," in *Molecular Aspects of Plant Beneficial Microbes in Agriculture*, Vol. 438, eds V. Sharma, R. Salwan, and L. Tawfeeq (Cambridge, MA: Academic Press), 77–87.

Mondal, T. K., Bhattacharya, A., Laxmikurmaran, M., and Ahuja, P. S. (2004). Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell Tiss.Org. Cult.* 76, 195–254. doi: 10.1023/B:TICU.0000009254.87882.71

Montañez, A., Blanco, A. R., Barlocco, C., Beracochea, M., and Sicardi, M. (2012). Characterization of cultivable putative endophytic plant growth promoting bacteria associated with maize cultivars (*Zea mays* L.) and their inoculation effects in vitro. *Appl. Soil Ecol.* 58, 21–28. doi: 10.1016/j.apsoil.2012.02.009

Nabrdalik, M., Moliszewska, E., and Wierzba, S. (2018). Importance of endophytic strains pantoea agglomerans in the biological control of rhizoctonia solani. *Ecol. Chem. Eng. S* 25, 331–342. doi: 10.1515/eces-2018-0023

Naqqash, T., Imran, A., Hameed, S., Shahid, M., Majeed, A., Iqbal, J., et al. (2020). First report of diazotrophic *Brevundimonas* spp. as growth enhancer and root colonizer of potato. *Sci. Rep.* 10:12893. doi: 10.1038/s41598-020-69782-6

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., et al. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Front. Microbiol.* 6:745. doi: 10.3389/fmicb.2015.00745

O'Toole, G. A., and Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30, 295–304. doi: 10.1046/j.1365-2958.1998.01062.x

Passari, A. K., Mishra, V. K., Leo, V. V., Gupta, V. K., and Singh, B. P. (2016). Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. *Microbiol. Res.* 193, 57–73. doi: 10.1016/j.micres.2016.09.006

Passari, A. K., Mishra, V. K., Saikia, R., Gupta, V. K., and Singh, B. P. (2015). Isolation, abundance and phylogenetic affiliation of endophytic actinomycetes associated with medicinal plants and screening for their in vitro antimicrobial biosynthetic potential. *Front. Microbiol.* 6:273. doi: 10.3389/fmicb.2015.00273

Patel, P. K., Zhang, D., Borthakur, D., Hazarika, M., Boruah, P., Barooah, R., et al. (2019). Quality green tea (*Camellia sinensis* L.) clones marked through novel traits. *Beverages* 5:63. doi: 10.3390/beverages5040063

Patten, C. L., and Glick, B. R. (1996). Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* 42, 207–220. doi: 10.1139/m96-032

Payne, S. M. (1994). Detection, isolation, and characterization of siderophores. *Meth. Enzymol.* 235, 329–344. doi: 10.1016/0076-6879(94)35151-1

Pisarska, K., and Pietr, S. (2015). Biodiversity of dominant cultivable endophytic bacteria inhabiting tissyes of six different cultivar of Maize (*Zea mays* L. ssp *mays*). *Pol. J. Microbiol.* 64, 163–170.

Quadt-Hallmann, A., Benhamou, N., and Kloepper, J. W. (1997). Bacterial endophytes in cotton: mechanisms of entering the plant. *Can. J. Microbiol.* 43, 577–582. doi: 10.1139/m97-081

Rambaut, A. (2018). *FigTree v1.4.4. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh*. Available online at: http://tree.bio.ed.ac.uk/software/figtree/ (accessed May 12, 2021).

Rodríguez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* 17, 319–339. doi: 10.1016/S0734-9750(99)00014-2

Rosenberg, M. (2006). Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS Microbiol. Lett.* 262, 129–134. doi: 10.1111/j.1574-6968.2006.00291.x

Rosenblueth, M., and Martínez-romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Mol. Plant Microbe Interact.* 19, 827–837. doi: 10.1094/MPMI-19-0827

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278, 1–9. doi: 10.1111/j.1574-6968.2007.00918.x

Santoyo, G., Moreno-hagelsieb, G., Orozco-mosqueda, C., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008

Sarani, B. T. M. (2020). *State / Region Wise and Month Wise Tea Production Data for the Year 2020-21*. Available online at: www.teaboard.gov.in/pdf/Production_20202_21_Apr_Dec_and_2020_Jan_Dec_pdf7013.pdf (accessed June 26, 2021).

Schulz, B., Wanke, U., Draeger, S., and Aust, H. J. (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol. Res.* 97, 1447–1450. doi: 10.1016/S0953-7562(09)80215-3

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Sessitsch, A., and Nowak, J. (2008). Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN : from the rhizosphere to inflorescence tissues. *FEMS Microbiol. Ecol.* 63, 84–93. doi: 10.1111/j.1574-6941.2007.00410.x

Sessitsch, A., Reiter, B., and Berg, G. (2004). Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. *Can. J. Microbiol.* 50, 239–249. doi: 10.1139/w03-118

Sgroy, V., Cassán, F., Masciarelli, O., Del Papa, M. F., Lagares, A., and Luna, V. (2009). Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. *Appl. Microbiol. Biotechnol.* 85, 371–381. doi: 10.1007/s00253-009-2116-3

Sharangi, A. B. (2009). Medicinal and therapeutic potentialities of tea (*Camellia sinensis* L.) - A review. *Food Res. Int.* 42, 529–535. doi: 10.1016/j.foodres.2009.01.007

Sharma, N., and Singhvi, R. (2017). Effects of chemical fertilizers and pesticides on human health and environment: a review. *Int. J. Agr. Environ. Biotechnol.* 10:675. doi: 10.5958/2230-732x.2017.00083.3

Siddiqui, M. W., Sharangi, A. B., Singh, J. P., Thakur, P. K., Ayala-Zavala, J. F., Singh, A., et al. (2016). Antimicrobial properties of teas and their extracts in vitro. *Crit. Rev. Food Sci. Nutr.* 56, 1428–1439. doi: 10.1080/10408398.2013.769932

Singh, P., Kumar, V., and Agrawal, S. (2014). Evaluation of phytase producing bacteria for their plant growth promoting activities. *Int. J. Microbiol.* 2014:426483. doi: 10.1155/2014/426483

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Sturz, A. V., Christie, B. R., and Matheson, B. C. (1998). Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can. J. Microbiol.* 44, 162–167. doi: 10.1139/w97-146

Verma, P., Yadav, A. N., Khannam, K. S., Panjiar, N., Kumar, S., Saxena, A. K., et al. (2015). Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India. *Ann. Microbiol.* 65, 1885–1899. doi: 10.1007/s13213-014-1027-4

Win, P. M., Matsumura, E., and Fukuda, K. (2018). Diversity of tea endophytic fungi: cultivar- and tissue preferences. *Appl. Ecol. Environ. Res.* 16, 677–695. doi: 10.15666/aeer/1601_677695

Woźniak, M., Gałązka, A., Tyśkiewicz, R., and Jaroszuk-ściseł, J. (2019). Endophytic bacteria potentially promote plant growth by synthesizing different metabolites and their phenotypic/physiological profiles in the biolog gen iii microplate^{TM} test. *Int. J. Mol. Sci.* 20:5283. doi: 10.3390/ijms20215283

Xie, S., Feng, H., Yang, F., Zhao, Z., Hu, X., Wei, C., et al. (2019). Does dual reduction in chemical fertilizer and pesticides improve nutrient loss and tea yield and quality? A pilot study in a green tea garden in Shaoxing, Zhejiang Province, China. *Environ. Sci. Pollut. Res.* 26, 2464–2476. doi: 10.1007/s11356-018-3732-1

Xu, M., Sheng, J., Chen, L., Men, Y., Gan, L., Guo, S., et al. (2014). Bacterial community compositions of tomato (*Lycopersicum esculentum* Mill.) seeds and plant growth promoting activity of ACC deaminase producing *Bacillus subtilis* (HYT-12-1) on tomato seedlings. *World J. Microbiol. Biotechnol.* 30, 835–845. doi: 10.1007/s11274-013-1486-y

Yu, Z., Kang, X., Liu, H., Liu, Y., Li, Y., Yu, X., et al. (2018). Endophytes isolated from ginger rhizome exhibit growth promoting potential for *Zea mays*. *Arch. Agron. Soil Sci.* 64, 1302–1314. doi: 10.1080/03650340.2018.1430892

Yuen, S. H., and Pollard, A. G. (1954). Determination of nitrogen in agricultural materials by the nessler reagent. II.—micro-determinations in plant tissue and in soil extracts. *J. Sci. Food Agr.* 5, 364–369. doi: 10.1002/jsfa.2740050803

Zhang, Y. F., He, L. Y., Chen, Z. J., Wang, Q. Y., Qian, M., and Sheng, X. F. (2011). Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*. *Chemosphere* 83, 57–62. doi: 10.1016/j.chemosphere.2011.01.041

Zhu, B., Chen, M., Lin, L., Yang, L., Li, Y., and An, Q. (2012). Genome sequence of *Enterobacter* sp. strain SP1, an endophytic nitrogen-fixing bacterium isolated from sugarcane. *J. Bacteriol.* 194, 6963–6964. doi: 10.1128/JB.01933-12

Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczmarski, D., Higley, P., et al. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.* 68, 2198–2208. doi: 10.1128/AEM.68.5.2198-2208.2002

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 October 2021
doi: 10.3389/fmicb.2021.732989

Drought Sensitivity of Sugarcane Cultivars Shapes Rhizosphere Bacterial Community Patterns in Response to Water Stress

*Qi Liu[1,2,3], Sasa Xie[1,2,3], Xiaowen Zhao[1,2,3], Yue Liu[1,2,3], Yuanjun Xing[1,2,3], Jicao Dao[1,2,3], Beilei Wei[1,2,3], Yunchang Peng[1,2,3], Weixing Duan[4] and Ziting Wang[1,2,3]**

[1] Guangxi Key Laboratory of Sugarcane Biology, Nanning, China, [2] State Key Laboratory for Conservation & Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, China, [3] College of Agronomy, Guangxi University, Nanning, China, [4] Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China

OPEN ACCESS

Edited by:
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Reviewed by:
Humaira Yasmin, COMSATS University, Islamabad Campus, Pakistan
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***Correspondence:**
Ziting Wang
zitingwang@gxu.edu.cn

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: 29 June 2021
Accepted: 16 September 2021
Published: 21 October 2021

Citation:
Liu Q, Xie S, Zhao X, Liu Y, Xing Y, Dao J, Wei B, Peng Y, Duan W and Wang Z (2021) Drought Sensitivity of Sugarcane Cultivars Shapes Rhizosphere Bacterial Community Patterns in Response to Water Stress. Front. Microbiol. 12:732989. doi: 10.3389/fmicb.2021.732989

Rhizosphere bacteria, the main functional microorganisms inhabiting the roots of terrestrial plants, play important roles in regulating plant growth and environmental stress resistance. However, limited information is available regarding changes occurring within the structure of the root microbial community and the response mechanisms of host plants that improve adaptability to drought stress. In this study, we conducted an experiment on two sugarcane varieties with different drought tolerance levels under drought and control treatments and analyzed the rhizosphere bacterial communities using 16S rRNA high-throughput sequencing. Correlation analysis results clarified the influence of various factors on the rhizosphere bacterial community structure. Drought stress reduced the diversity of the bacterial community in the rhizosphere of sugarcane. Interestingly, the bacterial community of the drought-sensitive sugarcane cultivar GT39 changed more than that of the drought-tolerant cultivar ZZ9. In addition, ZZ9 had a high abundance of drought-resistant bacteria in the rhizosphere under optimal soil water conditions, whereas GT39 accumulated a large number of drought-resistant bacteria only under drought stress. GT39 mainly relied on Actinobacteria in its response to drought stress, and the abundance of this phylum was positively correlated with soil acid phosphatase and protease levels. In contrast, ZZ9 mainly relied on Bacilli in its response to drought stress, and the abundance of this class was positively correlated with only soil acid phosphatase levels. In conclusion, drought stress can significantly reduce the bacterial diversity and increase the abundance of drought-resistant bacteria in the sugarcane rhizosphere. The high abundance of drought-resistant bacteria in the rhizosphere of drought-tolerant cultivars under non-drought conditions is an important factor contributing to the high drought adaptability of these cultivars. Moreover, the core drought-resistant bacteria of the sugarcane rhizosphere and root exudates jointly affect the resistance of sugarcane to drought.

Keywords: drought stress, sugarcane, rhizosphere bacterial community, environmental factor, drought response

INTRODUCTION

Plants grow in dynamic environments to which they exhibit remarkable adaptations (Butt et al., 2019; Kundariya et al., 2020). However, adverse environmental changes can cause abiotic stresses, which not only affect the normal growth and development of plants but also threaten their survival (Gupta and Huang, 2014). Drought stress is one of the most common environmental stresses and is capable of reducing major crop yields by 50%–80% (Buchanan et al., 2015; Griffiths and Paul, 2017). In response to environmental stress, plants can form symbiotic relationships with microorganisms. Soil microorganisms are reportedly more sensitive than plants to changes in the soil environment (Waldrop and Firestone, 2006; Lee et al., 2019). Plant roots interact with many soil microbes to form a unique rhizosphere microbial community that participates in the responses to environmental conditions and allows plants to achieve optimal growth and development (Jordan et al., 2016; Meijuan et al., 2018). Although the mechanisms associated with plant responses to drought stress have been extensively studied in terms of morphology, physiology, and genetics, the effects of drought on soil microorganisms remain poorly understood (Zhao et al., 2016; Aimin et al., 2018; Li P. et al., 2019). Investigation of these effects could provide insights into the resistance of plants to environmental stress (Jurburg et al., 2017; Krause et al., 2018).

Plants employ various strategies to overcome drought stress, including a combination of stress avoidance and regulation of drought tolerance, depending on their genotypes (Chaves et al., 2002; Verma et al., 2020). For instance, plants can better resist drought by regulating stomatal closure and expressing drought-related genes (Cao et al., 2017; Koech et al., 2019). Plant roots, owing to their roles in nutrient and water absorption, are important organs for sensing and responding to soil water stress (Iquebal et al., 2019; Teramoto et al., 2020). The interaction between plants and the soil microorganisms that colonize the rhizosphere and root system is considered a key factor in the rapid adaptation of plants to soil environmental stress (Giehl and von Wirén, 2014; Pascale et al., 2020). Plant growth-promoting bacteria have been shown to enhance the water absorption capacity of plant roots under water shortage conditions (Sade et al., 2010; Khan et al., 2020). Specifically, drought stress can induce the growth of certain gram-negative bacteria and increase osmotic pressure, maintain turgor pressure, and protect the macromolecular structures of plant tissues (Welsh, 2000; Schimel et al., 2007). Yasmin et al., found that plant growth-promoting bacteria could reduce malondialdehyde content in plants under drought stress, promote the synthesis of proline and related hormones, and significantly improve plant drought resistance (Yasmin et al., 2020, 2021). However, the changes in the rhizosphere microbial community that occur under drought stress depend on the effects of the host plants and surrounding soil (Naylor and Coleman-Derr, 2018). Differences in plant varieties and genotypes lead to varying levels of drought resistance. For instance, drought-resistant plants have evolved different strategies for adapting to drought compared with water-sensitive plants (Hilker et al., 2016; Yao et al., 2018), and they can rapidly activate drought resistance mechanisms at the physiological and molecular levels in response to drought stress (Zhao et al., 2016; Yao et al., 2018). In addition, the health of host plants and the nutrient conditions in the soil surrounding the roots can alter the microbial composition of the rhizosphere by influencing root structure and exudates (Benitez et al., 2017; Wen et al., 2020). Further, the availability of soil nutrients, such as nitrogen, regulates the diversity of rhizosphere microorganisms (Howard et al., 1995; Treseder, 2008; Kavamura et al., 2013). However, quantifying the effects of various factors on rhizosphere microbes under drought stress is complex, and this requires further exploration.

Sugarcane is an important crop for bioenergy production. It contributes toward 80% of the raw materials used in the global sugar industry, and it is cultivated in more than 110 countries (Garsmeur et al., 2018; Hajar-Azhari et al., 2018). Sugarcane is highly sensitive to water deficit during vegetative growth. Specifically, extreme water shortage reduces the accumulation of sugarcane stalk biomass by up to 60% (Gentile et al., 2015; Ghannoum, 2016). Most sugarcane studies have focused on improving sugar yield and breeding abiotic stress-resistant cultivars (Siraree et al., 2017). However, a large knowledge gap remains regarding the stress resistance of sugarcane rhizosphere microbes, which has been explored in other plants (Zhao et al., 2020b). For example, the rhizosphere microorganisms of different core communities constructed from unique varieties of tomato, citrus, and chickpea plants functioned to improve their environmental adaptability (Demers et al., 2015; Navid et al., 2015; Jin et al., 2018). Similarly, genetically diverse rice species responded to drought stress by utilizing specific rhizosphere microbial communities (Andreo-Jimenez et al., 2019). We hypothesized that the diversity of the rhizosphere microbial community of different sugarcane varieties resulted from the long-term selection and shaping of the rhizosphere environment by the host sugarcane, which could help it adapt to adversity. Thus, certain sugarcane varieties could respond better to water shortage under drought stress.

The current study, therefore, sought to analyze the major changes in sugarcane rhizosphere microbial diversity under drought stress. We intended to provide insights into not only the general responses of soil microbes to environmental stress but also the sugarcane-specific rhizosphere bacterial community. To this end, we selected ZZ9, a sugarcane cultivar with strong drought resistance, and GT39, a drought-sensitive cultivar, to evaluate their microbial communities under drought and normal water conditions. The primary questions that were addressed in this study were as follows: (1) What changes in the sugarcane rhizosphere bacterial community structure are induced by drought stress? (2) What are the main effects of sugarcane cultivar drought tolerance on the rhizosphere bacterial community? (3) What is the response pathway of drought-resistant bacteria in the sugarcane rhizosphere under drought stress?

MATERIALS AND METHODS

Cultivar Selection and Field Experiment Design

This study was performed in the Sugarcane Variety R&D and Breeding Base, Fusui, China (between 107°310′ and 108°060′ E and 22°170′ and 22°570′ N; 83 m a.s.l.) of Guangxi University. Experiments were carried out in the summer of 2018. The experimental site is located in a subtropical monsoon climate, with long summers and short winters. The average annual temperature at the experimental site is 22°C. In this region, the annual sunshine time is approximately 2600 h, and the annual precipitation is 1050–1300 mm. The soil used in the experiments was collected from the top layer (0–20 cm) of a long-term sugarcane cultivation field with the following physicochemical characteristics: pH, 6.15; organic matter content, 19.47 g/kg; total nitrogen content, 100.5 g/kg; total phosphorus content, 22.4 g/kg; total potassium content, 7.11 g/kg; alkaline hydrolyzed nitrogen, 136 mg/kg; available phosphorus, 83 mg/kg; and available potassium, 77.1 mg/kg. The sugarcane varieties selected were Zhongzhe9 (ZZ9) and Guitang39 (GT39). GT39 is a high-yield species bred through sexual hybridization. Its female parent is Yuetang93/159, and its male parent is ROC22. GT39 is more sensitive to water and fertilizer conditions than its parents (Wang et al., 2013). The female parent of ZZ9 is ROC22, and the male parent is Yunzhe89-7. Under drought conditions, single index analysis and comprehensive membership function evaluation of stomatal characteristics, physiological changes, and molecular mechanisms showed that ZZ9 is more resistant to drought than its parents (Yin et al., 2020). Because the two cultivars exhibit a large difference in sensitivity to water, we used GT39 as a water-sensitive variety and ZZ9 as a drought-resistant (drought-tolerant) variety in these experiments.

The sugarcane was planted in flowerpots and cultivated in a glass greenhouse. The average temperature in the greenhouse was 22°C, consistent with the outside temperature, and the plants were only exposed to natural light. The upper diameter of the plastic pots was 35 cm, the lower diameter was 25 cm, and the height was 35 cm; three drainage holes, with a diameter of approximately 1 cm, were drilled in the bottom of each pot (**Supplementary Figure 1**). A total of 60 pots were used for planting, 30 for GT39 and 30 for ZZ9, each with an average of two to three sugarcane plants (**Supplementary Figure 2**). When the plants sprouted 2–3 leaves, 12 pots of each cultivar were selected for follow-up experiments; of these pots, six per cultivar were subjected to drought stress (Drought, D), and the remaining six were watered normally (Control, C). Soil water content was measured at a depth of 10–15 cm using a TDR-100 soil moisture meter (Spectrum Technologies, Inc., Aurora, IL, United States). Irrigation was based on the weekly water requirements of sugarcane, and regular watering was carried out in the early stage of planting and continued for the control plants. For drought treatments, drought stress was induced by stopping irrigation. In a previous study, the chlorophyll content of the sugarcane leaves decreased under soil water deficit, and this was more pronounced in varieties with low drought tolerance (Luo and Liang, 2005; Qin et al., 2017). Hence, in this study, chlorophyll content was used to indicate the level of drought stress.

Soil Sampling and Assessment of Physical and Chemical Properties

Soil samples were collected from the soil attached to the surface of the sugarcane roots; the soil tightly attached to the roots was regarded as rhizosphere soil. At the end of the drought stress period (day 31), two plants with uniform growth were selected from each group, and the root system was dug out with surrounding soil (surface area of approximately 20 cm^2 around the plant). Large soil lumps and stones were removed, loose soil was gently shaken off, and the rhizosphere soil attached to the root surface was collected with a brush. The collected soil was sieved through a 2 mm sieve. Three pots for each group were sampled for a total of 12 soil samples collected from the four treatment groups (GT39D, GT39C, ZZ9D, and ZZ9C; D represents drought, and C represents the control). Each soil sample was divided into two sub-samples. One was stored at −40°C and was used to measure the physical and chemical properties of the soil, and the other was stored at −80°C for the extraction of rhizosphere microbial DNA, which was done within 24 h (Zhao et al., 2020a).

The chemical properties of the soil were determined as previously described (Bao, 2000). Soil organic carbon (SOC) was determined by the potassium dichromate sulfuric acid oxidation method, total nitrogen (TN) was measured using the semi-micro Kelvin method, and available phosphorus (AP) was determined by molybdenum antimony colorimetry. Measurements were conducted on soil collected from a depth of approximately 10–15 cm. Leaf chlorophyll content was measured with a chlorophyll meter (SPAD-502 Plus; Spectrum Technologies, Aurora, IL, United States). Three leaves from three plants for each treatment group were selected for chlorophyll content determination. The water potential of the leaves was measured with a dew point water potential meter (WP4; Decagon Devices, Inc., Pullman, WA, United States) between 11:30 and 12:00 using the youngest fully expanded leaves. To determine leaf water potential, we selected five points from the tip to the base of each leaf, and the measurement was repeated on three different plants for each treatment group. The root-shoot ratio was calculated as the root dry weight divided by the stem dry weight. Soil enzyme activity was determined as previously described (Shao and Li, 2016). Soil catalase (S-CAT) activity was measured using a volumetric method, and soil acid phosphatase (S-ACP), soil urease (S-UE), and acid protease (S-ACPT) activities were measured using colorimetry. For biomass determination, each plant was divided into shoots (including all aerial components) and roots, and their biomasses were determined after drying at 80°C.

DNA Extraction, Bacterial 16S Gene Polymerase Chain Reaction Amplification, and Sequencing

Soil DNA was extracted using the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, United States) according to the manufacturer's instructions. Microbial DNA was extracted from a 1 g sample of fresh soil, and the extraction was

performed three times for each sample. The concentration and quality of DNA samples were measured with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The primer pair F515 (5′-GTG CCA GCM GCC GCG GTA A-3′) and R806 (5′-GGA CTA CHV GGG TWT CTA AT-3′), targeting the V3–V4 hypervariable regions of the bacterial 16S rRNA gene, was used for polymerase chain reaction (PCR) (Peiffer et al., 2013). The reverse primer contained a 12 bp error-correcting barcode unique to each sample. Primers were synthesized by Invitrogen (Carlsbad, CA, United States). Each reaction was conducted in a volume of 50 μL, containing 25 μL of 2 × Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 μL of each primer (10 M), and 3 μL of DNA (20 ng/μL) template, using an S1000 thermal cycler (Bio-Rad Laboratories, Inc., Foster City, CA, United States). The following cycling conditions were used: 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; with a final elongation step at 72°C for 10 min. The PCR products were sequenced by Magigene Technology (Guangzhou, China) on the Illumina HiSeq 2500 platform.

Quality filtering of the paired-end raw reads was performed under specific conditions to obtain high-quality, clean reads using Trimmomatic (V0.33[1]) as the quality-control process. Paired-end clean reads were merged using FLASH (V1.2.11[2]) based on the overlap between paired-end reads; spliced sequences with at least 10 overlapping reads generated from the opposite ends of the same DNA fragment and with the maximum allowable error ratio in the overlap region of 0.1 were designated Raw Tags (Caporaso et al., 2010). Sequences were assigned to each sample based on their unique barcode and primer using Mothur (V1.35.1[3]). Then, the barcodes and primers were removed to obtain effective clean tags. Further sequence analysis was performed using Usearch (V10[4]) to filter and eliminate noise from the data by clustering similar sequences with less than 3% dissimilarity, and the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (VirtualBox Version 1.1.0) was used to select 16S rRNA operational taxonomic units (OTUs) from the combined reads of clustered OTUs with 97% similarity (Edgar, 2010). The 16S rRNA gene sequences obtained in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive database[5] under accession number PRJNA655948.

Statistical and Bioinformatics Analyses

The species diversity of each sample was analyzed based on three alpha diversity indices: Chao1, Shannon, and Fisher. All sample indexes were calculated using QIIME (V1.9.1) (Caporaso et al., 2010), and the correlation between the alpha diversity index and environmental factors was analyzed using the corrplot package (Wei et al., 2017) in R (V3.6.3). Beta diversity analysis was used to evaluate differences in terms of species complexity. Weighted and unweighted UniFrac beta diversity indexes were calculated by QIIME. R and local Perl scripts (Perl version 5.6.1) (Tisdall, 2001) were used to generate the sample distance heatmap based on the Bray-Curtis dissimilarity matrix. The relationship between bacterial communities and environmental factors was analyzed using the Mantel test. Heat trees (R package1) (Yu et al., 2017) were used to show the effect of the variables (variety and water stress) on the main flora, and edgeR (Robinson et al., 2010) was used to determine the most differentially abundant bacterial communities between the two treatments. The Mantel test, principal coordinates analysis (PCoA), and distance-based redundancy analysis (dbRDA) were performed using the vegan package in R v3.6.3 (Oksanen et al., 2013).

Molecular ecological network analysis (MENA)[6] was used to demonstrate the relationship between the rhizosphere bacterial communities of the two sugarcane cultivars and environmental factors (Li Y. et al., 2019). Networks were constructed for the root-associated area and the soil communities based on the relative abundances of OTUs, resulting in two networks. All analyses were performed using the MENA pipeline, and networks were graphed using Cytoscape 2.8.2 (Shannon et al., 2003). We also characterized the modularity of each network created in this study. A module is a group of nodes (OTUs) that are highly connected within the group with few connections outside the group (Newman, 2006).

RESULTS

Physical and Chemical Properties of Rhizosphere Soil and the Physiological State of Sugarcane

The SOC, TN, and AP contents in the rhizosphere soil were higher under the control treatment than under drought treatment. Soil organic carbon and TN were significantly correlated with cultivar and water treatment, whereas AP was significantly correlated only with drought stress (**Table 1**). Soil enzyme activities were significantly correlated with both cultivar and water content. The S-CAT content in the GT39 rhizosphere soil was higher than that in the ZZ9 soil under both the control and drought treatments, whereas the S-ACPT content was higher in the ZZ9 rhizosphere soil than in the GT39 soil. S-UE and SOC showed no significant variability among the four different treatments. Analysis of the physiological indexes of the two cultivars under control and drought treatment revealed that the root-shoot ratio, chlorophyll content, plant biomass, and root biomass under control and drought treatments were higher in ZZ9 than in GT39 (**Table 2**). In addition, water treatment had a significant effect on all physiological indexes, including leaf water potential, chlorophyll content, and plant biomass; this was significantly correlated with the cultivar. This confirms the high drought resistance of ZZ9 compared with that of GT39.

[1]http://www.usadellab.org/cms/?page=trimmomatic

[2]https://ccb.jhu.edu/software/FLASH/

[3]http://www.mothur.org

[4]https://www.drive5.com/usearch/

[5]https://www.ncbi.nlm.nih.gov/sra

[6]http://ieg4.rccc.ou.edu/mena/

TABLE 1 | Physical and chemical properties of soil under different treatments.

Line	Treatment	Soil water content (%)	SOC (g·kg^{-1})	TN (g·kg^{-1})	AP (g·kg^{-1})	S-CAT (U·g^{-1})	S-ACP (nmol·d^{-1}·g^{-1})	S-UE (U·g^{-1})	S-ACPT (U·g^{-1})
GT39	Control	17.55b	8.03b	107.90c	22.63b	21.89d	1.42a	2.17c	1.02a
	Drought	4.98a	5.70a	55.60a	21.40a	15.36c	2.01c	1.64b	1.81b
ZZ9	Control	18.23b	11.27c	94.52b	22.27b	12.86b	1.59b	1.62b	2.12c
	Drought	5.01a	7.24ab	57.35a	21.31a	12.24a	2.77d	1.38a	2.17c
ANOVA	Line	*P = 0.842*	*P < 0.001*	*P < 0.001*	*P = 0.180*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*
	Treatment	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*
	Line × Treatment	*P < 0.468*	*P = 0.070*	*P < 0.001*	*P = 0.407*	*P < 0.001*	*P < 0.001*	*P = 0.003*	*P < 0.001*

Values are mean of three soil samples.
Different letters indicate significant differences (ANOVA, P < 0.05, Tukey's HSD post hoc analysis) among tillage treatments.
Soil water content water content, SOC soil organic carbon, TN total nitrogen, AP available phosphorous, S-CAT solid-catalase, S-ACP solid-acid phosphatase, S-UE soil urease, S-ACPT solid -acid protease.
Significant at 0.001 level.

TABLE 2 | Plant traits of different varieties under different water treatments.

Line	Treatment	Root shoot ratio	Leaf water potential (pd·MPa^{-1})	Chlorophyll content (SPAD)	Plant biomass (g)	Root biomass (g)
GT39	Control	0.0999a	−0.276c	28.23c	433.99b	43.36a
	Drought	0.1064ab	−1.286a	11.33a	375.27a	39.92a
ZZ9	Control	0.1200b	−0.185d	29.43c	477.29c	57.28b
	Drought	0.1482c	−0.863b	14.83b	433.28b	64.22b
ANOVA	Line	*P = 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P = 0.373*
	Treatment	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*
	Line × Treatment	*P = 0.014*	*P < 0.001*	*P = 0.032*	*P = 0.223*	*P = 0.023*

Values are mean of three soil samples.
Different letters indicate significant differences (ANOVA, P < 0.05, Tukey's HSD post hoc analysis) among tillage treatments.
Significant at 0.001 level.

Diversity of the Bacterial Community in the Sugarcane Rhizosphere

Chao1, Shannon, and Fisher indices were used to analyze the α-diversity of sugarcane rhizosphere bacterial communities under the different treatments (**Figure 1A**). The results showed significant differences in rhizosphere bacterial diversity between ZZ9 and GT39 under control and drought treatments; the rhizosphere bacterial diversity was greater in the GT39 rhizosphere than in the ZZ9 rhizosphere. A significant difference in rhizosphere bacterial richness was observed between the control and drought treatments for GT39 but not for ZZ9. The Shannon index was significantly different between the two cultivars; however, there was no significant difference in the Shannon index for the different water treatments within the same cultivar. The Fisher index showed a significant difference among the four treatments, which indicated the presence of different species of rhizosphere bacteria in the four treatments. Pearson correlation analysis revealed a significant correlation between α-diversity and soil enzyme activities: there was a significant positive correlation with S-CAT and S-UE and a significant negative correlation with S-ACP and S-ACPT (**Figure 1B**).

Principal coordinates analysis (PCoA) based on the weighted (composition) and unweighted UniFrac (membership) distance was conducted for the rhizosphere bacterial communities in the different treatments (**Figures 2A,B**). The phylogenetic membership distribution of the rhizosphere bacterial communities in the four treatments was dispersed. ZZ9C and ZZ9D were less affected by drought stress, whereas GT39C and GT39D were distributed on both sides of the first principal coordinate (PCo1) and were significantly affected by drought stress. Variety had a stronger effect on the phylogenetic composition of the GT39 rhizosphere bacteria and a weaker effect on that of the ZZ9 rhizosphere bacteria. We analyzed the relationships between environmental factors and the bacterial community using the Mantel test (**Figures 2C,D**). The results showed that the phylogenetic membership of the sugarcane rhizosphere bacterial community was correlated only with S-ACP ($P < 0.05$), whereas the phylogenetic composition was significantly correlated with S-ACPT, S-CAT, and S-UE ($P < 0.01$).

Effect of Drought Stress on the Bacterial Community of the Sugarcane Rhizosphere

A class-based population structure analysis of the rhizosphere bacteria under different treatments revealed the dominant microflora in the rhizosphere environment based on their relative abundance (**Figure 3A**). The main groups of sugarcane rhizosphere bacteria were Bacilli, Alphaproteobacteria, Actinobacteria, Betaproteobacteria, Sphingobacteriia, and Flavobacteria. In the control treatment, the species compositions of the rhizosphere bacterial communities of GT39 and ZZ9

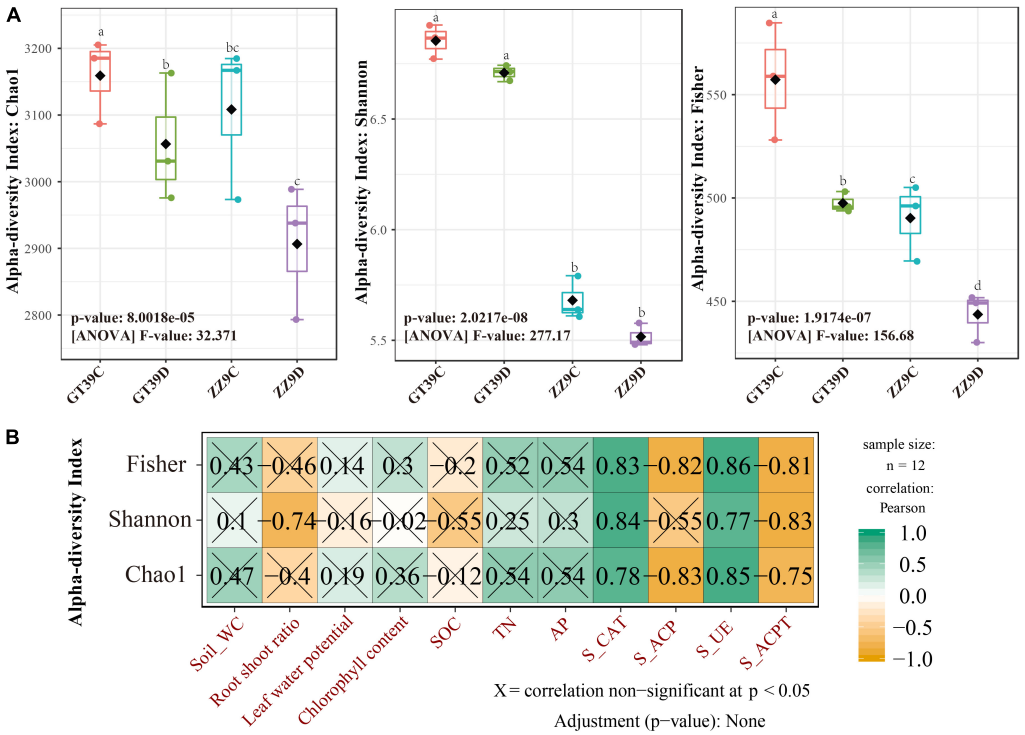


FIGURE 1 | Analysis of differences in microbial diversity based on water treatment and variety differences. **(A)** Measured values of rhizobacteria alpha diversity in Chao 1 abundance, Shannon diversity, and Fisher among different water treatments and cultivars. **(B)** Analysis of the relationship between bacterial alpha diversity and environmental factors using Pearson Correlation.

were similar, but their abundances were quite different. Compared with that in GT39C, the relative abundance of Alphaproteobacteria and Actinobacteria in GT39D was significantly increased, whereas that of Betaproteobacteria, Sphingobacteriia, and Flavobacteria was significantly decreased. In ZZ9D, the relative abundances of Bacilli and Actinobacteria were increased, whereas the abundances of Betaproteobacteria and Sphingobacteriia were decreased. dbRDA analysis of the correlation between the major bacteria in the community and environmental factors (**Figure 3B**) revealed that drought stress accounted for 82.6% of the changes in bacterial flora, whereas the difference in drought tolerance of the two cultivars accounted for 16.5% of the changes. Bacteroidetes and Proteobacteria were closely related to S-UE and S-CAT, whereas Alphaproteobacteria was closely related to S-ACP and S-ACPT.

The heat tree showed the effects of different variables (cultivar and drought stress) on the main bacterial flora, and edgeR revealed the bacterial groups that were most affected by the two variables (**Figure 4**). In the ZZ9 rhizosphere, Bacilli was the main group that responded to drought stress, whereas in the GT39 rhizosphere, Proteobacteria and Actinomycetes showed a marked response to drought stress. Comparison of the control and drought treatments revealed that Actinobacteria, Gammaproteobacteria, and Flavobacteria were mainly affected. Compared with the control, drought stress was positively correlated with the abundance of Actinobacteria, Deltaproteobacteria, and Alphaproteobacteria and negatively correlated with that of Gammaproteobacteria, Flavobacteriia, and other groups.

Response of Drought-Tolerant Strains of Rhizosphere Bacteria to the Different Sugarcane Cultivars

The changes in the rhizosphere bacterial community related to drought resistance and environmental factors in the two sugarcane cultivars under drought conditions were assessed using MENA. To identify the factors that were strongly correlated with the rhizosphere bacterial community, we separately analyzed the modules with the most node connections. The modules that showed a high correlation of microbiota with environmental factors were Modules 15 and 18 (**Supplementary Figures 3, 4**). The summary points for GT39 (total nodes = 260, total links = 1750) were fewer than those for the drought-tolerant variety, ZZ9 (total nodes = 341, total links = 1908). However, the GT39 node centralization degree and density were higher than those for ZZ9 (**Table 3**). The GT39 (drought-sensitive) microbial community had a more complex degree of association (**Table 3**).

The GT39 analysis further demonstrated that the environmental factors of soil water content, chlorophyll content, S-UE, S-ACP, S-CAT, S-ACPT, and TN were located in the center of the network and were closely connected to the bacterial flora (**Figure 5**). In Module 18, the main bacterial groups

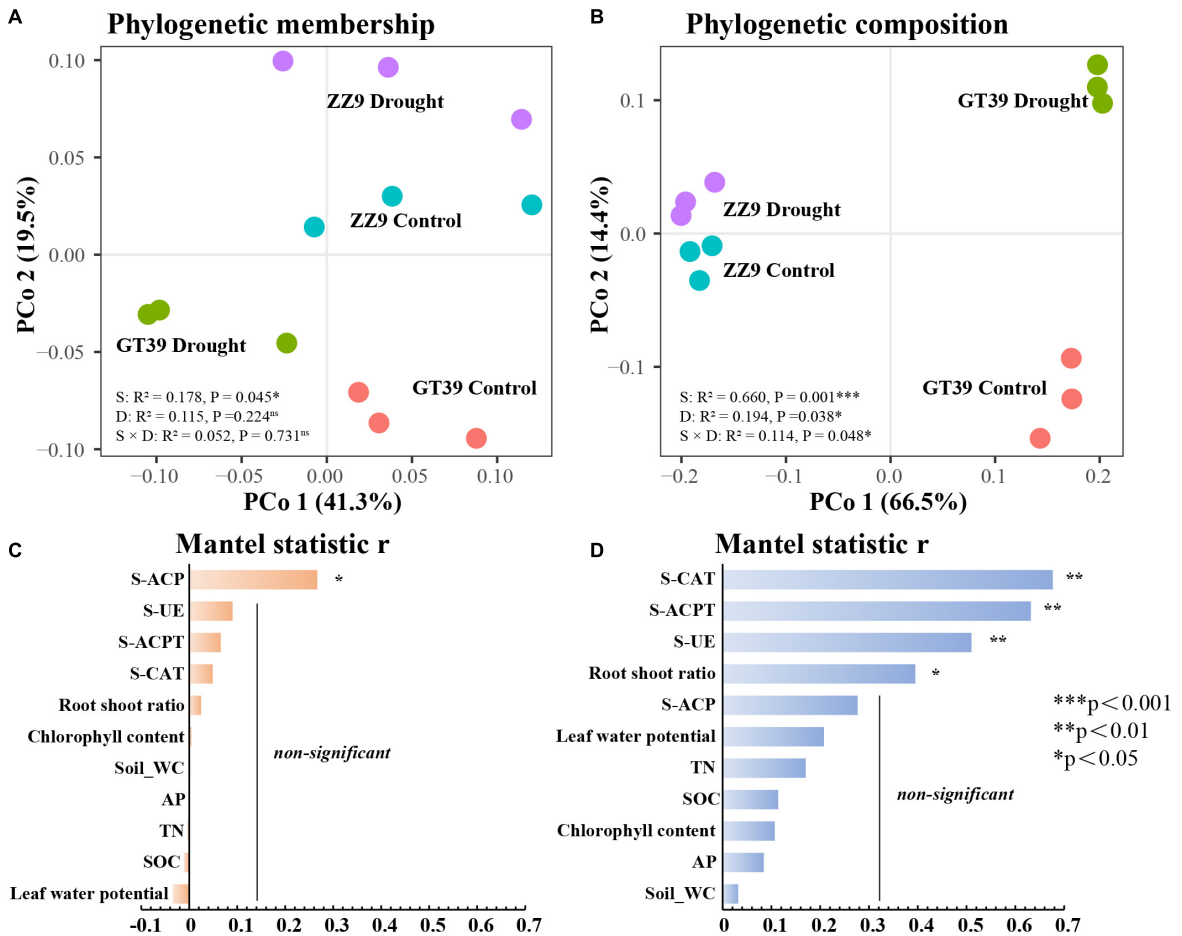


FIGURE 2 | PCoA analysis of beta diversity based on Unifrac distance. **(A)** PCoA analysis of β diversity based on unweighted UinFrace distance, where PCo1 axis shows the effect of water treatment, and PCo2 represents the effect of variety differences on microbial community β diversity. **(B)** PCoA based on weighted UniFrac distance According to the analysis, the PCo1 axis shows the main influencing factors, and the PCo2 represents the effect of water treatment on the microbial community β diversity. **(C,D)** Correlation between environmental factors and correlation between bacterial beta-diversity and environmental factors in two sugarcane cultivars using Mantel test. **(C)** Mantel statistic analysis of the correlation between β diversity of ZZ9 varieties and environmental factors. **(D)** Mantel statistic analysis of the correlation between β diversity of GT39 varieties and environmental factors.

related to environmental factors were Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Actinobacteria. Chlorophyll content showed a direct negative correlation with Alphaproteobacteria and Actinobacteria, whereas chlorophyll content and soil water content were positively correlated with Betaproteobacteria and Gammaproteobacteria (**Figure 5**). In the drought treatment, the abundance of Actinobacteria and Alphaproteobacteria changed significantly, and Actinobacteria were closely and positively correlated with S-ACPT and S-ACP.

In the network analysis of ZZ9, the environmental factors in the center were primarily soil water content, chlorophyll content, TN, S-ACP, AP, and SOC, whereas S-UE, S-CAT, and the root-shoot ratio were relatively close to the edge (**Figure 6**). The core key nodes in Module 15 were concentrated, and Bacilli, Actinobacteria, Alphaproteobacteria, Sphingobacteriia and others were highly connected to the environmental factors in the central region. The bacterial flora negatively correlated with chlorophyll content were Alphaproteobacteria, Actinobacteria, and Bacilli. The abundance of these three classes increased significantly in drought-treated ZZ9, and a close connecting line was observed between Bacilli and S-ACP, indicating a high degree of correlation.

DISCUSSION

Drought Stress Reduced the Bacterial Diversity in the Sugarcane Rhizosphere

Differences in host plants and drought stress are two important factors affecting rhizosphere bacteria (Naylor et al., 2017). In the present study, we found that drought stress reduced the bacterial diversity in the rhizosphere of sugarcane, especially in the drought-sensitive cultivar GT39, and a significant correlation was detected between this change in bacterial diversity and soil enzyme activities (**Figure 1**). Drought stress stimulates the host plant response to a varying degree, depending on the

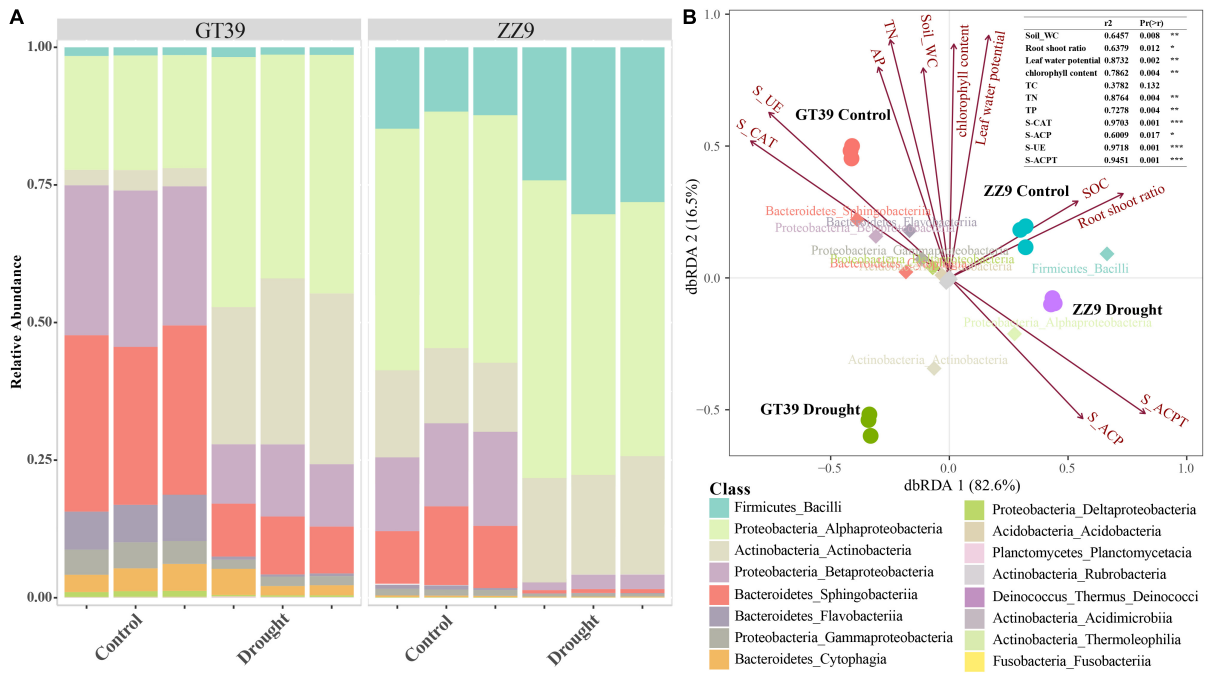


FIGURE 3 | (A) A class-based population structure analysis (relative abundance) of the rhizosphere microbial bacterial communities of ZZ9 and GT39, and the main active populations are displayed. **(B)** The main active populations and environmental factors affecting the community are analyzed by dbRDA (environmental factors are represented by arrows).

drought tolerance of the host plant, which in turn affects the rhizosphere microorganisms (Larkunthod et al., 2018; de Vries et al., 2020). The phylogenetic membership and composition of the rhizosphere bacterial community of the drought-tolerant cultivar ZZ9 were more stable than those of the drought-sensitive cultivar GT39 (**Figures 2A,B**). The effect of cultivar type on rhizosphere microorganisms has been widely studied (Liljeroth and Bååth, 1988; Babu S. et al., 2015). Zhao et al., reported that sugarcane with strong stress resistance can resist drought and other stress conditions by utilizing rhizosphere microorganisms in an arid environment (Liu et al., 2020; Zhao et al., 2020b).

However, the rhizosphere is a complex environment occupied by microorganisms, which themselves are very sensitive to changes triggered by drought stress, including root exudates, soil conditions, and other factors (Wang et al., 2020). The rhizosphere soils of sugarcane cultivars with different drought tolerances differed significantly in nutrient composition and enzyme activities (**Table 1**). According to the Pearson's correlation analysis, only soil enzyme activities were significantly correlated ($P < 0.05$) with the α-diversity of the rhizosphere bacterial community (**Figure 1B**). The deficiency in soil nutrients caused by drought stress is initiated by the decrease in the enzyme cycle rate, which is responsible for nutrient cycling and decomposition under drought stress (Hueso et al., 2012). He et al., showed that drought stress reduces TN in the soil, slows down the degradation of organophosphorus, and affects the absorption and release rates of C by plants (van der Molen et al., 2011; He and Dijkstra, 2014). Rhizosphere microorganisms maintain the soil nutrient supply by increasing the metabolic activity in the rhizosphere soil environment, promoting the effective mineralization of soil nutrients, and decomposing organic matter (Tarkka et al., 2008). S-ACP, S-UE, and S-ACPT are important enzymes involved in soil nutrient cycling (Shukla and Varma, 2010). Moreover, the changes in the bacterial communities in the sugarcane rhizosphere were significantly correlated with soil enzyme activities (**Figures 2C,D**). Therefore, we concluded that the bacterial community of drought-tolerant sugarcane cultivars is more stable under drought stress, whereas the rhizosphere bacterial community of drought-sensitive sugarcane cultivars is less stable and that this is significantly correlated with soil enzyme activities.

Differences in the Drought Tolerance of Sugarcane Cultivars Led to Different Core Drought-Resistant Bacterial Strains

The response of the rhizosphere bacterial community to stress is closely related to the adaptability of plants to adversity (Gao et al., 2019). Analysis of the relative abundance of 16S rRNA in the rhizosphere revealed similar species compositions but significantly different relative abundances of different bacterial strains between the drought-tolerant ZZ9 cultivar and the drought-sensitive GT39 cultivar (**Figure 3**). A previous study by Kalinowski et al., showed that plant adaptation to stress can change the bacterial composition of the rhizosphere (Kalinowski and Halden, 2012), which was confirmed in our

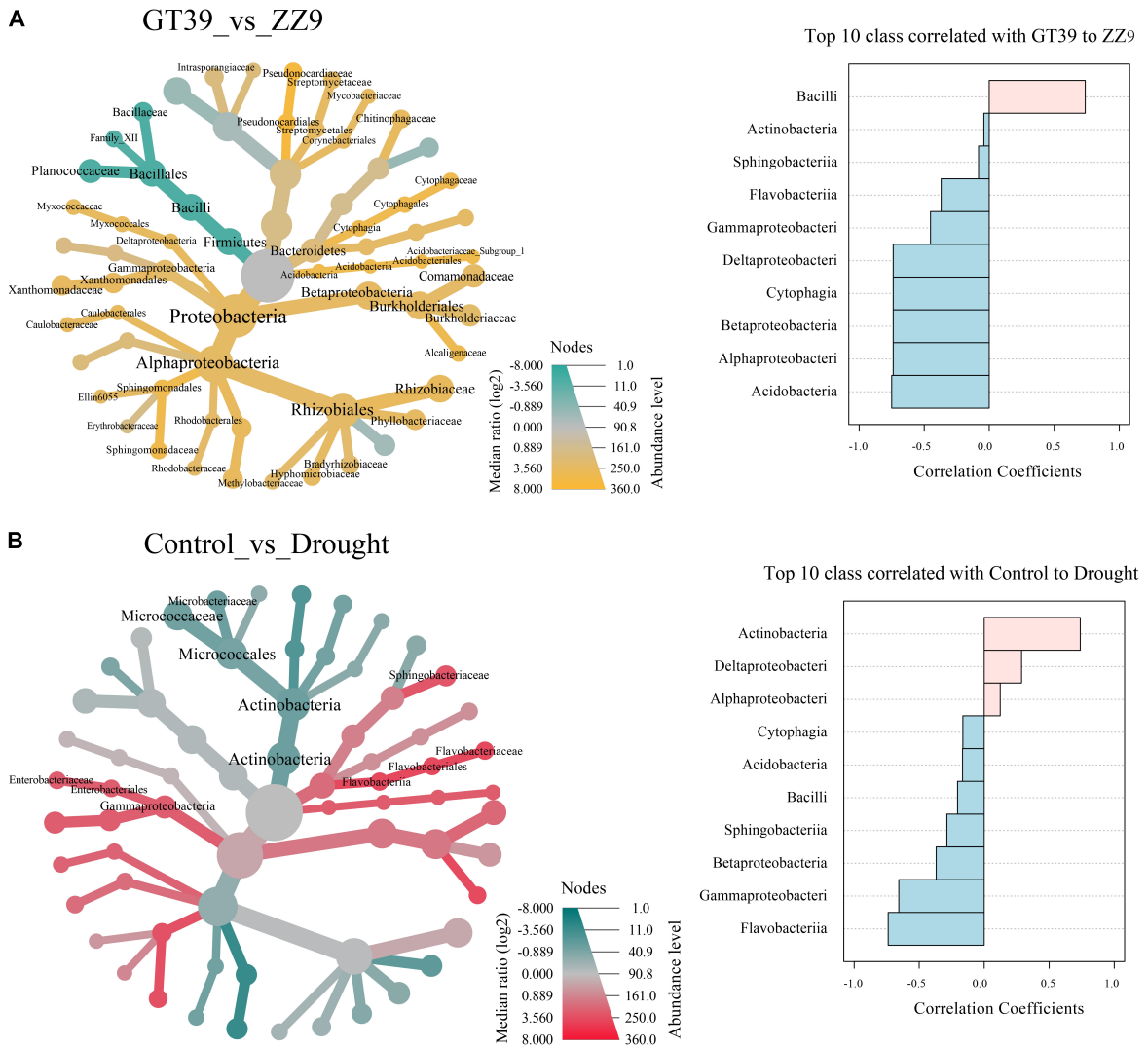


FIGURE 4 | Visualization of classification differences in tree heat maps. **(A)** The influence of the differences between the GT39 and ZZ9 varieties on the rhizosphere flora. EdgeR represents the top ten major flora with a greater correlation with the drought tolerance of the two varieties, and pink and blue indicate the drought resistance of the variety. Relevance. **(B)** Control the differential effects of watering and drought stress on the rhizobacteria, edgeR represents the top ten major flora associated with water stress, pink and blue represent the correlation with the degree of water stress

experiments. Under drought stress, the relative abundances of Alphaproteobacteria and Actinobacteria significantly increased in the water-sensitive cultivar, whereas it was already high in the drought-resistant cultivar under control conditions. Exposure to drought stress increased the abundance of Bacilli (**Figure 3A**). We conclude that the rhizosphere bacteria with increased relative abundance under drought stress are usually those with some level of drought tolerance and are able to maintain the rhizosphere environment under stress (Naylor and Coleman-Derr, 2018; Yasmin et al., 2021). The changes in Alphaproteobacteria and Actinobacteria abundance were positively correlated with drought stress, whereas the increase in the relative abundance of Bacilli had a positive correlation with the drought tolerance attributes of the drought-tolerant variety ZZ9 (**Figure 4**). A previous study found that Alphaproteobacteria can make full use of sugarcane rhizosphere secretions under environmental stress to improve the rhizosphere environment, and this class dominates the rhizosphere community of sugarcane (Da Costa et al., 2018). Actinomycetes and Bacilli are known plant growth-promoting bacteria that boost the adaptation of host plants under drought stress (Naylor et al., 2017). A rich community of drought-resistant bacteria exist in the rhizosphere of the drought-tolerant sugarcane cultivar under optimal soil conditions, and the abundance of these bacteria increased in the rhizosphere of drought-sensitive GT39 under drought stress.

Drought stress reduced the activities of various tested soil enzymes, such as S-ACP, S-UE, and enzymes involved in the N cycle, and it reduced the nutrient supply of plants (Sardans and Peñuelas, 2005). Rhizosphere bacteria can stimulate the decomposition of organic matter; enhance the solubility

TABLE 3 | Topological characteristics of two varieties Network analysis.

Network indexes	GT39-Network	ZZ9-Network
Total nodes	260	341
Total links	1750	1908
R square of power-law	0.645	0.706
Average degree (avgK)	13.462	11.191
Average clustering coefficient (avgCC)	0.419	0.406
Average path distance (GD)	4.145	6.003
Geodesic efficiency (E)	0.334	0.258
Harmonic geodesic distance (HD)	2.991	3.873
Maximal degree	55	59
Nodes with max degree	OTU_847;s-acp	s-acp
Centralization of degree (CD)	0.162	0.141
Maximal betweenness	2124.394	5634.936
Nodes with max betweenness	OTU_3435	OTU_751
Centralization of betweenness (CB)	0.055	0.088
Maximal stress centrality	139230	281349
Nodes with max stress centrality	OTU_433	OTU_177
Centralization of stress centrality (CS)	3.593	4.385
Maximal eigenvector centrality	0.179	0.183
Nodes with max eigenvector centrality	OTU_81	s-acp
Centralization of eigenvector centrality (CE)	0.146	0.159
Density (D)	0.052	0.033
Reciprocity	1	1
Transitivity (Trans)	0.596	0.587
Connectedness (Con)	0.685	0.667
Efficiency	0.929	0.955
Hierarchy	0	0
Lubness	1	1

of C, N, and P; and promote the growth of host plant roots (Dijkstra et al., 2013; Li et al., 2013). We believe that there is a close correlation between rhizosphere bacteria and rhizosphere enzyme activities under drought stress owing to the interaction between the two, which promotes soil nutrient cycling (**Figures 2D**, **3B**). For example, Da Costa et al., showed that members of Alphaproteobacteria could efficiently utilize the C in the rhizosphere exudates of sugarcane (Da Costa et al., 2018). Actinomycetes widely exist in soil ecosystems and can affect the decomposition and formation of soil humus to regulate the balance of soil nutrients (Yandigeri et al., 2012). *Bacillus*, a genus of plant growth-promoting bacteria, can induce nutrient absorption and the growth of host plants and stimulate the defense mechanisms of host plants under stress conditions (Sivasakthi et al., 2014). S-ACPT, S-ACP, S-UE, and other enzymes that are closely related to rhizosphere bacteria are key enzymes involved in the N and P cycles and soil protein decomposition (Cao et al., 2003). This explains the strong correlation between drought-responsive flora and soil physicochemical properties revealed by the dbRDA analysis (**Figure 3B**).

In conclusion, drought-resistant bacterial strains are dominant in the rhizosphere of drought-tolerant sugarcane cultivars, whereas the abundance of drought-tolerant bacteria in drought-sensitive sugarcane cultivars increases only when they are subjected to drought stress. Drought-tolerant bacteria, together with soil enzyme activities, maintain nutrient cycling in rhizosphere soil to ensure an adequate nutrient supply to sugarcane roots under drought stress.

Response of Drought-Resistant Bacteria in the Sugarcane Rhizosphere to Drought Stress

Molecular ecological network analysis (MENA) was used to elucidate the ecological community structure and interactions among sugarcane rhizosphere bacteria under drought stress. Durán et al., found that the complexity of plant root microbial networks is related to plant survival, and the interaction with the rhizosphere microflora is key to the environmental adaptability of host plants (Durán et al., 2018). We found that the complexity of the sugarcane rhizosphere bacterial ecological network under drought stress was higher in the drought-tolerant sugarcane cultivar ZZ9 than in the drought-sensitive cultivar GT39 (**Figures 5**, **6** and **Table 3**). The high connectivity of the complex rhizosphere microflora network is the main factor that renders this network more resistant to environmental stress than a simple network environment (Santolini and Albert-László, 2018). In addition, the rhizosphere bacterial OTUs of the two cultivars showed different high density-associated regions under drought stress, and significantly more strains of rhizosphere bacteria were associated with drought-tolerant ZZ9 than with drought-sensitive GT39 (**Supplementary Figures 3, 4**). Therefore, we concluded that the difference in the rhizosphere bacterial community between the two cultivars is a factor that contributes to the difference in drought tolerance between the two sugarcane cultivars. However, the resistance of host plants to environmental stress is also an important factor affecting the structure of the rhizosphere bacterial community (Berendsen et al., 2012). It is because of this close correlation that abiotic environmental factors, such as drought stress, may affect both of them simultaneously (Wardle et al., 2004).

The experimental results showed significant differences between GT39 and ZZ9 under drought stress, not only in the physiological characteristics of drought resistance (**Table 2**), but also in the response of the core drought-resistant bacteria (**Figures 5**, **6**). The differences in the rhizosphere exudates of the host plants are the main driving force of the observed change in rhizosphere bacteria under drought stress (Li X. et al., 2019). The drought-responsive Actinobacteria of GT39 were positively correlated with S-ACP and S-ACPT (**Figure 5**), whereas Bacilli, which were the dominant rhizosphere bacteria of ZZ9 under drought stress, were positively correlated with S-ACP (**Figure 6**). As important elements of root exudates in the rhizosphere region, S-ACP and S-ACPT play important roles in the oxidative metabolism of soil nutrients, affecting the mineralization of phosphorus in rhizosphere soil and the transformation of soil protein components, respectively (Mijangos et al., 2006; Badri and Vivanco, 2009; Babu A. G. et al., 2015). Based on the close correlation between the

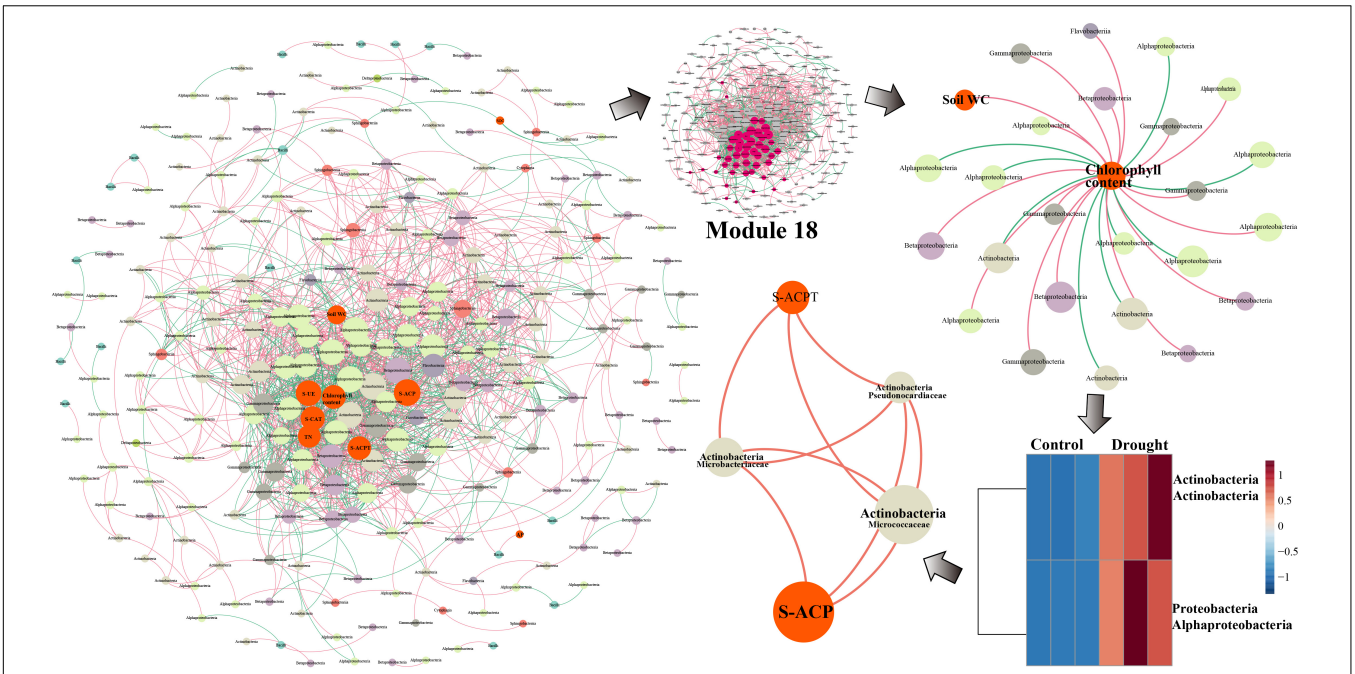


FIGURE 5 | The network analysis of MENA shows that there is a significant interaction between the rhizobacteria community OUT under normal water and drought stress in the sugarcane variety GT39. The node indicates that the bacteria OUT in the microbial network with significant interactions are determined by the degree of connection. It is colored according to the system-level classification membership. The red line connecting the nodes indicates positive interaction, and the green line indicates negative interaction. Among them, GT39 (drought sensitive) mainly depends on Actinobacteria flora in response to drought stress Groups are closely related to S-ACP and S-ACPT.

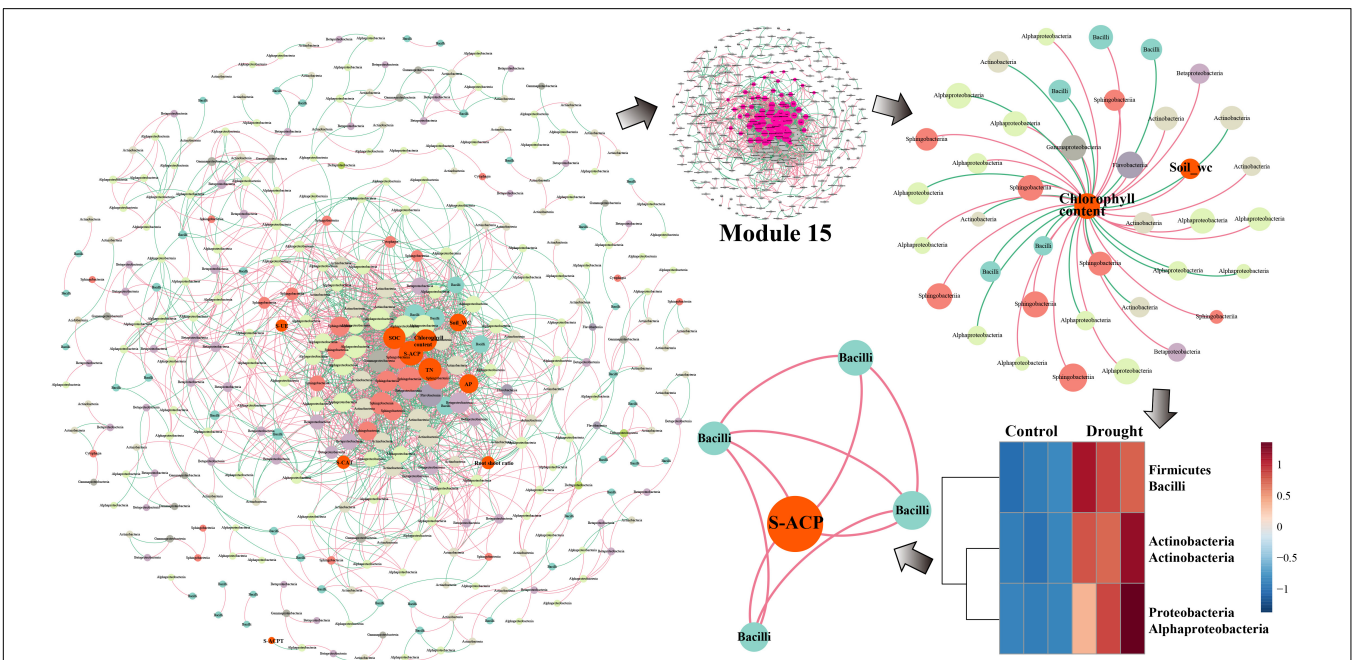


FIGURE 6 | Network analysis of MENA, the analysis shows that there are different degrees of association between the bacterial communities of rhizosphere of sugarcane variety ZZ9. The nodes represent the bacteria OUT that have significant interaction in the microbial network, the size of which is determined by the degree of connection, and they are classified according to the system level. The relationship is colored. The red line connecting the nodes indicates positive interaction, and the green line indicates negative interaction. The rhizosphere bacterial community of the drought-tolerant variety ZZ9 mainly relies on the Bacilli flora (PGPR) to respond to drought stress. S-ACP is closely related.

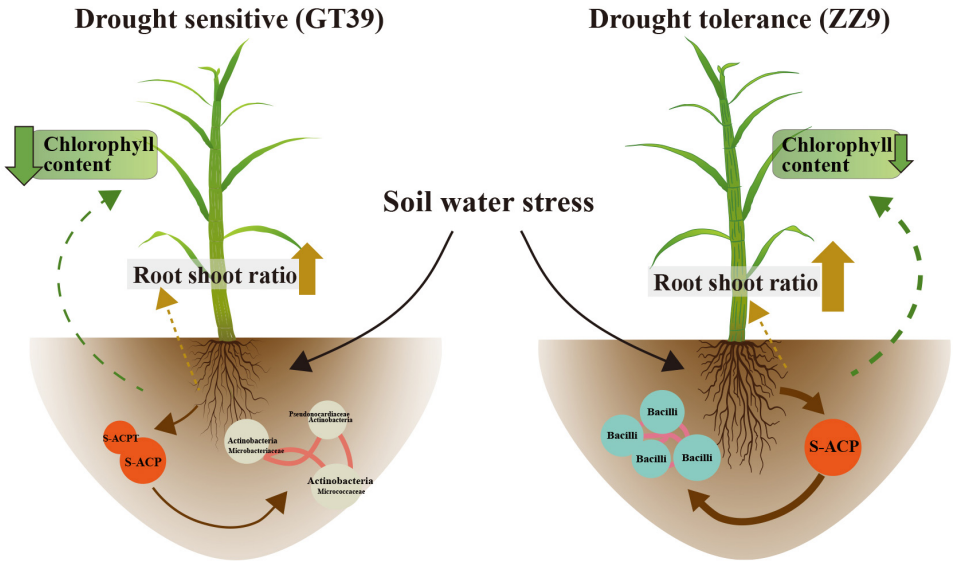


FIGURE 7 | Changes in chlorophyll content and root exudates of the two sugarcane varieties GT39 and ZZ9 under water stress conditions and their interaction with the main rhizosphere bacterial communities.

components of the rhizosphere environment, we believe that the changes in rhizosphere exudates that occur in response to soil nutrient status under drought stress affect the response of drought-resistant rhizosphere bacteria (Canarini et al., 2016; Naylor and Coleman-Derr, 2018). In addition, many studies have shown that Actinobacteria can also effectively regulate the C and N contents in the soil under drought stress, maintain the health status of rhizosphere soil, and effectively alleviate abiotic stresses, such as salt, alkali, and drought (Badri and Vivanco, 2009). Bacilli are also believed to promote phosphate solubilization and are an important regulator of AP content in rhizosphere soil (Dias et al., 2009; Mujahid et al., 2015). Therefore, the drought resistance of sugarcane is achieved by both self-regulation and the response of drought-resistant bacteria in the rhizosphere. Drought-resistant bacteria affect soil nutrient cycling under the influence of root exudates, and both jointly regulate the nutrient supply of sugarcane root in water-deficient environments (**Figure 7**).

CONCLUSION

We investigated the changes in the rhizosphere bacterial communities of two sugarcane cultivars with different drought resistance responses. This difference in drought resistance did not lead to significant differences in the rhizosphere bacterial community structure between the two sugarcane cultivars under drought stress. However, the diversity of the rhizosphere bacteria was decreased in both sugarcane cultivars under drought stress, and the change was greater in the rhizosphere of the drought-sensitive sugarcane cultivar than in the rhizosphere of the drought-resistant sugarcane cultivar. In addition, we found that the drought-tolerant cultivar was better adapted to drought owing to the high abundance of drought-resistant bacteria in its rhizosphere under normal water conditions, whereas the abundance of drought-resistant bacteria in the rhizosphere of the drought-sensitive cultivar began to increase only after exposure to drought stress. Thus, drought-sensitive cultivars require more time to respond to drought stress and are therefore more vulnerable to damage. The resistance of sugarcane to drought stress is related to both physiological adjustments and the regulation of rhizosphere bacteria. Under the influence of rhizosphere exudates, the core drought-resistant bacteria in the sugarcane rhizosphere regulate the nutrient status of rhizosphere soil and improve the drought resistance of sugarcane.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA655948.

AUTHOR CONTRIBUTIONS

QL, ZW, XZ, and YL contributed to the design of the experiments, data analysis, and manuscript writing. QL, SX, and YL contributed to the experimentation. QL and ZW contributed to the data interpretation. YP and WD participated in the revision of the article. YX, JD, and BW cultivated sugarcane growth during the experiment and participated in the determination of experimental indicators. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31901466).

ACKNOWLEDGMENTS

We are grateful to the Guangxi Key Laboratory of Sugarcane Biology for providing the experimental site. We also thank the reviewers and the editors for their constructive comments on this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.732989/full#supplementary-material

REFERENCES

Aimin, Z., Liu, E., Liu, J., Feng, S., Gong, S., and Wang, J. (2018). Characterization of increased cuticular wax mutant and analysis of genes involved in wax biosynthesis in Dianthus spiculifolius. *Hortic. Res.* 5:40.

Andreo-Jimenez, B., Vandenkoornhuyse, P., Van, A. L., Heutinck, A., and Bouwmeester, H. (2019). Plant host and drought shape the root associated fungal microbiota in rice. *PeerJ* 7:e7463. doi: 10.7717/peerj.7463

Babu, A. G., Kim, S. W., Yadav, D. R., Hyum, U., Adhikari, M., and Lee, Y. S. (2015). *Penicillium menonorum*: a novel fungus to promote growth and nutrient management in cucumber plants. *Mycobiology* 43, 49–56. doi: 10.5941/myco.2015.43.1.49

Babu, S., Bidyarani, N., Chopra, P., Monga, D., Kumar, R., Prasanna, R., et al. (2015). Evaluating microbe-plant interactions and varietal differences for enhancing biocontrol efficacy in root rot disease challenged cotton crop. *Eur. J. Plant Pathol.* 142, 345–362. doi: 10.1007/s10658-015-0619-6

Badri, D. V., and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant Cell Environ.* 32, 666–681. doi: 10.1111/j.1365-3040.2009.01926.x

Bao, S. (2000). *Soil and agricultural chemistry analysis*. Beijing: China agriculture press.

Benitez, M.-S., Osborne, S. L., and Lehman, R. M. (2017). Previous crop and rotation history effects on maize seedling health and associated rhizosphere microbiome. *Sci. Rep.* 7, 1–13.

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Buchanan, B. B., Gruissem, W., and Jones, R. L. (2015). *Biochemistry and Molecular Biology of Plants*. Hoboken, NJ: John Wiley & Sons.

Butt, H., Piatek, A., Li, L., Reddy, A. S. N., and Mahfouz, M. M. (2019). Multiplex CRISPR mutagenesis of the serine/arginine-rich (SR) gene family in rice. *Genes* 10:596. doi: 10.3390/genes10080596

Canarini, A., Andrew, M., and Dijkstra, F. A. (2016). Drought effects on *Helianthus annuus* and Glycine max metabolites: from phloem to root exudates. *Rhizosphere* 2, 85–97. doi: 10.1016/j.rhisph.2016.06.003

Cao, H., Sun, H., Yang, H., Sun, B., and Zhao, Q. (2003). A review: soil enzyme activity and its indication for soil quality. *Chinese J. Appl. Environ. Biol.* 9, 105–109.

Cao, M.-J., Zhang, Y.-L., Liu, X., Huang, H., Zhou, X. E., Wang, W.-L., et al. (2017). Combining chemical and genetic approaches to increase drought resistance in plants. *Nat. Commun.* 8, 1–12.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osório, M. L., et al. (2002). How plants cope with water stress in the field? Photosynthesis and growth. *Ann. Bot.* 89, 907–916. doi: 10.1093/aob/mcf105

Da Costa, D. P., Dias, A. C., Cotta, S. R., Vilela, D., De Andrade, P. A., Pellizari, V. H., et al. (2018). Changes of bacterial communities in the rhizosphere of sugarcane under elevated concentration of atmospheric CO 2. *GCB Bioenergy* 10, 137–145. doi: 10.1111/gcbb.12476

de Vries, F. T., Griffiths, R. I., Knight, C. G., Nicolitch, O., and Williams, A. (2020). Harnessing rhizosphere microbiomes for drought-resilient crop production. *Science* 368, 270–274. doi: 10.1126/science.aaz5192

Demers, J. E., Gugino, B. K., and Jimenez-Gasco, M. D. M. (2015). Highly diverse endophytic and soil fusarium oxysporum populations associated with field-grown tomato plants. *Appl. Environ. Microbiol.* 81, 81–90. doi: 10.1128/aem.02590-14

Dias, A. C., Costa, F. E., Andreote, F. D., Lacava, P. T., Teixeira, M. A., Assumpçao, L. C., et al. (2009). Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. *World J. Microbiol. Biotechnol.* 25, 189–195. doi: 10.1007/s11274-008-9878-0

Dijkstra, F. A., Carrillo, Y., Pendall, E., and Morgan, J. A. (2013). Rhizosphere priming: a nutrient perspective. *Front. Microbiol.* 4:216. doi: 10.3389/fmicb.2013.00216

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973.e–983.e.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Gao, J., Luo, Y., Wei, Y., Huang, Y., Zhang, H., He, W., et al. (2019). Effect of aridity and dune type on rhizosphere soil bacterial communities of *Caragana microphylla* in desert regions of northern China. *PLoS one* 14:e0224195. doi: 10.1371/journal.pone.0224195

Garsmeur, O., Droc, G., Antonise, R., Grimwood, J., Potier, B., Aitken, K., et al. (2018). A mosaic monoploid reference sequence for the highly complex genome of sugarcane. *Nat. Commun.* 9, 1–10.

Gentile, A., Dias, L. I., Mattos, R. S., Ferreira, T. S. H., and Menossi, M. (2015). MicroRNAs and drought responses in sugarcane. *Front. Plant Ence* 6:1–13. doi: 10.3389/fpls.2015.00058

Ghannoum, O. (2016). How can we breed for more water use-efficient sugarcane? *J. Exp. Bot.* 67, 557–559. doi: 10.1093/jxb/erw009

Giehl, R. F., and von Wirén, N. (2014). Root nutrient foraging. *Plant Physiol.* 166, 509–517. doi: 10.1104/pp.114.245225

Griffiths, C. A., and Paul, M. J. (2017). Targeting carbon for crop yield and drought resilience. *J. Sci. Food Agric.* 97, 4663–4671. doi: 10.1002/jsfa.8501

Gupta, B., and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genom.* 2014:701596.

Hajar-Azhari, S., Wan-Mohtar, W. A. A. Q. I, Ab Kadir, S., Abd Rahim, M. H., and Saari, N. (2018). Evaluation of a Malaysian soy sauce koji strain Aspergillus oryzae NSK for γ-aminobutyric acid (GABA) production using different native sugars. *Food Sci. Biotechnol.* 27, 479–488.

He, M., and Dijkstra, F. A. (2014). Drought effect on plant nitrogen and phosphorus: a meta-analysis. *New Phytol.* 204, 924–931. doi: 10.1111/nph.12952

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., et al. (2016). Priming and memory of stress responses in organisms lacking a nervous system. *Biol. Rev.* 91, 1118–1133. doi: 10.1111/brv.12215

Howard, A., Comber, S., Kifle, D., Antai, E., and Purdie, D. (1995). Arsenic speciation and seasonal changes in nutrient availability and micro-plankton abundance in Southampton water, UK. *Estuar. Coast. Shelf Sci.* 40, 435–450. doi: 10.1006/ecss.1995.0030

Hueso, S., García, C., and Hernández, T. (2012). Severe drought conditions modify the microbial community structure, size and activity in amended and unamended soils. *Soil Biol. Biochem.* 50, 167–173. doi: 10.1016/j.soilbio.2012.03.026

Iquebal, M. A., Sharma, P., Jasrotia, R. S., Jaiswal, S., Kaur, A., Saroha, M., et al. (2019). RNAseq analysis reveals drought-responsive molecular pathways with candidate genes and putative molecular markers in root tissue of wheat. *Sci. Rep.* 9, 1–18.

Jin, X., Yunzeng, Z., Pengfan, Z., and Riera, N. (2018). The structure and function of the global citrus rhizosphere microbiome. *Nat. Commun.* 9: 4894.

Jordan, V., Yvan, M. N. L., Audrey, D., Maximilien, G. A. M., Daniel, M., and Claire, P. C. (2016). Fluorescent *Pseudomonas* strains with only few plant-beneficial properties are favored in the maize rhizosphere. *Front. Plant Sci.* 7:1212. doi: 10.3389/fpls.2016.01212

Jurburg, S. D., Nunes, I., Stegen, J. C., Roux, X. Le, Priemé, A., Sørensen, S. J., et al. (2017). Autogenic succession and deterministic recovery following disturbance in soil bacterial communities. *Sci. Rep.* 7:45691.

Kalinowski, T., and Halden, R. U. (2012). Can stress enhance phytoremediation of polychlorinated biphenyls? *Environ. Eng. Sci.* 29, 1047–1052. doi: 10.1089/ees.2012.0089

Kavamura, V. N., Taketani, R. G., Lançoni, M. D., Andreote, F. D., Mendes, R., and de Melo, I. S. (2013). Water regime influences bulk soil and rhizosphere of *Cereus jamacaru* bacterial communities in the Brazilian caatinga biome. *PLoS One* 8:e73606. doi: 10.1371/journal.pone.0073606

Khan, N., Bano, A. M., and Babar, A. (2020). Impacts of plant growth promoters and plant growth regulators on rainfed agriculture. *PLoS One* 15:e0231426. doi: 10.1371/journal.pone.0231426

Koech, R. K., Malebe, P. M., Nyarukowa, C., Mose, R., Kamunya, S. M., Joubert, F., et al. (2019). Functional annotation of putative QTL associated with black tea quality and drought tolerance traits. *Sci. Rep.* 9, 1–11.

Krause, S. M., Meima-Franke, M., Veraart, A. J., Ren, G., Ho, A., and Bodelier, P. L. (2018). Environmental legacy contributes to the resilience of methane consumption in a laboratory microcosm system. *Sci. Rep.* 8, 1–9.

Kundariya, H., Yang, X., Morton, K., Sanchez, R., Axtell, M. J., Hutton, S. F., et al. (2020). MSH1-induced heritable enhanced growth vigor through grafting is associated with the RdDM pathway in plants. *Nat. Commun.* 11, 1–14.

Larkunthod, P., Nounjan, N., Siangliw, J. L., Toojinda, T., Sanitchon, J., Jongdee, B., et al. (2018). Physiological responses under drought stress of improved drought-tolerant rice lines and their parents. *Notulae Bot. Horti Agrobotanici Cluj Napoca* 46, 679–687. doi: 10.15835/nbha46211188

Lee, S. A., Kim, Y., Kim, J. M., Chu, B., Joa, J.-H., Sang, M. K., et al. (2019). A preliminary examination of bacterial, archaeal, and fungal communities inhabiting different rhizocompartments of tomato plants under real-world environments. *Sci. Rep.* 9, 1–15.

Li, P., Wu, H., Jin, Y., Huang, X., Chen, Y., Yang, X., et al. (2019). Exploring the diversity and dynamic of bacterial community vertically distributed in tongguling national nature reserve in Hainan Island, China. *Braz. J. Microbiol.* 50, 729–737. doi: 10.1007/s42770-019-00078-2

Li, X., Jousset, A., de Boer, W., Carrión, V. J., Zhang, T., Wang, X., et al. (2019). Legacy of land use history determines reprogramming of plant physiology by soil microbiome. *ISME J.* 13, 738–751. doi: 10.1038/s41396-018-0300-0

Li, X., Mu, Y., Cheng, Y., Liu, X., and Nian, H. (2013). Effects of intercropping sugarcane and soybean on growth, rhizosphere soil microbes, nitrogen and phosphorus availability. *Acta Physiol. Plant.* 35, 1113–1119. doi: 10.1007/s11738-012-1148-y

Li, Y., Hu, Y., Song, D., Liang, S., Qin, X., and Siddique, K. H. (2019). The effects of straw incorporation with plastic film mulch on soil properties and bacterial community structure on the loess plateau. *Eur. J. Soil Sci.* 72, 979–994. doi: 10.1111/ejss.12912

Liljeroth, E., and Bååth, E. (1988). Bacteria and fungi on roots of different barley varieties (*Hordeum vulgare* L.). *Biol. Fertil. Soils* 7, 53–57.

Liu, Y., Yang, H., Liu, Q., Zhao, X., Xie, S., Wang, Z., et al. (2020). Effect of two different sugarcane cultivars on rhizosphere bacterial communities of sugarcane and soybean upon intercropping. *Front. Microbiol.* 11:596472. doi: 10.3389/fmicb.2020.596472

Luo, M., and Liang, J. (2005). Response of sugarcane to drought stress at seedling stage. *J. Trop. Crops* 026, 38–41.

Meijuan, Z., Yongjia, Z., Shijie, C., and Yong, D. (2018). Deciphering the bacterial composition in the rhizosphere of *Baphicacanthus cusia* (NeeS) Bremek. *Sci. Rep.* 8:15831.

Mijangos, I., Pérez, R., Albizu, I., and Garbisu, C. (2006). Effects of fertilization and tillage on soil biological parameters. *Enzyme Microb. Technol.* 40, 100–106. doi: 10.1016/j.enzmictec.2005.10.043

Mujahid, T. Y., Subhan, S. A., Wahab, A., Masnoon, J., Ahmed, N., and Abbas, T. (2015). Effects of different physical and chemical parameters on phosphate solubilization activity of plant growth promoting bacteria isolated from indigenous soil. *J. Pharm. Nutr. Sci.* 5, 64–70. doi: 10.6000/1927-5951.2015.05.01.10

Navid, B., Chantal, H., Yantai, G., Bunyamin, T. A., and Joan, D. K. (2015). Genotype-specific variation in the structure of root fungal communities is related to chickpea plant productivity. *Appl. Environ. Microbiol.* 81, 2368–2377. doi: 10.1128/aem.03692-14

Naylor, D., and Coleman-Derr, D. (2018). Drought stress and root-associated bacterial communities. *Front. Plant Sci.* 8:2223. doi: 10.3389/fpls.2017.02223

Naylor, D., DeGraaf, S., Purdom, E., and Coleman-Derr, D. (2017). Drought and host selection influence bacterial community dynamics in the grass root microbiome. *ISME J.* 11, 2691–2704. doi: 10.1038/ismej.2017.118

Newman, M. E. (2006). Modularity and community structure in networks. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8577–8582.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O'hara, R., et al. (2013). *Community Ecology Package. R package version, 2.0-2.*

Pascale, A., Proietti, S. I, Pantelides, S., and Stringlis, I. A. (2020). Modulation of the root microbiome by plant molecules: the basis for targeted disease suppression and plant growth promotion. *Front. Plant Sci.* 10:1741. doi: 10.3389/fpls.2019.01741

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Qin, Q., Zhu, J., Shi, Z., Yu, T., and Cao, K. (2017). Association of leaf anatomical structure characteristics with photosynthetic capacity and drought tolerance in seven sugarcane varieties. *Acta Plant Physiol.* 053, 705–712.

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Sade, N., Gebretsadik, M., Seligmann, R., Schwartz, A., Wallach, R., and Moshelion, M. (2010). The role of tobacco Aquaporin1 in improving water use efficiency, hydraulic conductivity, and yield production under salt stress. *Plant Physiol.* 152, 245–254. doi: 10.1104/pp.109.145854

Santolini, M., and Albert-László, B. (2018). Predicting perturbation patterns from the topology of biological networks. *Proc. Natl. Acad. Sci. U.S.A.* 115, E6375–E6383.

Sardans, J., and Peñuelas, J. (2005). Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. *Soil Biol. Biochem.* 37, 455–461. doi: 10.1016/j.soilbio.2004.08.004

Schimel, J., Balser, T. C., and Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Eology* 88, 1386–1394. doi: 10.1890/06-0219

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shao, W., and Li, G. (2016). Research progress of soil enzymes function and its determination method. *Northern Hortic.* 000, 188–193.

Shukla, G., and Varma, A. (2010). *Soil Enzymology*. Berlin: Springer Science & Business Media.

Siraree, A., Banerjee, N., Kumar, S., Khan, M., Singh, P., Sharma, S., et al. (2017). Identification of marker-trait associations for morphological descriptors and yield component traits in sugarcane. *Physiol. Mol. Biol. Plants* 23, 185–196. doi: 10.1007/s12298-016-0403-x

Sivasakthi, S., Usharani, G., and Saranraj, P. (2014). Biocontrol potentiality of plant growth promoting bacteria (PGPR)-*Pseudomonas* fluorescens and *Bacillus subtilis*: a review. *Afr. J. Agric. Res.* 9, 1265–1277.

Tarkka, M., Schrey, S., and Hampp, R. (2008). "Plant associated soil micro-organisms," in *Molecular Mechanisms of Plant and Microbe Coexistence*, eds C. S. Nautiyal and P. Dion (Berlin: Springer), 3–51. doi: 10.1007/978-3-540-75575-3_1

Teramoto, S., Takayasu, S., Kitomi, Y., Arai-Sanoh, Y., Tanabata, T., and Uga, Y. (2020). High-throughput three-dimensional visualization of root system architecture of rice using X-ray computed tomography. *Plant Methods* 16, 1–14.

Tisdall, J. (2001). *Beginning Perl for Bioinformatics: An Introduction to Perl for Biologists*. Sebastopol, CA: O'Reilly Media, Inc.

Treseder, K. K. (2008). Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecol. Lett.* 11, 1111–1120. doi: 10.1111/j.1461-0248.2008.01230.x

van der Molen, M. K., Dolman, A. J., Ciais, P., Eglin, T., Gobron, N., Law, B. E., et al. (2011). Drought and ecosystem carbon cycling. *Agric. For. Meteorol.* 151, 765–773.

Verma, A., Niranjana, M., Jha, S., Mallick, N., and Agarwal, P. (2020). QTL detection and putative candidate gene prediction for leaf rolling under moisture stress condition in wheat. *Sci. Rep.* 10, 1–13.

Waldrop, M., and Firestone, M. (2006). Response of microbial community composition and function to soil climate change. *Microb. Ecol.* 52, 716–724. doi: 10.1007/s00248-006-9103-3

Wang, L., Xiang, L. I., Tang, S., Huang, H., Jing, Y., Tan, F., et al. (2013). Characteristic study of a new sugarcane variety GT39. *Agric. Sci. Technol.* 14, 1550–1553.

Wang, S., Cheng, J., and Liao, Y. (2020). Response of Rhizosphere microecology to plants, soil and microbes. *Acta Microsc.* 29:43150.

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setälä, H., Van Der Putten, W. H., and Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science* 304, 1629–1633. doi: 10.1126/science.1094875

Wei, T., Simko, V., Levy, M., Xie, Y., Jin, Y., and Zemla, J. (2017). Package 'corrplot'. *Statistician* 56:e24.

Welsh, D. (2000). Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiol. Rev.* 24, 263–290. doi: 10.1111/j.1574-6976.2000.tb00542.x

Wen, T., Zhao, M., Liu, T., Huang, Q., Yuan, J., and Shen, Q. (2020). High abundance of Ralstonia solanacearum changed tomato rhizosphere microbiome and metabolome. *BMC Plant Biol.* 20:166. doi: 10.1186/s12870-020-02365-9

Yandigeri, M. S., Meena, K. K., Singh, D., Malviya, N., Singh, D. P., Solanki, M. K., et al. (2012). Drought-tolerant endophytic actinobacteria promote growth of wheat (*Triticum aestivum*) under water stress conditions. *Plant Growth Regul.* 68, 411–420. doi: 10.1007/s10725-012-9730-2

Yao, J., Sun, D., Cen, H., Xu, H., Weng, H., Yuan, F., et al. (2018). Phenotyping of *Arabidopsis* drought stress response using kinetic chlorophyll fluorescence and multicolor fluorescence imaging. *Front. Plant Sci.* 9:603. doi: 10.3389/fpls.2018.00603

Yasmin, H., Bano, A., Wilson, N. L., Nosheen, A., Naz, R., Hassan, M. N., et al. (2021). Drought tolerant *Pseudomonas* sp. showed differential expression of stress-responsive genes and induced drought tolerance in *Arabidopsis thaliana*. *Physiol. Plant.* [Epub ahead of print].

Yasmin, H., Rashid, U., Hassan, M. N., Nosheen, A., Naz, R., Ilyas, N., et al. (2020). Volatile organic compounds produced by *Pseudomonas* pseudoalcaligenes alleviated drought stress by modulating defence system in Maize (*Zea mays* L.). *Physiol. Plant.* 172, 896–911. doi: 10.1111/ppl.13304

Yin, S., Contest, W., Xiaoyu, H., Wenyan, L., Wenlan, L., and Baoshan, C. (2020). Response of new series of mid-cane varieties to drought stress and evaluation of drought resistance. *S. Agric. J.* 51:200.

Yu, G., Smith, D. K., Zhu, H., Guan, Y., and Lam, T. T. Y. (2017). ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol. Evol.* 8, 28–36. doi: 10.1111/2041-210x.12628

Zhao, P., Liu, P., Yuan, G., Jia, J., Li, X., Qi, D., et al. (2016). New insights on drought stress response by global investigation of gene expression changes in Sheepgrass (*Leymus chinensis*). *Front. Plant Sci.* 7:954. doi: 10.3389/fpls.2016.00954

Zhao, X., Liu, Q., Xie, S., Jiang, Y., Yang, H., Wang, Z., et al. (2020b). Response of soil fungal community to drought-resistant Ea-DREB2B transgenic sugarcane. *Front. Microbiol.* 11:2329.

Zhao, X., Jiang, Y., Liu, Q., Yang, H., Wang, Z., and Zhang, M. (2020a). Effects of drought-tolerant Ea-DREB2B transgenic sugarcane on bacterial communities in soil. *Front. Microbiol.* 11: 704.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fmicb.2021.754837

Identification of the Important Genes of *Bradyrhizobium diazoefficiens* 113-2 Involved in Soybean Nodule Development and Senescence

Songli Yuan[1†], Shunxin Zhou[1†], Yong Feng[2], Chanjuan Zhang[1], Yi Huang[1], Zhihui Shan[1], Shuilian Chen[1], Wei Guo[1], Hongli Yang[1], Zhonglu Yang[1], Dezhen Qiu[1], Haifeng Chen[1] and Xinan Zhou[1]**

[1] Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture and Rural Affairs of PRC, Oil Crops Research Institute of Chinese Academy of Agriculture Sciences, Wuhan, China, [2] Shenzhen Branch, Guangdong Laboratory of Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture and Rural Affairs, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Yuan-Ming Zhang, Huazhong Agricultural University, China
Esther Menendez, University of Evora, Portugal

***Correspondence:**
Haifeng Chen
chenhaifeng@caas.cn
Xinan Zhou
zhouxinan@caas.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *07 August 2021*
Accepted: *04 October 2021*
Published: *11 November 2021*

Citation:
Yuan S, Zhou S, Feng Y, Zhang C, Huang Y, Shan Z, Chen S, Guo W, Yang H, Yang Z, Qiu D, Chen H and Zhou X (2021) Identification of the Important Genes of Bradyrhizobium diazoefficiens 113-2 Involved in Soybean Nodule Development and Senescence. Front. Microbiol. 12:754837. doi: 10.3389/fmicb.2021.754837

Legume nodule development and senescence directly affect nitrogen fixation efficiency and involve a programmed series of molecular events. These molecular events are carried out synchronously by legumes and rhizobia. The characteristics and molecular mechanisms of nitrogen fixation at soybean important developmental stages play critical roles in soybean cultivation and fertilizer application. Although the gene expression of soybean were analyzed in nodules at five important soybean developmental stages, information on the expression of rhizobial genes in these nodule samples is limited. In the present study, we investigated the expression of *Bradyrhizobium diazoefficiens* 113-2 genes in the nodule samples from five developmental stages of soybean (Branching stage, flowering stage, fruiting stage, pod stage and harvest stage). Similar gene expression patterns of *B. diazoefficiens* 113-2 were existed during optimal symbiotic functioning, while different expression patterns were found among early nodule development, nitrogen fixation progress and nodule senescence. Besides, we identified 164 important different expression genes (DEGs) associated with nodule development and senescence. These DEGs included those encoding nod, nif, fix proteins and T3SS secretion system-related proteins, as well as proteins involved in nitrogen metabolism, ABC transporters and two-component system pathways. Gene Ontology, KEGG pathway and homology analysis of the identified DEGs revealed that most of these DEGs are uncharacterized genes associated with nodule development and senescence, and they are not core genes among the rhizobia genomes. Our results provide new clues for the understanding of the genetic determinants of soil rhizobia in nodule development and senescence, and supply theoretical basis for the creation of high efficiency soybean cultivation technology.

Keywords: soybean symbiotic nitrogen fixation, soybean developmental stages, rhizobial gene expression, rhizobial DEGs, nodule development and senescence

INTRODUCTION

The symbiotic relationship between legume and soil rhizobia leads to the formation of root nodule and fixation of atmospheric nitrogen (Ferguson et al., 2010). Depending on the persistence of the nodule meristem and morphology characteristics of nodules, nodules in legumes can be divided into determinate nodules and indeterminate nodules (Hirsch, 1992). Nodules formed in *Glycine max* and *Lotus japonicus* are determinate nodules, they have non-persistent meristems and predominately contain one specific form of the bacterial development, which is determined by the nodule's age (Karr and Emerich, 1988; Karr et al., 1990). Indeterminate nodules, such as those in *Medicago truncatula* and *Astragalus sinicus*, contain persistent meristems and a mixture of bacteroid forms in all stages of development (Vasse et al., 1990). In both determinate and indeterminate nodules, nodule development directly affects nitrogen fixation activity, and the premature senescence of nodules negatively regulates the efficiency of symbiotic nitrogen fixation (Serova et al., 2018). Delaying nodule senescence is a practical measure to increase nitrogen fixation amount and solve the problem of late defertilization in legume crops. Therefore, studies on the root nodule development and senescence are of great significance in the efficient use of nitrogen sources and legume crop yield.

Legume nodule development is initiated by an exchange of chemical signals between legumes and soil rhizobia, and included lots of protein-protein interactions between legumes and soil rhizobia proteins (Zhang et al., 2018). The leguminous molecular events involved in nodulation and nodule development are quite well understood (Li Q. G. et al., 2013; Mergaert et al., 2020; Roy et al., 2020). The characteristics and the corresponding detection methods, the key leguminous genes currently known to be involved in the regulation of nodule senescence and various abiotic factors affecting nodule senescence were summarized in our previous review (Zhou et al., 2021). Except for legumes, the soil rhizobia also play critical roles in symbiotic nitrogen fixation progress (Lindström and Mousavi, 2020). Because Nod factors (NFs), surface polysaccharides and secreted proteins are three main determinants of host specificity in most rhizobia, the rhizobial genes that affect the biological synthesis of these signaling molecules were explored in nodulation, including *nod*, *nif*, *fix* genes, surface polysaccharides biosynthesis genes and secretion system-related genes (Wang et al., 2014; Li et al., 2020). Besides, it has been demonstrated that several rhizobial genes play key roles in nodule senescence. *SmgshB* is a critical gene for the synthesis of glutathione, which is a carbon source that can ensure the normal development of nodules and can suppress the early senescence of nodules (Cheng et al., 2017; Yang et al., 2020). *lrpL-acdS* genes, which encode an ACC deaminase enzyme, are associated with nitrogen fixation and delayed nodule senescence (Tittabutr et al., 2015). The sigma factors *RpoH1* and *RpoH2* are involved in different stress responses and negatively regulate nodule senescence (Martínez-Salazar et al., 2009). *Anabaena variabilis* flavodoxin, a protein involved in the response to oxidative stress, can significantly delay nodule senescence in alfalfa (Redondo et al., 2009). Therefore, both leguminous genes and rhizobial genes are critical to nodule development and senescence.

Soybean (*Glycine max*) is an important leguminous crop and has been grown worldwide for edible oil, food and feed material (Palander et al., 2005). Large amounts of N from the atmosphere fixed by soybean symbiotic nitrogen fixation can be widely available for growth of soybean and other intercropping and rotation crops (Biswas and Gresshoff, 2014). Branching stage, flowering stage, fruiting stage, pod stage and harvest stage are five important developmental stages for soybean cultivation and fertilizer application studies, and the nitrogen fixation characteristics of these stages have been well studied (Fehr et al., 1971). In our previous study, the expression of nodule genes at above-mentioned five stages was assessed quantitatively using RNA-Seq, we only analyzed the important different expression genes (DEGs) of soybean in nodule development and senescence, but not of *Bradyrhizobium diazoefficiens* 113-2 (Yuan et al., 2017).

B. diazoefficiens 113-2 was collected from paddy fields in Hengyang area of Hunan Province, China in 1972 by Xuejiang Zhang, and its genome shared a large synteny blocks and a high ANI value with that of *B. diazoefficiens* USDA110 (Li et al., 2020). In this report, we investigated the expression of *B. diazoefficiens* 113-2 genes in the nodule samples from above-mentioned five developmental stages of soybean and identified 164 important DEGs associated with nodule development and senescence. These DEGs included those encoding nod, nif, fix proteins and T3SS secretion system-related proteins, as well as proteins involved in nitrogen metabolism, ABC transporters and two-component system pathways. Our results firstly connected the action mode of rhizobial genes during nodule development with the developmental stages of soybean, which should provide new clues for the understanding of the genetic determinants of soil rhizobia in nodule development and senescence, and shed new light on the molecular mechanisms of nitrogen fixation at above-mentioned five developmental stages of soybean.

RESULTS

Expression Analysis of the Genes of *B. diazoefficiens* 113-2

We previously performed RNA-Seq analysis for five different nodule samples from five important development stages of soybean (Yuan et al., 2017) and sequenced the genome of *B. diazoefficiens* 113-2 (Li et al., 2020). In this report, we want to investigate the expression of the genes of *B. diazoefficiens* 113-2 in nodule development and senescence by using the above-mentioned RNA-Seq data. The genome and gene mapping details of *B. diazoefficiens* 113-2 were shown in **Supplementary Table 1**, and the results of sequencing saturation, reads coverage and reads random analyses were displayed in **Supplementary Figures 1–3**. As shown in **Table 1**, 2234 (about 25.38%) genes of *B. diazoefficiens* 113-2 were not identified in these five nodule samples, and more than 30% of the genes (except for Pod stage_N) contained meaningless fpkm values (0∼1). For the rest genes, most of the fpkm values were between 1 and 1,000, and

TABLE 1 | The numbers of *B. diazoefficiens* 113-2 genes in different fpkm value groups in five nodule samples.

	fpkm value				
	>10,000	1,000∼10,000	1∼1,000	0∼1	NA
Branching stage_N	16 (0.18%)	223 (2.53%)	2087 (23.71%)	4241 (48.19%)	2234 (25.38%)
Flowering stage_N	18 (0.20%)	181 (2.06%)	2362 (26.84%)	4006 (45.52%)	2234 (25.38%)
Fruiting stage_N	18 (0.20%)	208 (2.36%)	3293 (37.42%)	3048 (34.63%)	2234 (25.38%)
Pod stage_N	18 (0.20%)	210 (2.39%)	5260 (59.77%)	1079 (12.26%)	2234 (25.38%)
Harvest stage_N	25 (0.28%)	190 (2.16%)	3632 (41.27%)	2720 (30.91%)	2234 (25.38%)

a very small number of the fpkm values were above 10,000 (**Table 1**). Besides, the numbers of genes with meaningful fpkm values (>1) at one or more nodule samples were shown in **Figure 1A**. There are 966 genes consistently expressed at five nodule samples, 1085 genes were consistently found at four nodule samples, and 1252, 1552, and 1771 genes expressed at three, two and only one nodule sample, respectively. The detail fpkm information was shown in **Supplementary Table 2**.

To study the correlation of the expressions of *B. diazoefficiens* 113-2 genes among the five nodule samples, we calculated the Pearson correlation coefficients of each two nodule samples based on the total gene expression levels (**Figure 1B**). The Pearson correlation coefficients between Branching stage_N and the other four nodule samples were 0.54∼0.63, indicating that the expression of the genes of *B. diazoefficiens* 113-2 in Branching stage_N was very different from other four nodule samples. The Pearson correlation coefficients between harvest stage_N and flowering stage_N, fruiting stage_N or pod stage_N were less than 0.9, suggesting that relative lower correlation between these nodule samples. While the Pearson correlation coefficients among flowering stage_N, fruiting stage_N and pod stage_N were more than 0.9, indicating that relative higher correlation were existed in these three nodule samples.

Different Expression Gene Identification Between Different Nodule Samples

To screen the important genes of *B. diazoefficiens* 113-2 involved in nodule development and senescence, a false discovery rate (FDR) $\leq$ 0.001 and $|\log2\ \text{ratio}| \geq 1$ were used to identify the DEGs in the ten Groups. The numbers of up-regulated and down-regulated DEGs in these ten Groups were shown in **Figure 2A**, and the ID information on these DEGs was provided in **Supplementary Table 3**. The Group (Harvest vs. Branching) possessed the highest number of DEGs (36 up-regulated, 47 down-regulated), followed by Pod vs. Branching Group (33 up-regulated, 47 down-regulated) and Fruiting vs. Branching Group (34 up-regulated, 31 down-regulated). Besides, Harvest vs. Flowering Group (27 up-regulated, 25 down-regulated), Harvest vs. Pod Group (28 up-regulated, 13 down-regulated) and Harvest vs. Fruiting Group (19 up-regulated, 18 down-regulated) also had relative high number of DEGs. There were relatively low DEG numbers among the three Groups (Fruiting vs. Flowering, Pod vs. Flowering and Pod vs. Fruiting).

According to different reference developmental stages N, above-mentioned ten Groups were divided into four Classes (**Figure 2A**). The number of DEGs consistently found in all four Classes was 15 and the total number of DEGs identified during the soybean nodule development was 164 (**Figure 2B**).

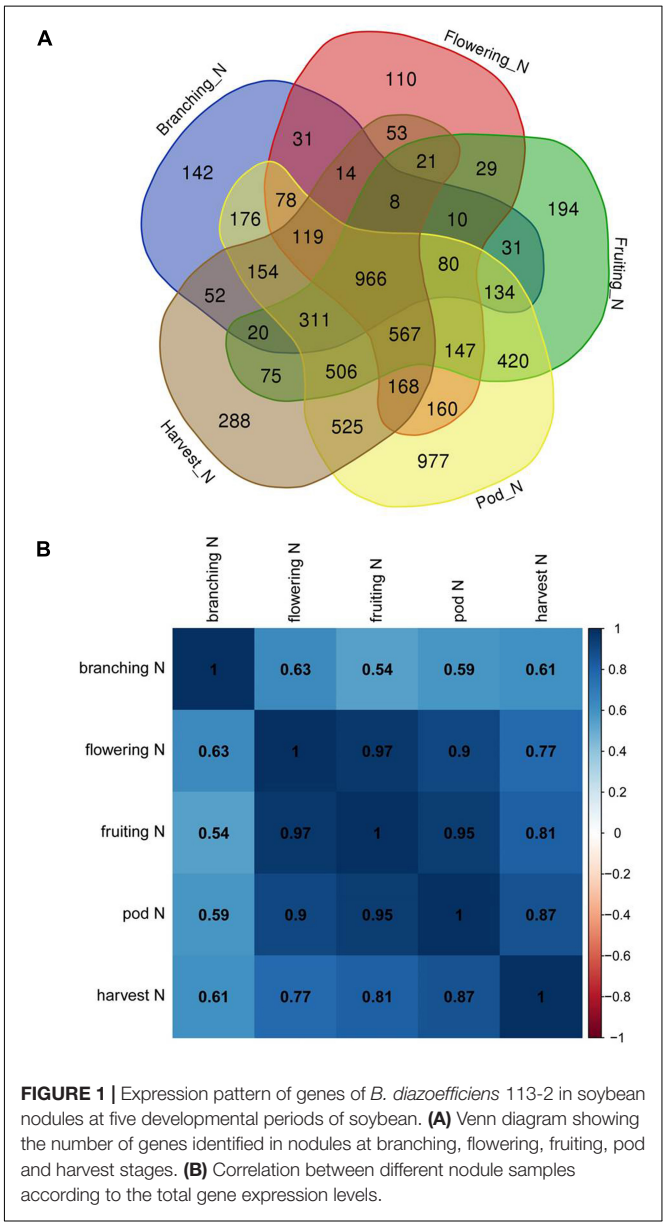


FIGURE 1 | Expression pattern of genes of *B. diazoefficiens* 113-2 in soybean nodules at five developmental periods of soybean. **(A)** Venn diagram showing the number of genes identified in nodules at branching, flowering, fruiting, pod and harvest stages. **(B)** Correlation between different nodule samples according to the total gene expression levels.

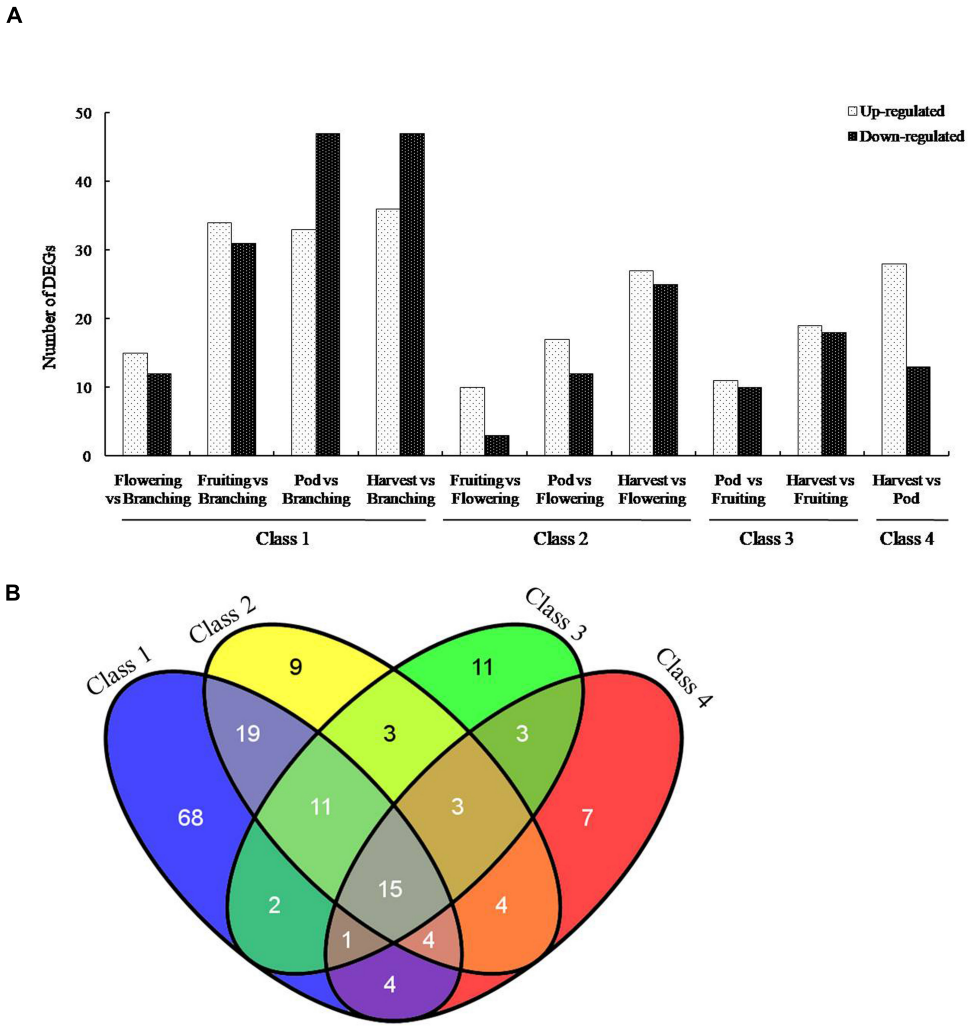


FIGURE 2 | Genes differentially expressed in soybean nodules at five developmental periods of soybean. **(A)** Differentially expressed genes (DEGs) of *B. diazoefficiens* 113-2 between different nodule samples from five developmental periods of soybean. These DEGs were separated into two groups according to whether they were significantly up-regulated or down-regulated. Class 1, Flowering vs. Branching∪Fruiting vs. Branching∪Pod vs. Branching ∪ Harvest vs. Branching. Class 2, Fruiting vs. Flowering ∪Pod vs. Flowering∪ Harvest vs. Flowering. Class 3, Pod vs. Fruiting∪Harvest vs. Fruiting. Class 4, Harvest vs. Pod. **(B)** Venn diagrams showing the number of DEGs in each gene set for the four classes.

DEG Annotation and Expression Pattern Analysis

The above-mentioned 164 DEGs encoded peptides with 41∼3105 amino acid residues, and their detail sequence information was shown in **Supplementary Table 4**. These DEGs were annotated to evaluate their potential functions. Among them, five DEGs-encoding nodulation proteins (NodG, NolG, NoeK, and NoeJ), nine DEGs-encoding Nif proteins (NifB, NifD, NifE, NifH, NifK, NifN, NifQ, NifW, and NifX), three DEGs-encoding Fix proteins (FixA, FixC, and FixK), three DEGs-encoding transcriptional regulator proteins and nine DEGs-encoding ABC transporter-related proteins were identified. Besides, 27 DEGs were involved in type-III secretion system (T3SS), eight DEGs located in membrane and many DEGs encoded various proteases (**Supplementary Table 5**).

To investigate the expression profile of the 164 DEGs during nodule development and senescence, we analyzed the RNA-Seq results of these DEGs at above-mentioned five nodule samples, and the result showed that these DEGs had various expression patterns (**Figure 3**). 64 DEGs reached their peaks at branching stage_N or flowering stage_N, and most of them were down-regulated during nodule development and senescence (**Figure 3A**), indicating that these DEGs mainly play roles in the early stages of nodule development. 45 DEGs reached their peaks at flowering stage_N or fruiting stage_N, and had relative low expression at branching stage_N or harvest stage_N (**Figure 3B**), suggesting that these DEGs mainly participate in nitrogen fixation progress. About 55 DEGs reached their peaks at harvest stage_N or pod stage_N, and most of them were up-regulated during nodule development and senescence

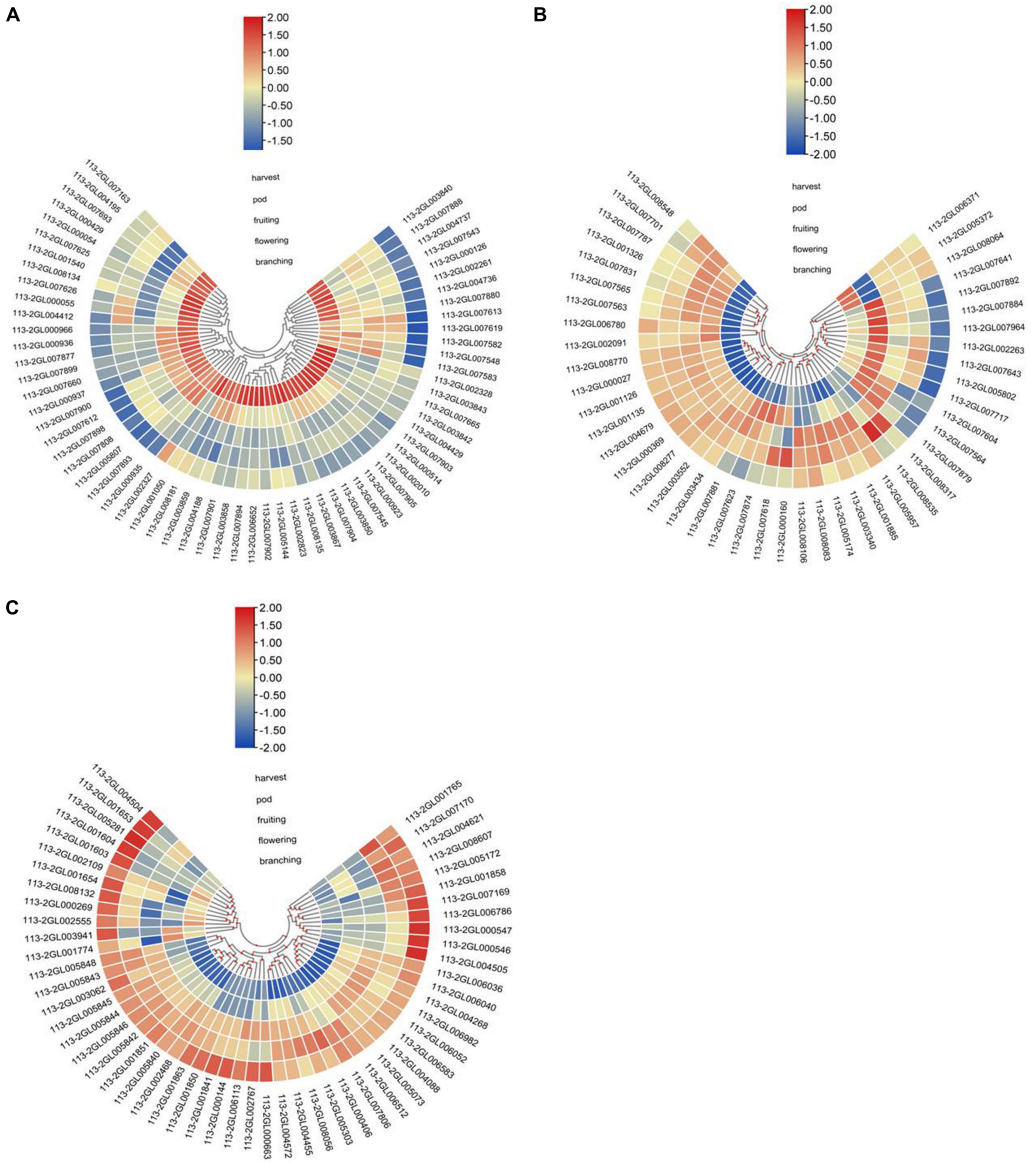


FIGURE 3 | Heatmap plot showing the expression profile of the DEGs during nodule development and senescence. **(A)** Heatmap of DEGs mainly expressed in early nodule development. **(B)** Heatmap of DEGs maily expressed in nitrogen fixation progress. **(C)** Heatmap of DEGs mainly expressed in nodule senescence. These five nodule samples include the nodules at branching stage (1), flowering stage (2), fruiting stage (3), pod stage (4) and harvest stage (5), and the pheatmap packages in R were used to product this heatmap.

(**Figure 3C**), indicating that these DEGs mainly play roles in nodule senescence.

DEG Functional Ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analysis

To evaluate the potential functions of the 164 DEGs, we used Gene Ontology (an internationally standardized gene function classification system) to classify these DEGs. Among these DEGs, 62 genes had no functional GO term, and a total of 150 GO terms were assigned for the rest genes, while about 2/3 (101 out of 150) functional GO terms were specific (only for one gene) (**Supplementary Table 6**). The rest49 Go terms were listed in **Figure 4** and were divided into three categories: cellular components, biological process and molecular function. The cellular components mainly included integral component of membrane and membrane. The main biological processes of the DEGs were oxidation-reduction process, nitrogen fixation, metabolic process, transport and regulation of transcription. The molecular functions associated with the DEGs mainly focused on oxidoreductase activity, catalytic activity, ATP binding, nucleic acid binding and DNA binding.

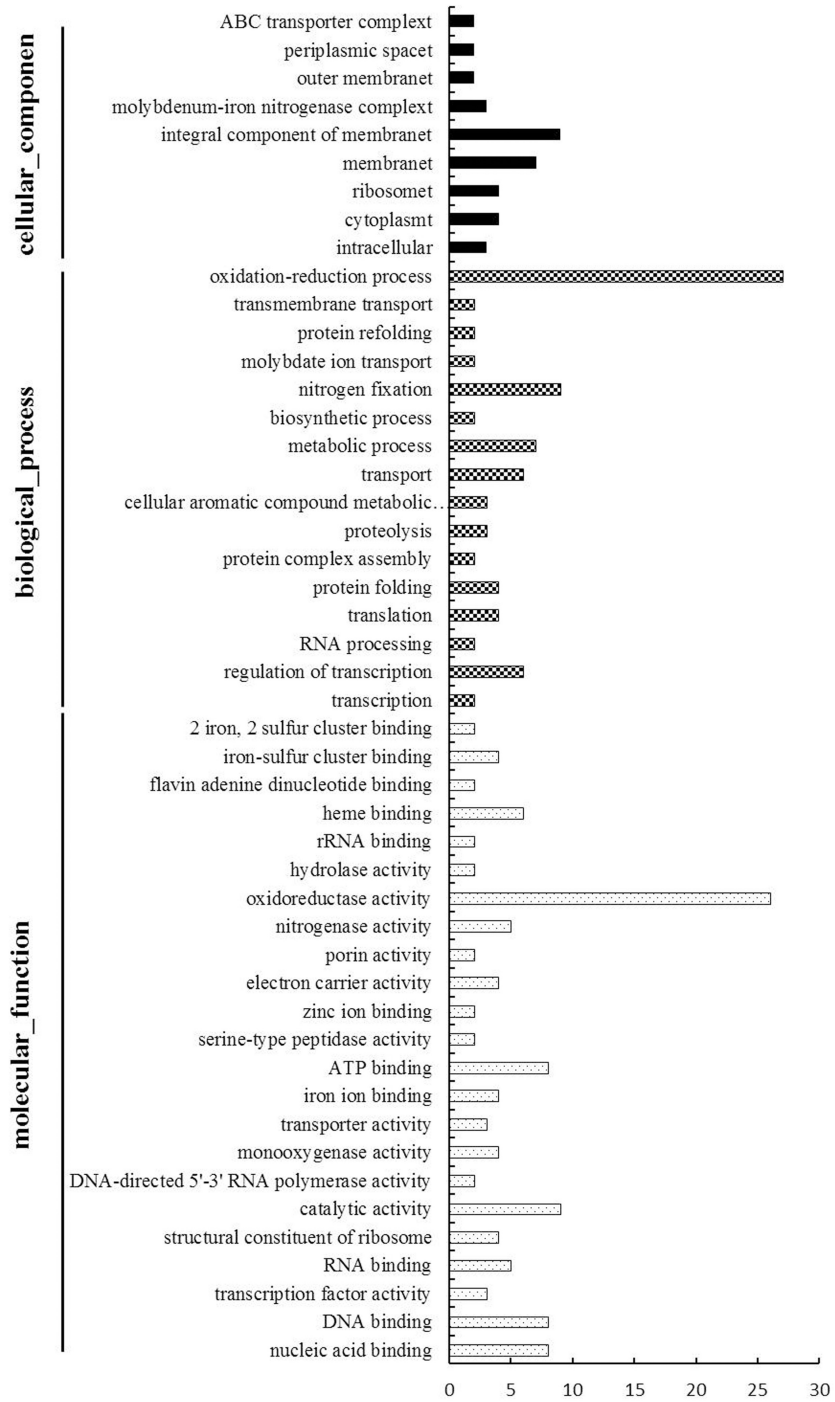


FIGURE 4 | Gene Ontology-based functional annotation of DEGs of *B. diazoefficiens* 113-2 between different nodules from different developmental periods of soybean. Three GO domains (biological process, cellular components, and molecular function) are shown and the numbers of genes in each term were shown in histograms.

KEGG is the major public database for pathway enrichment analysis (Kanehisa et al., 2016). In this report, we used KEGG database to classify these DEGs. Among these DEGs, 74 genes had no classified KEGG pathways, and a total of 149 KEGG pathway subgroups were assigned for the rest genes, while about 76% (113 out of 149) pathway subgroups were specific (only for one gene) (**Supplementary Table 7**). The other 36 pathway subgroups associated with the DEGs were shown in **Figure 5**. Most of these pathways were metabolic-related pathways. The pathways with the greatest numbers were metabolic pathway, followed by

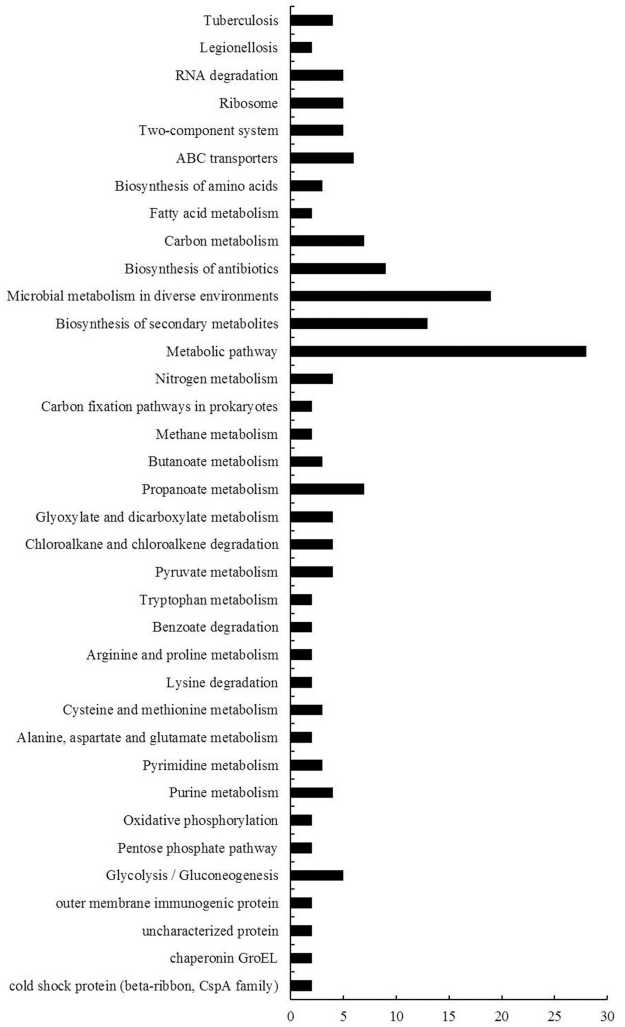


FIGURE 5 | KEGG pathway enrichment analyses of DEGs of *B. diazoefficiens* 113-2 between different nodules from different developmental periods of soybean. 36 KEGG pathways were analyzed and the x- and y-axes represent pathway categories and the number of genes in each pathway, respectively.

microbial metabolism in diverse environments, biosynthesis of secondary metabolites and biosynthesis of antibiotics.

DEGs Associated With Nitrogen Metabolism, ABC Transporters and Two-Component System Pathways

To investigate the regulation of the DEGs of 113-2 during nodule development and senescence, we analyzed nitrogen metabolism (K00910), ABC transporters (K02010) and Two-Component system (K02020) pathways (obtained by KEGG)[1] in more detail (**Figure 6**). Two KEGG gene sets in K00910, seven in K02010 and six in K02020 were differentially expressed during nodule development and senescence. One gene that matched K00910 pathway gene *NirK* (*113-2GL002109*) was up-regulated in Harvest vs. Branching, Harvest vs. Flowering, Harvest vs. Fruiting and Harvest vs. Pod, suggesting that this gene has relative higher expression in harvest stage nodules and plays key roles in nodule senescence. Among the three DEGs that matched k02010 pathway gene *nifDKH*, *113-2GL007881* has different expression patterns with *113-2GL007904* or *113-2GL007905* in Flowering vs. Branching, Fruiting vs. Branching, Pod vs. Branching and Harvest vs. Branching groups, indicating that *nifDKH* gene may play diverse roles in nodulation and nodule development. For K02010 pathway gene sets, three genes that matched *modC*, *modA, gltI, and aatJ* were down-regulated in all of the detected groups, the other gene that matched *pstS* was up-regulated in Pod vs. Branching and Pod vs. Flowering groups, and the rest two genes *lptG* and *urtA* were down-regulated then up-regulated during nodule development and senescence, suggesting that they may have roles in both nodulation and nodule senescence. Three K02020 pathway gene sets (*pstS*, *gltI* and *aatJ*) were the same as that of K02010 pathway, and among the rest three gene sets, one DEG that matched *fixK* (*113-2GL006786*) was up-regulated in all of the detected groups, one gene that matched *ccoN* (*113-2GL006780*) was up-regulated in most of the detected groups and only down-regulated in Fruiting vs. Flowering group, while the rest one DEG that matched *atoB* (*113-2GL005802*) was down-regulated in most of the detected groups and only up-regulated in Flowering vs. Branching group.

DEGs Encoding Nod and T3SS Secretion System-Related Proteins

In most rhizobia, NFs, surface polysaccharides and secreted proteins are the main determinants of host specificity (Putnoky et al., 1988; Lorkiewicz, 1997; Li et al., 2014). To examine whether these three signaling molecules also play roles during nodule development and senescence, we explored DEGs that required for the biological synthesis of these signaling molecules between different nodules from different developmental periods of soybean, including five *Nod* genes, eight *nif* genes, three *fix* genes and 27 T3SS-related genes (**Figure 7**). 16 DEGs (two *Nod* genes, seven *nif* genes, one *fix* gene and six T3SS-related genes) were down-regulated in all of the detected groups, suggesting that these genes mainly play roles in nodule development rather than nodule senescence. Ten DEGs (one *Nod* gene, one *fix* gene and eight T3SS-related genes) were up-regulated in all of the detected groups, and most of these genes (except for *113-2GL007701*) have relative higher expression in pod stage nodules or harvest stage nodules, meaning that these genes may participate in regulating nodule senescence. Seven DEGs (*113-2GL007881*, *113-2GL007641*, *113-2GL007564*, *113-2GL007604*, *113-2GL007623*, *113-2GL007879,* and *113-2GL007892*) have highest expression in flowering stage nodules or fruiting stage nodules, reflecting that these genes may be critical to nitrogen fixation progress. Four DEGs (*113-2GL001050*, *113-2GL008064*, *113-2GL002555,* and *113-2GL004188*) were down-regulated then up-regulated during nodule development and senescence, suggesting that these genes may play roles in both in the early stages of nodule development and nodule senescence. Five genes (*113-2GL000406*, *113-2GL005174*,

[1]http://www.kegg.jp/kegg/kegg1.html

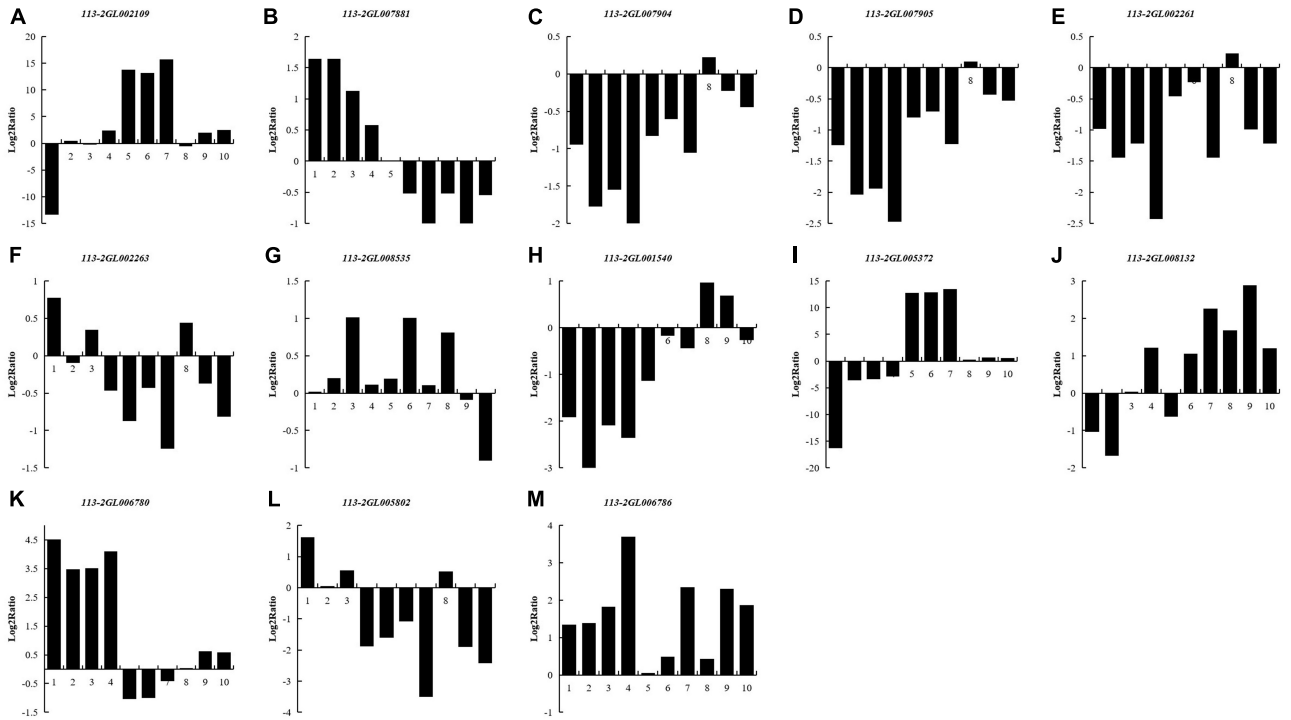


FIGURE 6 | List of the DEGs associated with three pathways between nodules from different developmental periods of soybean. Nitrogen metabolism (Ko00910) **(A–D)**: NirK (113-2GL002109), nifDKH (113-2GL007881, 113-2GL007904 and 113-2GL007905); ABC transporters (Ko02010) **(E–J)**: modC (113-2GL002261), modA (113-2GL002263), pstS (113-2GL008535), gltI, aatJ (113-2GL001540), lptG (113-2GL005372), urtA (113-2GL008132); Two-component system (Ko02020) **(G,H,K–M)**: pstS (113-2GL008535), gltI, aatJ (113-2GL001540), ccoN (113-2GL006780), atoB (113-2GL005802) and fixK (113-2GL006786). 1, branching stage vs. flowering stage; 2, branching stage vs. fruiting stage; 3, branching stage vs. pod stage; 4, branching stage vs. harvest stage; 5, flowering stage vs. fruiting stage; 6, flowering stage vs. pod stage; 7, flowering stage vs. harvest stage; 8, fruiting stage vs. pod stage; 9, fruiting stage vs. harvest stage; 10, pod stage vs. harvest stage.

113-2GL006780, *113-2GL007717,* and *113-2GL008548*) have relative high expression in flowering, fruiting or pod stage nodules, indicating that they mainly play roles in nitrogen fixation progress. The rest one gene (*113-2GL007717*) was only down-regulated in Harvest/Flowering group.

Orthologs of the DEGs

To further study the roles of these rhizobial DEGs during nodule development and senescence, we identified their orthologs in 113-2 itself, USDA110, *Mesorhizobium loti* MAFF303099, *Mesorhizobium huakuii* 7653R and *Sinorhizobium meliloti* 2011. Four homologous pairs (*113-2GL000406*/*113-2GL001850*, *113-2GL004736*/*113-2GL006371*, 113-2GL004195/113-2GL002328, and 113-2GL003941/113-2GL001604) were existed in these 164 DEGs. 16 genes have no orthologs, and most of them have no COG functions (**Supplementary Table 8**), indicating that these DEGs were mainly strain-specific. 84 DEGs have only orthologs in USDA110, suggesting that these DEGs were mainly species-specific or host specific, and among these DEGs, about 13.1% (11 out of 84) have orthologs in 113-2 itself and more than one orthologs in USDA110 (**Table 2**). Among the rest 62 DEGs, 35 DEGs have one or more orthologs in all of the five rhizobia, 11 DEGs have orthologs in four rhizobia and 16 DEGs have orthologs in three rhizobial, reflecting that not all of these nodule development and senescence-related DEGs are core genes among these four rhizobial (**Table 3**). Among the core 35 DEGs, about 60% (21 out 35) were mainly expressed in the early stages of nodule development, six DEGs showed highest expression in the progress of nitrogen fixation, and eight DEGs may play critical roles in nodule senescence, indicating that these core genes have diverse roles during nodule development and senescence (**Table 3**). Among these four rhizobia, 113-2 or USDA110 and *M. loti* MAFF303099 can form determinate nodules with soybean and *Lotus*, respectively, and nine DEGs of 113-2 have orthologs only in USDA110 and *M. loti* MAFF303099 (**Table 3**). Besides, we selected four gene groups with more orthologs to perform phylogeny analyses, and the results revealed closer phylogenetic relationships between the two strains in the same genus (**Supplementary Figures 4–7**).

Our previous study has indicated that 113-2 has an extremely close phylogenetic relationship to USDA110 (Li et al., 2020). An interspecies protein interactome was constructed and 290 USDA110 proteins associated with soybean symbiosis were provided (Zhang et al., 2018). In this report, about 203 USDA110 proteins were searched from the orthologs of the DEGs (**Tables 2**, **3**). We compared these two USDA110 protein groups and discovered six same proteins (bll7322, blr1971, blr1091, blr1516, blr0462, and bll7600), which were detected to be expressed in rhizoidal of root nodules during symbiosis in previous studies (Pessi et al., 2007; Delmotte et al., 2010;

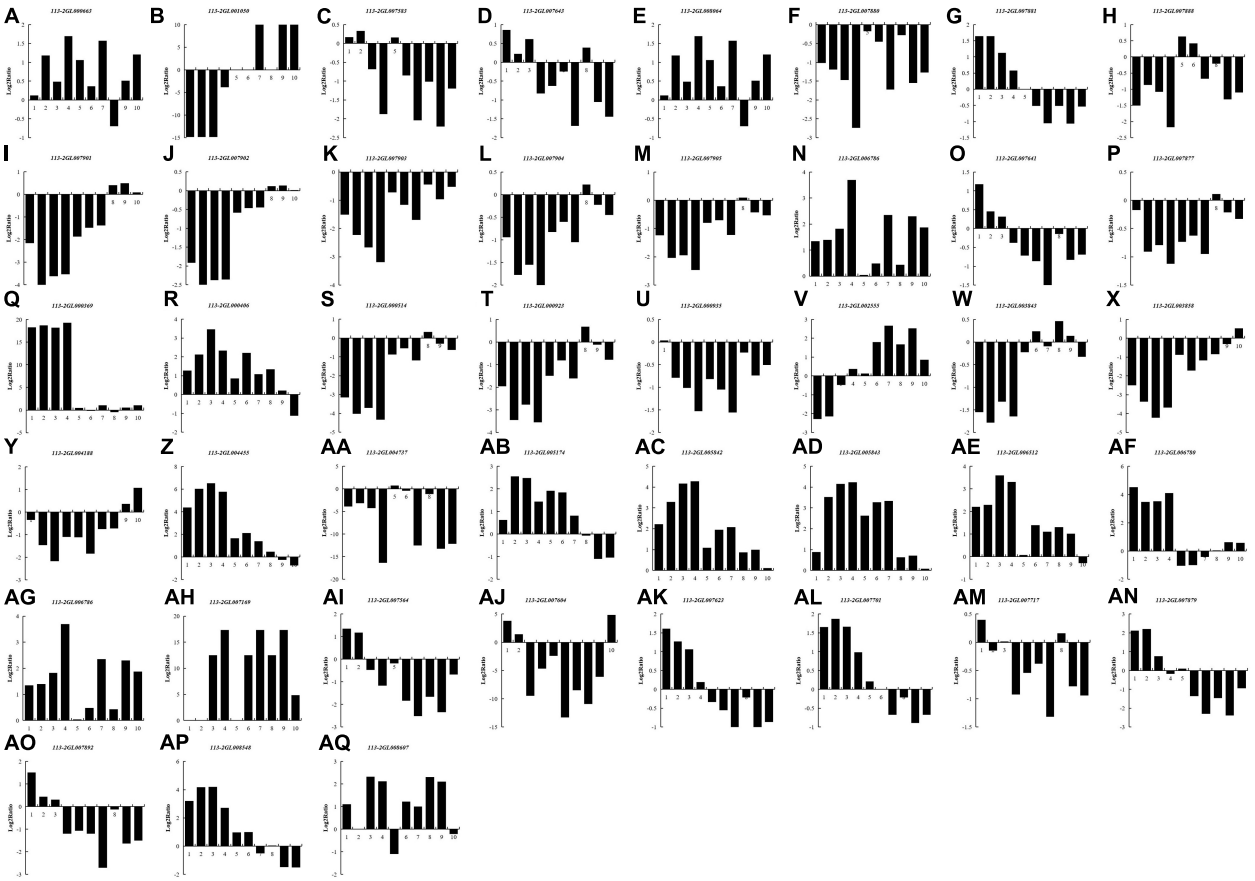


FIGURE 7 | The Log2Ratio values of the DEG encoding Nod-related genes and T3SS secretion system-related genes during nodule development and senescence. *Nod* genes: **(A–E)**; *Nif* genes: **(F–M)**; *Fix* genes: **(N–P)**; T3SS-related genes: **(Q–AQ)**. 1, branching stage vs. flowering stage; 2, branching stage vs. fruiting stage; 3, branching stage vs. pod stage; 4, branching stage vs. harvest stage; 5, flowering stage vs. fruiting stage; 6, flowering stage vs. pod stage; 7, flowering stage vs. harvest stage; 8, fruiting stage vs. pod stage; 9, fruiting stage vs. harvest stage; 10, pod stage vs. harvest stage.

Čuklina et al., 2016; Zhang et al., 2018). Then we predict the interaction proteins of the six DEGs (orthologs of the six USDA110 proteins) according to the resources from the constructed protein interactome (Zhang et al., 2018), and the results were showed in **Table 4**. These rhizoidal proteins have more than two predicted interacting proteins in soybean, especially for bll7322 or 113-2GL000406 or 113-2GL001850, and blr1971 or 113-2GL007693; they have 21 and 25 predicted interacting proteins in soybean, respectively.

DISCUSSION

Symbiotic nitrogen fixation system is of great significance in agriculture and ecology, and nodule development and senescence directly affects nitrogen fixation efficiency. Nodule development and senescence involves a programmed series of molecular events that are carried out synchronously by both legumes and rhizobia (Vasse et al., 1990; Cermola et al., 2000; Oldroyd et al., 2011; Roux et al., 2014; Alemneh et al., 2020). The nitrogen fixation characteristics of five soybean developmental stages (Branching stage, flowering stage, fruiting stage, pod stage and harvest stage) have been well studied (Fehr et al., 1971), the expression of nodule genes at these developmental stages was assessed quantitatively using RNA-Seq, and the important DEGs of soybean that associated with nodule development and senescence were identified in our previous study (Yuan et al., 2017). In the present study, we firstly investigated the expression of rhizobial genes in the nodule samples from above-mentioned five developmental stages of soybean by using our previous RNA-Seq data (Yuan et al., 2017)and identified 164 important rhizobial DEGs associated with nodule development and senescence.

The Gene Expression of *B. diazoefficiens* 113-2 at Different Nodule Samples From Different Soybean Developmental Stages

Previous comparative transcriptomic analyses have explored the gene expression patterns of rhizobia during chemoautotrophic growth (Franck et al., 2008) or symbiotic non-growth (Vercruysse et al., 2011), under different stresses (Ampe et al., 2003; Chang et al., 2007; Cytryn et al., 2007), in determinate

TABLE 2 | Analysis of species-specific or host specific DEGs.

DEGs	113-2	USDA110	COG annotion
113-2GL000027		*bll0805*	CubicO group peptidase, beta-lactamase class C family
113-2GL000144		*blr0694*	Serine protease, subtilase family
113-2GL000369		*blr0497*	–
113-2GL000546		*bll0333*	Glucose dehydrogenase
113-2GL000547		*bll0332*	Cytochrome c, mono- and diheme variants
113-2GL000923	*113-2GL007956*	*blr1693,bll8244*	–
113-2GL000935		*bll8232*	L-lysine 2,3-aminomutase (EF-P beta-lysylation pathway)
113-2GL000936		*bll8231*	Gamma-glutamyltranspeptidase
113-2GL000937		*bll8230*	–
113-2GL000966		*bll8195*	Retron-type reverse transcriptase
113-2GL001135		*bll7952*	DNA-binding beta-propeller fold protein YncE
113-2GL001326		*bll7787*	–
113-2GL001540	*113-2GL005055,113-2GL005658*	*bll4384,bll7600,blr3849*	ABC-type amino acid transport/signal transduction system, periplasmic component/domain
113-2GL001603		*bll7540*	Fe-S oxidoreductase
113-2GL001604	*113-2GL003941*	*bll7539,blr5311*	–
113-2GL001653		*bll7492*	Predicted DNA-binding protein, contains Ribbon-helix-helix (RHH) domain
113-2GL001765		*bll7395*	Uncharacterized protein
113-2GL001774		*bll7385*	Cellulose biosynthesis protein BcsQ
113-2GL001885		*blr7289*	Flavin-dependent oxidoreductase, luciferase family
113-2GL002261		*blr6953*	ABC-type molybdate transport system, ATPase component
113-2GL002263		*blr6951*	ABC-type molybdate transport system, periplasmic component
113-2GL002327		*blr6889*	DNA-binding transcriptional regulator, MarR family
113-2GL002328	*113-2GL004195, 113-2GL004285*	*bll5076,bll4983,bll6888*	–
113-2GL002767		*bll6479*	ABC-type branched-chain amino acid transport system, periplasmic component
113-2GL002823		*bll6433*	–
113-2GL003062		*bll6129*	Uncharacterized protein
113-2GL004088		*bll5176*	–
113-2GL004268		*bsl5002*	–
113-2GL004412	*113-2GL004612, 113-2GL002719, 113-2GL004272, 113-2GL004615, 113-2GL001425, 113-2GL004090*	*blr4994,blr5174,bll6524, blr4699,bll4867,blr4701, blr7695*	Opacity protein and related surface antigens
113-2GL004429	*113-2GL005811*	*bll4853,blr3712*	Outer membrane protein assembly factor BamA
113-2GL004621		*bsr4694*	–
113-2GL004679		*blr4646*	CBS domain
113-2GL004737		*bll4594*	Fatty-acid desaturase
113-2GL005073		*bll4367*	Glyoxylase or a related metal-dependent hydrolase, beta-lactamase superfamily II
113-2GL005144		*bll4305*	Ribonuclease G or E
113-2GL005174		*bll4278*	–
113-2GL005281		*blr4186*	Glyoxylase or a related metal-dependent hydrolase, beta-lactamase superfamily II
113-2GL005303		*bsl4167*	–
113-2GL005372		*bll4106*	Lipopolysaccharide export LptBFGC system, permease protein LptF
113-2GL005840		*blr3683*	Chaperonin GroEL (HSP60 family)
113-2GL005842		*blr3681*	Predicted metal-dependent hydrolase, TIM-barrel fold
113-2GL005843		*blr3680*	–
113-2GL005844		*blr3679*	–
113-2GL005845		*blr3678*	Ferredoxin-NADP reductase
113-2GL005846		*blr3677*	–

(Continued)

TABLE 2 | (Continued)

DEGs	113-2	USDA110	COG annotion
113-2GL005848		*blr3675*	D-arabinose 1-dehydrogenase, Zn-dependent alcohol dehydrogenase family
113-2GL006036		*blr3477*	Aspartate/methionine/tyrosine aminotransferase
113-2GL006040		*blr3474*	Arginine/lysine/ornithine decarboxylase
113-2GL006052		*blr3464*	Uncharacterized protein
113-2GL006113		*blr3404*	Heme-degrading monooxygenase HmoA and related ABM domain proteins
113-2GL006583		*bll2956*	Sugar lactone lactonase YvrE
113-2GL007613	*113-2GL000934, 113-2GL007832, 113-2GL007909, 113-2GL000934, 113-2GL007752, 113-2GL007853, 113-2GL007932, 113-2GL004410*	*blr2165,blr7550,blr1829, blr1911,blr4868,blr1716, blr1702,bll4642,blr1740, blr8233,bll8293,blr2076, blr1807*	Transposase
113-2GL006652		*blr2887*	Uncharacterized protein
113-2GL006786	*113-2GL007581, 113-2GL006786, 113-2GL006050*	*bll3466,bll2109,bll2757*	cAMP-binding domain of CRP or a regulatory subunit of cAMP-dependent protein kinases
113-2GL006982		*bll2590*	Nucleotide-binding universal stress protein, UspA family
113-2GL007163		*bll2460*	Outer membrane receptor for ferrienterochelin and colicins
113-2GL007170		*blr2455*	Isocitrate lyase
113-2GL007564		*blr2132*	–
113-2GL007565		*blr2131*	Lysine/ornithine N-monooxygenase
113-2GL007582		*blr2108*	Non-ribosomal peptide synthetase component F
113-2GL007583		*blr2106*	Mannose-6-phosphate isomerase, cupin superfamily
113-2GL007612		*blr2077*	O-acetylhomoserine/O-acetylserine sulfhydrylase, pyridoxal phosphate-dependent
113-2GL007619		*bll2067*	–
113-2GL007623		*bll2063*	Acyl-CoA dehydrogenase related to the alkylation response protein AidB
113-2GL007660		*bll2012*	Opacity protein and related surface antigens
113-2GL007665		*bll2003*	Carbohydrate-selective porin OprB
113-2GL007693		*blr1971*	Dipeptidyl aminopeptidase/acylaminoacyl peptidase
113-2GL007717		*bll1944*	Opacity protein and related surface antigens
113-2GL007787	*113-2GL000704*	*bll0184,blr1879*	DnaJ-class molecular chaperone with C-terminal Zn finger domain
113-2GL007806	*113-2GL001935, 113-2GL001936*	*blr7243,blr1853,blr7242*	Cytochrome P450
113-2GL007808		blr1851	Uncharacterized protein YjbI, contains pentapeptide repeats
113-2GL007874		bll1777	Alkyl hydroperoxide reductase subunit AhpC (peroxiredoxin)
113-2GL007880		blr1770	–
113-2GL007884	113-2GL008289	blr1311,bll1766	Outer membrane protein W
113-2GL007893		bll1754	–
113-2GL007894		blr1753	Ferredoxin subunit of nitrite reductase or a ring-hydroxylating dioxygenase
113-2GL007900		blr1748	–
113-2GL007901		blr1747	Predicted Fe-Mo cluster-binding protein, NifX family
113-2GL007903		blr1745	Nitrogenase molybdenum-iron protein, alpha and beta chains
113-2GL008083		blr1496	Uncharacterized conserved protein YjbJ, UPF0337 family
113-2GL008134		bll1447	Superfamily II DNA and RNA helicase
113-2GL008317		bll1285	–
113-2GL008548		blr1081	1,2-phenylacetyl-CoA epoxidase, catalytic subunit
113-2GL008607	113-2GL006492	bll1028, blr3042	DNA-directed RNA polymerase specialized sigma subunit, sigma24 family

nodules and indeterminate nodules (Li Y. et al., 2013), and in different zones of the indeterminate nodule (Roux et al., 2014). For the bacteroids in soybean symbiosis, several transcriptomic analyses of bacteroids focused on symbiotic gene region (Hauser et al., 2006) and single time point during optimal symbiotic functioning (Chang et al., 2007; Pessi et al., 2007;

TABLE 3 | Orthologs of the selected DEGs in 113-2, USDA110, *M. loti* MAFF303099, *M. huakuii* 7653R, and *S. meliloti* 2011.

DEGs	Orthologs of the DEGs					Expression in soybean symbiosis
	113-2	USDA110	MAFF303099	7653R	SM2011	(according to Figure 3)
113-2GL000054		*bll0779*	*MAFF_RS22785*	*MCHK_RS30795*	*SM2011_RS15560*	Early stages of nodule development
113-2GL000055		*blr0778*	*MAFF_RS28315*	*MCHK_RS02430*	*SM2011_RS06535*	Early stages of nodule development
113-2GL000126		*bll0710*	*MAFF_RS20750*	*MCHK_RS28780*	*SM2011_RS15760*	Early stages of nodule development
113-2GL000160		*blr0678*	*MAFF_RS19575*	*MCHK_RS27375*	*SM2011_RS15250*	Nodule senescence
113-2GL000269		*blr0573*	*MAFF_RS17030*	*MCHK_RS25150*	*SM2011_RS31340*	Nodule senescence
113-2GL000406	*113-2GL001850*	*bll7322,blr0462*	*MAFF_RS17955*	*MCHK_RS26065*	*SM2011_RS30815*	Nodule senescence
113-2GL000429		*bll0442*	*MAFF_RS16950*	*MCHK_RS25060*	*SM2011_RS29850*	Early stages of nodule development
113-2GL000514	*113-2GL002082*	*bsr7117, blr0365*	*MAFF_RS13260*	*MCHK_RS21570*	*SM2011_RS24865*	Early stages of nodule development
113-2GL000663	*113-2GL005801*	*bll0225,blr3725*	*MAFF_RS16145*		*SM2011_RS31010*	Nodule senescence
113-2GL001050		*blr8272,bll8126*			*SM2011_RS09965*	Early stages of nodule development
113-2GL001126	*113-2GL004687*	*blr4637,blr7961*		*MCHK_RS12390, MCHK_RS18535*	*SM2011_RS03045*	Nitrogen fixation
113-2GL001654		*blr7491*		*MCHK_RS11600*		Nodule senescence
113-2GL001841		*blr7327*	*MAFF_RS27500*			Nodule senescence
113-2GL001851		*blr7321*	*MAFF_RS27480*		*SM2011_RS04580*	Nodule senescence
113-2GL001863		*bll7310*	*MAFF_RS27520*	*MCHK_RS18460*	*SM2011_RS04570, SM2011_RS01895*	Nodule senescence
113-2GL002091		*bsl7109*			*SM2011_RS33980*	Nitrogen fixation
113-2GL002109		*blr7089*		*MCHK_RS17580*	*SM2011_RS03405*	Nodule senescence
113-2GL002468		*bll6756*	*MAFF_RS00585*		*SM2011_RS21450*	Nodule senescence
113-2GL003340	*113-2GL001478*	*bll7648,bll5866*	*MAFF_RS18965*	*MCHK_RS27010*		Nitrogen fixation
113-2GL003434		*blr5774*		*MCHK_RS17320*		Nitrogen fixation
113-2GL003840		*bll5412*	*MAFF_RS02150*	*MCHK_RS09810*	*SM2011_RS21190*	Early stages of nodule development
113-2GL003842		*bll5410*	*MAFF_RS02160*	*MCHK_RS09820*	*SM2011_RS21200*	Early stages of nodule development
113-2GL003843		*bll5409*	*MAFF_RS02165*	*MCHK_RS09825*	*SM2011_RS21205*	Early stages of nodule development
113-2GL003850		*bll5402*	*MAFF_RS02200, MAFF_RS02105*		*SM2011_RS21230, SM2011_RS21150*	Early stages of nodule development
113-2GL003858		*bll5394*	*MAFF_RS02240*	*MCHK_RS09900*	*SM2011_RS21270*	Early stages of nodule development
113-2GL003859		*bll5393*	*MAFF_RS02245*	*MCHK_RS09905*	*SM2011_RS21275*	Early stages of nodule development
113-2GL003867		*bll5385*	*MAFF_RS02285*	*MCHK_RS09945*	*SM2011_RS21315*	Early stages of nodule development
113-2GL004505		*bll4784*	*MAFF_RS27120*	*MCHK_RS01750*	*SM2011_RS26345*	Nodule senescence
113-2GL004572	*113-2GL005733*	*blr4723,blr3787*		*MCHK_RS07395*		Nodule senescence
113-2GL004736	*113-2GL001679, 113-2GL006371, 113-2GL008136*	*bsl4595,bsr7468, bsl4595,bsl1445, bsr3154*	*MAFF_RS33280, MAFF_RS00310, MAFF_RS11065, MAFF_RS11510 MAFF_RS25580, MAFF_RS11485*	*MCHK_RS19875, MCHK_RS07180, MCHK_RS19840, MCHK_RS32390 MCHK_RS19440, MCHK_RS31800*	*SM2011_RS02010, SM2011_RS24070, SM2011_RS00515, SM2011_RS26245 SM2011_RS25125, SM2011_RS24855*	Early stages of nodule development
113-2GL005802		*bll0226,blr3724*	*MAFF_RS16140*	*MCHK_RS24370, MCHK_RS02525*	*SM2011_RS31015*	Nitrogen fixation
113-2GL005957		*blr3566*	*MAFF_RS21725*	*MCHK_RS29825*		Nitrogen fixation
113-2GL006512		*bll3022*	*MAFF_RS15360*	*MCHK_RS23560*		Nodule senescence
113-2GL006780		*blr2763*	*MAFF_RS27090, MAFF_RS26180*	*MCHK_RS01720*	*SM2011_RS02090, SM2011_RS01660, SM2011_RS03325*	Nitrogen fixation
113-2GL007543		*blr2149*	*MAFF_RS26000*			Early stages of nodule development
113-2GL007545		*blr2147*	*MAFF_RS25985*			Early stages of nodule development
113-2GL007548		*blr2145*	*MAFF_RS25975*			Nitrogen fixation
113-2GL007604	*113-2GL007555, 113-2GL003457,*	*bll3527,bll1900, bll5000,blr1706*	*MAFF_RS25425*	*MCHK_RS31900*	*SM2011_RS30325*	Nitrogen fixation

(Continued)

TABLE 3 | (Continued)

DEGs	Orthologs of the DEGs					Expression in soybean symbiosis
	113-2	USDA110	MAFF303099	7653R	SM2011	(according to Figure 3)
	113-2GL008356, 113-2GL007792, 113-2GL004069, 113-2GL000973, 113-2GL007670, 113-2GL007896, 113-2GL007767, 113-2GL007630, 113-2GL007942, 113-2GL004713, 113-2GL007823	*bll1997*				
113-2GL007618		*blr2068*	*MAFF_RS25050*			Nodule senescence
113-2GL007625	*113-2GL002237, 113-2GL007625, 113-2GL001613, 113-2GL004032, 113-2GL003601*	*blr5226,blr6978, blr5625,blr5226, bll2060,bsr7532*	*MAFF_RS33345, MAFF_RS09905, MAFF_RS10535, MAFF_RS36295, MAFF_RS23815*	*MCHK_RS17610, MCHK_RS07245*	*SM2011_RS18355, SM2011_RS00340, SM2011_RS02030, SM2011_RS11615*	Early stages of nodule development
113-2GL007626	*113-2GL001611, 113-2GL004031, 113-2GL003600, 113-2GL002236, 113-2GL004689*	*blr5626,bll2059, blr6979,blr5227, blr4635,blr7533*	*MAFF_RS23810, MAFF_RS36300, MAFF_RS33340, MAFF_RS10540*	*MCHK_RS07240*	*SM2011_RS11610, SM2011_RS20400, SM2011_RS18350, SM2011_RS02025*	Early stages of nodule development
113-2GL007641		*blr2038*	*MAFF_RS24010*	*MCHK_RS32450*	*SM2011_RS02275*	Nitrogen fixation
113-2GL007643	*113-2GL006940*	*bll2623,blr2036*	*MAFF_RS13735*	*MCHK_RS22150*	*SM2011_RS04845*	Nitrogen fixation
113-2GL007877		*blr1774*	*MAFF_RS24000*	*MCHK_RS32460*	*SM2011_RS02265*	Early stages of nodule development
113-2GL007879		*blr1771*	*MAFF_RS24015*			Nitrogen fixation
113-2GL007881		*blr1769*	*MAFF_RS24195*	*MCHK_RS32325*	*SM2011_RS02280*	Nitrogen fixation
113-2GL007888		*blr1759*	*MAFF_RS23985*	*MCHK_RS32485*	*SM2011_RS02250*	Early stages of nodule development
113-2GL007892		*blr1755*	*MAFF_RS24355*			Nitrogen fixation
113-2GL007898		*bsr1750*	*MAFF_RS24040, MAFF_RS24300*			Early stages of nodule development
113-2GL007899		*bsr1749*	*MAFF_RS24035, MAFF_RS24295*			Early stages of nodule development
113-2GL007902		*blr1746*	*MAFF_RS24215*	*MCHK_RS32305*	*SM2011_RS02420*	Early stages of nodule development
113-2GL007904		*blr1744*	*MAFF_RS24205*	*MCHK_RS32315*	*SM2011_RS02290*	Early stages of nodule development
113-2GL007905		*blr1743*	*MAFF_RS24200*	*MCHK_RS32320*	*SM2011_RS02285*	Early stages of nodule development
113-2GL007964	*113-2GL005342*	*blr1686,blr4134*			*SM2011_RS08430*	Nitrogen fixation
113-2GL008056		*bll1523*	*MAFF_RS15765*	*MCHK_RS33030, MCHK_RS23965*	*SM2011_RS28455*	Nodule senescence
113-2GL008064		*blr1516*			*SM2011_RS15300*	Early stages of nodule development
113-2GL008106		*bsl1473*	*MAFF_RS37185*		*SM2011_RS11135*	Nitrogen fixation
113-2GL008132	*113-2GL003149*	*blr1448,blr6053*	*MAFF_RS04190*	*MCHK_RS11590*	*SM2011_RS14035*	Nodule senescence
113-2GL008135		*bsl1446*	*MAFF_RS26365*	*MCHK_RS00670*	*SM2011_RS17430*	Early stages of nodule development
113-2GL008535		*blr1091*	*MAFF_RS15660*	*MCHK_RS23865*		Nodule senescence

Franck et al., 2014). It is worth noting that a multiple time-point microarray study in *B. diazoefficiens* bacteroids isolated from soybean nodules from nodulation to nodule senescence, which revealed a shift gene expression patterns of *B. diazoefficiens* during symbiotic process, especially in nodule senescence (Franck et al., 2018). In this report, we also performed a multiple time-point transcriptomic ananlysis, the difference is that the RNA samples were collected from different nodule samples from five important developmental stages of soybean (Yuan et al., 2017). We firstly investigated the expression of rhizobial genes in symbiosis at above-mentioned five developmental stages of soybean, which should shed new light on the molecular mechanisms of nitrogen fixation during the growth and development of soybean.

TABLE 4 | The prediction of the interaction proteins of the selected DEGs.

DEGs	Orthologs in USDA110	References for orthologs	Potencial interaction proteins		
			Soybean	USDA110	113-2
113-2GL000406	bll7322	Zhang et al., 2018	Glyma.01G003800	bll7785	113-2GL002891
113-2GL001850			Glyma.02G017800		113-2GL001328
			Glyma.02G147200		
			Glyma.02G222300		
			Glyma.03G164400		
			Glyma.03G251900		
			Glyma.04G080800		
			Glyma.04G082000		
			Glyma.04G194700		
			Glyma.04G226700		
			Glyma.06G081900		
			Glyma.06G082500		
			Glyma.06G171200		
			Glyma.09G273200		
			Glyma.10G018200		
			Glyma.12G045400		
			Glyma.12G167500		
			Glyma.16G043900		
			Glyma.18G284300		
			Glyma.19G222600		
			Glyma.20G249300		
	blr0462	Pessi et al., 2007	Glyma.08G152600		
		Delmotte et al., 2010	Glyma.15G272000		
		Čuklina et al., 2016			
		Zhang et al., 2018			
113-2GL007693	blr1971	Pessi et al., 2007	Glyma.01G118900	blr1971	113-2GL007693
		Delmotte et al., 2010	Glyma.02G155300	bll5750	113-2GL003467
		Čuklina et al., 2016	Glyma.02G208700		
		Zhang et al., 2018	Glyma.03G004900		
			Glyma.03G195400		
			Glyma.04G007400		
			Glyma.04G081100		
			Glyma.05G204000		
			Glyma.07G079700		
			Glyma.07G173100		
			Glyma.08G067400		
			Glyma.09G278600		
			Glyma.10G280000		
			Glyma.11G102100		
			Glyma.11G237400		
			Glyma.14G176900		
			Glyma.14G206300		
			Glyma.15G058400		
			Glyma.16G117600		
			Glyma.18G056100		
			Glyma.18G060600		
			Glyma.18G210100		
			Glyma.19G173400		
			Glyma.19G205200		
			Glyma.20G005900		
113-2GL008535	blr1091	Pessi et al., 2007	Glyma.02G294900	blr1148	113-2GL008473

(Continued)

TABLE 4 | (Continued)

DEGs	Orthologs in USDA110	References for orthologs	Potencial interaction proteins		
			Soybean	USDA110	113-2
		Delmotte et al., 2010	Glyma.14G018700	blr3906	113-2GL005583
		Čuklina et al., 2016	Glyma.14G161800	blr4307	113-2GL005142
		Zhang et al., 2018	Glyma.14G162000	bll4945	113-2GL004326
113-2GL008064	blr1516	Pessi et al., 2007	Glyma.05G033800	blr1516	113-2GL008064
		Delmotte et al., 2010	Glyma.14G145000	bll3640	113-2GL005884
		Čuklina et al., 2016		blr4473	113-2GL004883
		Zhang et al., 2018		bll4736	113-2GL004557
				bll5710	113-2GL003508
113-2GL001540	bll7600	Delmotte et al., 2010	Glyma.17G228800	bll7600	113-2GL001540
		Čuklina et al., 2016	Glyma.18G277300		
		Zhang et al., 2018			

In the five soybean nodule samples, about 25.38% (2234 out of 8801) of *B. diazoefficiens* 113-2 genes were not detected, indicating that these genes may not participate in symbiotic progress. The pod stage nodules possessed the highest number of genes with meaningful fpkm values (>1), suggesting the initiation of a series of new processes, which might be associated with nodule senescence. The correlation analysis of the gene expressions in the five nodule samples showed that the expression of *B. diazoefficiens* 113-2 genes at Branching stage or harvest stage has relative lower correlation with the other soybean development stages, but high correlations were existed among flowering stage, fruiting stage and pod stage (**Figure 1B**), indicating that similar gene expression patterns of *B. diazoefficiens* 113-2 were found during optimal symbiotic functioning, but different expression patterns were existed among early nodule development, nitrogen fixation progress and nodule senescence. These results also revealed a shift gene expression pattern of *B. diazoefficiens* 113-2 in symbiosis during the growth and development of soybean.

DEGs of *B. diazoefficiens* 113-2 Associated With Nodule Development and Senescence

Previous study showed that > 800 USDA110 genes were up- or down-regulated at each time point of soybean nodule development (Franck et al., 2018), while in this report, the highest number of DEGs at the ten groups was 83 (**Figure 2A**). The major reason may be that we used our previous soybean nodule RNA-Seq data, in which the RNA samples were mixed with both soybean RNA and *B. diazoefficiens* 113-2 RNA (Yuan et al., 2017). Our main purpose is to screen the important DEGs that can interact with host and are mainly involved in nodule development and senescence. The gene expression of soil rhizobia inside of the host was different from free live state (Vercruysse et al., 2011), so we did not isolate *B. diazoefficiens* 113-2 from the nodule samples.

Lots of important rhizobial genes have been showed to participate in nodule development and senescence (Voroshilova et al., 2001; Tittabutr et al., 2015; Franck et al., 2018). Recently, we sequenced the genome of *B. diazoefficiens* 113-2 (Li et al., 2020) and identified 164 DEGs of *B. diazoefficiens* 113-2 during the soybean nodule development in this study. Among these DEGs, 49 DEGs had both no functional GO term and no classified KEGG pathway (**Supplementary Tables 6**, **7**), and about 13 DEGs were not clearly annotated (**Supplementary Table 5**), meaning that we identified many novel and uncharacterized genes associated with nodule development and senescence. About 62.2% (102 out of 164) of the DEGs have no orthologs in *M. loti* MAFF303099, *M. huakuii* 7653R and *S. meliloti*2011, and only 35 DEGs have orthologs in all of the three rhizobia (**Table 3**), indicating that most of the identified genes associated with nodule development and senescence are not core genes among the rhizobia genomes. Besides, *B. diazoefficiens* 113-2 and *M. loti* MAFF303099 can form determinate nodules with soybean and *Lotus*, respectively, but only nine DEGs of 113-2 have orthologs only in *M. loti* MAFF303099 (**Table 3**), suggesting that the nodule development and senescence in determinate nodules or indeterminate nodules was also mainly determined by host legume plants, which is similar to our previous study (Li et al., 2020).

The Potential Roles of Host Specificity-Related DEGs in Nodule Development and Senescence

In most rhizobia, the genes that affect the biosynthesis of nodulation factors, secretion system and surface polysaccharides are often play critical roles in host specificity (Horvath et al., 1986; Philip-Hollingsworth et al., 1989; Wang et al., 2014). In this report, five *Nod* genes, eight *nif* genes, three *fix* genes and 27 T3SS-related genes (no genes associated with surface polysaccharides biosynthesis) were identified (**Figure 7**). The *Nod* genes primarily played roles in nodulation (Moulin et al., 2004), and among the identified five *Nod* genes, only *Noek* (*113-2GL001050*) and *NolG* (*113-2GL008064*) may play roles in the early state of nodule development. *NoeJ* (*113-2GL007583*) and *NolG* (*113-2GL007643*) may participate in nitrogen fixation progress and *NodG* (*113-2000663*) may have roles in nodule senescence. The *nif* and *fix* genes are crucial for nitrogen

fixation progress (Fischer and Hennecke, 1984; Hennecke, 1990). NifH, NifD, NifK, NifE, and NifN proteins are the core components of nitrogenase (Raymond et al., 2004), and the *FixABCX* genes were found to encode a membrane complex participating in electron transfer to nitrogenase (Earl et al., 1987; Edgren and Nordlund, 2004). The two main regulatory cascades that can be found in rhizobia are the RpoN−NifA and the oxygen−responsive two−component FixL−FixJ system, together with FixK (Lindström and Mousavi, 2019), which was found to regulate positively and negatively nitrogen fixation genes in *Rhizobium meliloti* (Batut et al., 1989). Among the identified eight *Nif* genes, only *NifH* (*113-2GL007881*) and *NifW* (*113-2GL007879*) reached the expression peaks at flowering stage_N or fruiting stage_N, the rest six *nif* genes *NifB* (*113-2GL007888*), *NifD/K* (*113-2GL007904*, *113-2GL007095*), *NifE* (*113-2-GL007903*), *NifN* (*113-2GL007902*), *NifQ* (*113-2007880*), and *NifX* (*113-2GL007901*) may mainly involved in regulating the early nodule development. For the three *fix* genes, *FixA* (*113-2GL007641*) and *FixC* (*113-2GL007877*) had relative higher expression at branching stage_N and flower stage_N, implying that they may mainly play roles in nitrogen fixation progress, while *FixK* (*113-2GL006786*) may mainly have roles in nodule senescence. The rhizobia T3SS and its secreted effectors has been reported to modulate nodulation and host range (Okazaki et al., 2013). For the 27 T3SS-related genes, only seven DEGs had relative higher expression at branching stage_N, and the rest 20 genes may participate in nitrogen fixation progress or nodule senescence, meaning that the T3SS-related genes have roles in the whole nodule development and senescence rather than only in nodulation, which is similar to our previous study (Yuan et al., 2017). Together, these results revealed that the identified host specificity-related DEGs have diversified roles in symbiosis, meaning that host specific regulation maybe existed in the whole progress of nodule development and senescence, not only in nodulation.

In summary, we firstly combined the expression characteristics of rhizobial genes in symbiosis with the growth and development of soybean, and identified 164 important rhizobial DEGs associated with nodule development and senescence. Among these DEGs, many are firstly identified and showed to associate with nodule development and senescence. Besides, we focused on the DEGs encoding nod, nif, fix proteins and T3SS secretion system-related proteins, as well as proteins involved in nitrogen metabolism, ABC transporters and two-component system pathways. Our results supply valuable basises for studying the genetic determinants of soil rhizobia in symbiosis and the creation of high efficiency soybean cultivation technology.

MATERIALS AND METHODS

Genome and Gene Mapping

The clean reads for the five nodule samples at five developmental stages of soybean (branching stage, flowering stage, fruiting stage, pod stage and harvest stage) were obtained from our previous study (Yuan et al., 2017). The raw sequence reads have been submitted to NCBI under the assigned accession number PRJNA765164, and the BioSample accessions included SAMN21545854, SAMN21545855, SAMN21545856, SAMN21545857, and SAMN21545858. The information of the reference genome (*B. diazoefficiens* 113-2) was previously described (Li et al., 2020). We map clean reads to the reference genome using HISAT (Cock et al., 2010), and map clean reads to reference transcripts using Bowtie2 (Kim et al., 2015). The mapping details are shown in **Supplementary Table 1**.

Gene Expression Analysis and Correlation Analysis Between Samples

We calculate the gene expression level for each nodule sample with RSEM (Li and Dewey, 2011). Besides, in order to reflect the gene expression correlation between the nodule samples, we calculated the Pearson correlation coefficients for all gene expression levels between each two samples using *cor* function in R software, and reflected these coefficients in the form of heat maps. Heatmap was performed using TBtools software (Chen et al., 2020).

Screening of DEGs Between Different Nodule Samples

To screen the DEGs between nodule samples, in combination with the false discovery rata (FDR) $\leq$ 0.001 and $|\log2\ \text{ratio}| \geq 1$, the fold changes in gene expression were used to evaluate the significance of gene expression differences in the ten Groups which are classified according to our previous study (Yuan et al., 2017). Data of the DEGs in the ten Groups between five nodule samples are provided in **Supplementary Table 3**.

Gene Ontology Functional and Kyoto Encyclopedia of Genes and Genomes Pathway Analyses of DEGs

Gene Ontology (GO) is a database created by the Gene Ontology Consortium and is a classification system for genes' biological functions. The method of GO functional enrichment analysis first maps all DEGs to terms in the GO database[2] and the computing method as previously described (Yuan et al., 2017). Kyoto Encyclopedia of Genes and Genomes (KEGG) is a public pathway-related database,[3] and the computing method also were described in our previous study (Yuan et al., 2017).

Orthologs Analysis of the DEGs

Firstly, we performed Core/Pan gene analysis for *B. diazoefficiens* 113-2, *M. loti* MAFF303099, *M. huakuii* 7653R, and *S. meliloti* 2011. We used the CD-HIT 4.66[4] rapid clustering of similar proteins software (Edgar, 2004)with a threshold of 50% pairwise identity and 0.7 length difference cut off in amino acid to cluster the Core/Pan genes of these four strains, and obtained the pan gene pool. Then we used the identified DEGs as queries to search the orthologs in the pan gene pool.

[2]http://www.geneontology.org/

[3]https://www.kegg.jp/kegg/kegg1.html

[4]http://weizhong-lab.ucsd.edu/cd-hit/

Phylogenetic Analysis

Phylogenetic trees were conducted in MEGA X (Kumar et al., 2018). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SY, HC, and XZ designed this work. SY wrote the manuscript. SY, SZ, and YF performed most of the analysis, figures, and tables. CZ, YH, ZS, SC, WG, HY, ZY, and DQ contributed substantially to the completion of this work. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the National Natural Science Foundation of China (Grant No. 32071964), the National Key Research and Development Program of China (Grant No. 2020YFD1000903-10), and the Basic Scientific Research Service Fee Special of the Central Scientific Research Institute (Grant No. 1610172018001).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.754837/full#supplementary-material

REFERENCES

Alemneh, A. A., Zhou, Y., Ryder, M. H., and Denton, M. D. (2020). Mechanisms in plant growth-promoting rhizobacteria that enhance legume-rhizobial symbioses. *J. Appl. Microbiol.* 129, 1133–1156. doi: 10.1111/jam.14754

Ampe, F., Kiss, E., Sabourdy, F., and Batut, J. (2003). Transcriptome analysis of Sinorhizobium meliloti during symbiosis. *Genome Biol.* 4:R15. doi: 10.1186/gb-2003-4-2-r15

Batut, J., Daveran-Mingot, M. L., David, M., Jacobs, J., Garnerone, A. M., and Kahn, D. (1989). fixK, a gene homologous with fnr and crp from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in Rhizobium meliloti. *EMBO J.* 8, 1276–1286. doi: 10.1002/j.1460-2075.1989.tb03502.x

Biswas, B., and Gresshoff, P. M. (2014). The role of symbiotic nitrogen fixation in sustainable production of biofuels. *Int. J. Mol. Sci.* 15, 7380–7397. doi: 10.3390/ijms15057380

Cermola, M., Fedorova, E., Taté, R., Riccio, A., Favre, R., and Patriarca, E. J. (2000). Nodule invasion and symbiosome differentiation during Rhizobium etli-Phaseolus vulgaris symbiosis. *Mol. Plant Microbe Interact.* 13, 733–741. doi: 10.1094/MPMI.2000.13.7.733

Chang, W. S., Franck, W. L., Cytryn, E., Jeong, S., Joshi, T., Emerich, D. W., et al. (2007). An oligonucleotide microarray resource for transcriptional profiling of *Bradyrhizobium japonicum*. *Mol. Plant Microbe Interact.* 20, 1298–1307. doi: 10.1094/MPMI-20-10-1298

Chen, C., Chen, H., Zhang, Y., Thomas, H. R., Frank, M. H., He, Y., et al. (2020). TBtools: an Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* 13, 1194–1202. doi: 10.1016/j.molp.2020.06.009

Cheng, G., Karunakaran, R., East, A. K., Munoz-Azcarate, O., and Poole, P. S. (2017). Glutathione affects the transport activity of Rhizobium leguminosarum 3841 and is essential for efficient nodulation. *FEMS Microbiol. Lett.* 364:fnx045. doi: 10.1093/femsle/fnx045

Cock, P. J., Fields, C. J., Goto, N., Heuer, M. L., and Rice, P. M. (2010). The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. *Nucleic Acids Res.* 38, 1767–1771. doi: 10.1093/nar/gkp1137

Čuklina, J., Hahn, J., Imakaev, M., Omasits, U., Förstner, K. U., Ljubimov, N., et al. (2016). Genome-wide transcription start site mapping of *Bradyrhizobium japonicum* grown free-living or in symbiosis - a rich resource to identify new transcripts, proteins and to study gene regulation. *BMC Genomics* 17:302. doi: 10.1186/s12864-016-2602-9

Cytryn, E. J., Sangurdekar, D. P., Streeter, J. G., Franck, W. L., Chang, W. S., Stacey, G., et al. (2007). Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.* 189, 6751–6762. doi: 10.1128/JB.00533-07

Delmotte, N., Ahrens, C. H., Knief, C., Qeli, E., Koch, M., Fischer, H. M., et al. (2010). An integrated proteomics and transcriptomics reference data set provides new insights into the *Bradyrhizobium japonicum* bacteroid metabolism in soybean root nodules. *Proteomics* 10, 1391–1400. doi: 10.1002/pmic.200900710

Earl, C. D., Ronson, C. W., and Ausubel, F. M. (1987). Genetic and structural analysis of the Rhizobium meliloti fixA, fixB, fixC, and fixX genes. *J. Bacteriol.* 169, 1127–1136. doi: 10.1128/jb.169.3.1127-1136.1987

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113. doi: 10.1186/1471-2105-5-113

Edgren, T., and Nordlund, S. (2004). The fixABCX genes in Rhodospirillum rubrum encode a putative membrane complex participating in electron transfer to nitrogenase. *J. Bacteriol.* 186, 2052–2060. doi: 10.1128/JB.186.7.2052-2060.2004

Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. (1971). Stage of Development Descriptions for Soybeans, *Glycine Max* (L.) Merrill1. *Crop Sci.* 11, 929–931. doi: 10.2135/cropsci1971.0011183X001100060051x

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E., et al. (2010). Molecular analysis of legume nodule development and autoregulation. *J. Integr. Plant Biol.* 52, 61–76. doi: 10.1111/j.1744-7909.2010.00899.x

Fischer, H. M., and Hennecke, H. (1984). Linkage map of the Rhizobium japonicum nifl-I and nitDK operons encoding the polypeptides of the nitrogenase enzyme complex. *Mol. Gen. Genet.* 196, 537–540. doi: 10.1007/BF00436206

Franck, S., Franck, W. L., Birke, S. R., Chang, W.-S., Sangurdekar, D. P., Cytryn, E., et al. (2014). Comparative transcriptomic analysis of symbiotic *Bradyrhizobium japonicum*. *Symbiosis* 63, 123–135. doi: 10.1007/s13199-014-0294-y

Franck, S., Strodtman, K. N., Qiu, J., and Emerich, D. W. (2018). Transcriptomic Characterization of *Bradyrhizobium diazoefficiens* Bacteroids Reveals a Post-Symbiotic, Hemibiotrophic-Like Lifestyle of the Bacteria within Senescing Soybean Nodules. *Int. J. Mol. Sci.* 19:3918. doi: 10.3390/ijms19123918

Franck, W. L., Chang, W. S., Qiu, J., Sugawara, M., Sadowsky, M. J., Smith, S. A., et al. (2008). Whole-genome transcriptional profiling of *Bradyrhizobium japonicum* during chemoautotrophic growth. *J. Bacteriol.* 190, 6697–6705. doi: 10.1128/JB.00543-08

Hauser, F., Lindemann, A., Vuilleumier, S., Patrignani, A., Schlapbach, R., Fischer, H. M., et al. (2006). Design and validation of a partial-genome microarray for transcriptional profiling of the *Bradyrhizobium japonicum* symbiotic gene region. *Mol. Genet. Genomics* 275, 55–67. doi: 10.1007/s00438-005-0059-7

Hennecke, H. (1990). Nitrogen fixation genes involved in the *Bradyrhizobium japonicum*-soybean symbiosis. *FEBS Lett.* 268, 422–426. doi: 10.1016/0014-5793(90)81297-2

Hirsch, A. M. (1992). Tansley Review No. 40. Developmental Biology of Legume Nodulation. *New Phytol.* 122, 211–237. doi: 10.1111/j.1469-8137.1992.tb04227.x

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Török, I., Györgypal, Z., et al. (1986). Organization, structure and symbiotic function of Rhizobium meliloti nodulation genes determining host specificity for alfalfa. *Cell* 46, 335–343. doi: 10.1016/0092-8674(86)90654-9

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 44, D457–D462. doi: 10.1093/nar/gkv1070

Karr, D. B., and Emerich, D. W. (1988). Uniformity of the microsymbiont population from soybean nodules with respect to buoyant density. *Plant Physiol.* 86, 693–699. doi: 10.1104/pp.86.3.693

Karr, D. B., Suzuki, F., Waters, J. K., and Emerich, D. W. (1990). Further Evidence for the Uniformity of the Microsymbiont Population from Soybean Nodules. *J. Plant Physiol.* 136, 659–663. doi: 10.1016/S0176-1617(11)81340-4

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323

Li, Q. G., Zhang, L., Li, C., Dunwell, J. M., and Zhang, Y. M. (2013). Comparative genomics suggests that an ancestral polyploidy event leads to enhanced root nodule symbiosis in the Papilionoideae. *Mol. Biol. Evol.* 30, 2602–2611. doi: 10.1093/molbev/mst152

Li, Y., Tian, C. F., Chen, W. F., Wang, L., Sui, X. H., and Chen, W. X. (2013). High-resolution transcriptomic analyses of Sinorhizobium sp. NGR234 bacteroids in determinate nodules of Vigna unguiculata and indeterminate nodules of *Leucaena leucocephala*. *PLoS One* 8:e70531. doi: 10.1371/journal.pone.0070531

Li, R., Feng, Y., Chen, H., Zhang, C., Huang, Y., Chen, L., et al. (2020). Whole-Genome Sequencing of *Bradyrhizobium diazoefficiens* 113-2 and Comparative Genomic Analysis Provide Molecular Insights Into Species Specificity and Host Specificity. *Front. Microbiol.* 11:576800. doi: 10.3389/fmicb.2020.576800

Li, X., Deng, Z., Liu, Z., Yan, Y., Wang, T., Xie, J., et al. (2014). The genome of Paenibacillus sabinae T27 provides insight into evolution, organization and functional elucidation of nif and nif-like genes. *BMC Genomics* 15:723. doi: 10.1186/1471-2164-15-723

Lindström, K., and Mousavi, S. A. (2019). Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* 13, 1314–1335. doi: 10.1111/1751-7915.13517

Lindström, K., and Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* 13, 1314–1335.

Lorkiewicz, Z. (1997). Nodulation genes in the Rhizobium–plant signal exchange. *Acta Biochim. Pol.* 44, 1–12. doi: 10.18388/abp.1997_4434

Martínez-Salazar, J. M., Sandoval-Calderón, M., Guo, X., Castillo-Ramírez, S., Reyes, A., Loza, M. G., et al. (2009). The Rhizobium etli RpoH1 and RpoH2 sigma factors are involved in different stress responses. *Microbiology* 155, 386–397. doi: 10.1099/mic.0.021428-0

Mergaert, P., Kereszt, A., and Kondorosi, E. (2020). Gene Expression in Nitrogen-Fixing Symbiotic Nodule Cells in Medicago truncatula and Other Nodulating Plants. *Plant Cell* 32, 42–68. doi: 10.1105/tpc.19.00494

Moulin, L., Béna, G., Boivin-Masson, C., and Stêpkowski, T. (2004). Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the Bradyrhizobium genus. *Mol. Phylogenet. Evol.* 30, 720–732. doi: 10.1016/S1055-7903(03)00255-0

Okazaki, S., Kaneko, T., Sato, S., and Saeki, K. (2013). Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. *Proc. Natl. Acad. Sci. U. S. A.* 110, 17131–17136. doi: 10.1073/pnas.1302360110

Oldroyd, G. E., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Palander, S., Näsi, M., and Valkonen, E. (2005). Effect of age of growing turkeys on nutrient digestibility and energy value of cereal-soybean-based diets. *Arch. Anim. Nutr.* 59, 139–147. doi: 10.1080/17450390512331387945

Pessi, G., Ahrens, C. H., Rehrauer, H., Lindemann, A., Hauser, F., Fischer, H. M., et al. (2007). Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. *Mol. Plant Microbe Interact.* 20, 1353–1363. doi: 10.1094/MPMI-20-11-1353

Philip-Hollingsworth, S., Hollingsworth, R. I., Dazzo, F. B., Djordjevic, M. A., and Rolfe, B. G. (1989). The effect of interspecies transfer of Rhizobium host-specific nodulation genes on acidic polysaccharide structure and in situ binding by host lectin. *J. Biol. Chem.* 264, 5710–5714. doi: 10.1016/S0021-9258(18)83607-9

Putnoky, P., Grosskopf, E., Ha, D. T., Kiss, G. B., and Kondorosi, A. (1988). Rhizobium fix genes mediate at least two communication steps in symbiotic nodule development. *J. Cell Biol.* 106, 597–607. doi: 10.1083/jcb.106.3.597

Raymond, J., Siefert, J. L., Staples, C. R., and Blankenship, R. E. (2004). The Natural History of Nitrogen Fixation. *Mol. Biol. Evol.* 21, 541–554. doi: 10.1093/molbev/msh047

Redondo, F. J., De La Peña, T. C., Morcillo, C. N., Lucas, M. M., and Pueyo, J. J. (2009). Overexpression of flavodoxin in bacteroids induces changes in antioxidant metabolism leading to delayed senescence and starch accumulation in alfalfa root nodules. *Plant Physiol.* 149, 1166–1178. doi: 10.1104/pp.108.129601

Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., et al. (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J.* 77, 817–837. doi: 10.1111/tpj.12442

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. *Plant Cell* 32, 15–41. doi: 10.1105/tpc.19.00279

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Serova, T. A., Tsyganova, A. V., and Tsyganov, V. E. (2018). Early nodule senescence is activated in symbiotic mutants of pea (*Pisum sativum* L.) forming ineffective nodules blocked at different nodule developmental stages. *Protoplasma* 255, 1443–1459. doi: 10.1007/s00709-018-1246-9

Tittabutr, P., Sripakdi, S., Boonkerd, N., Tanthanuch, W., Minamisawa, K., and Teaumroong, N. (2015). Possible Role of 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity of Sinorhizobium sp. BL3 on Symbiosis with Mung Bean and Determinate Nodule Senescence. *Microbes Environ.* 30, 310–320. doi: 10.1264/jsme2.ME15120

Vasse, J., De Billy, F., Camut, S., and Truchet, G. (1990). Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.* 172, 4295–4306. doi: 10.1128/jb.172.8.4295-4306.1990

Vercruysse, M., Fauvart, M., Beullens, S., Braeken, K., Cloots, L., Engelen, K., et al. (2011). A comparative transcriptome analysis of Rhizobium etli bacteroids: specific gene expression during symbiotic nongrowth. *Mol. Plant Microbe Interact.* 24, 1553–1561. doi: 10.1094/MPMI-05-11-0140

Voroshilova, V. A., Boesten, B., Tsyganov, V. E., Borisov, A. Y., Tikhonovich, I. A., and Priefer, U. B. (2001). Effect of mutations in *Pisum sativum* L. genes blocking different stages of nodule development on the expression of late symbiotic genes in Rhizobium leguminosarum bv. viciae. *Mol. Plant Microbe Interact.* 14, 471–476. doi: 10.1094/MPMI.2001.14.4.471

Wang, S., Hao, B., Li, J., Gu, H., Peng, J., Xie, F., et al. (2014). Whole-genome sequencing of Mesorhizobium huakuii 7653R provides molecular insights into

host specificity and symbiosis island dynamics. *BMC Genomics* 15:440. doi: 10.1186/1471-2164-15-440

Yang, L., El Msehli, S., Benyamina, S., Lambert, A., Hopkins, J., Cazareth, J., et al. (2020). Glutathione Deficiency in Sinorhizobium meliloti Does Not Impair Bacteroid Differentiation But Induces Early Senescence in the Interaction With Medicago truncatula. *Front. Plant Sci.* 11:137. doi: 10.3389/fpls.2020.00137

Yuan, S. L., Li, R., Chen, H. F., Zhang, C. J., Chen, L. M., Hao, Q. N., et al. (2017). RNA-Seq analysis of nodule development at five different developmental stages of soybean (*Glycine max*) inoculated with *Bradyrhizobium japonicum* strain 113-2. *Sci. Rep.* 7:42248. doi: 10.1038/srep42248

Zhang, L., Liu, J. Y., Gu, H., Du, Y., Zuo, J. F., Zhang, Z., et al. (2018). *Bradyrhizobium diazoefficiens* USDA 110- *Glycine max* Interactome Provides Candidate Proteins Associated with Symbiosis. *J. Proteome Res.* 17, 3061–3074. doi: 10.1021/acs.jproteome.8b00209

Zhou, S., Zhang, C., Huang, Y., Chen, H., Yuan, S., and Zhou, X. (2021). Characteristics and Research Progress of Legume Nodule Senescence. *Plants* 10:1103. doi: 10.3390/plants10061103

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 November 2021
doi: 10.3389/fmicb.2021.756327

Unveiling Endophytic Bacterial Community Structures of Different Rice Cultivars Grown in a Cadmium-Contaminated Paddy Field

Chaoqun Chu[1†], Meiyu Fan[1†], Chongyang Song[1], Ni Li[2], Chao Zhang[2], Shaowei Fu[1], Weiping Wang[2] and Zhiwei Yang[1*]*

[1]College of Life Sciences, Capital Normal University, Beijing, China, [2]State Key Laboratory of Hybrid Rice, Hunan Hybrid Rice Research Center, Changsha, China

OPEN ACCESS

Edited by:
Gustavo Santoyo,
Universidad Michoacana de San Nicolás de Hidalgo, Mexico

Reviewed by:
Rungroch Sungthong,
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Kunming University of Science and Technology, China
Shenzheng Zeng,
Sun Yat-sen University, China

***Correspondence:**
Zhiwei Yang
yangzw@cnu.edu.cn
Weiping Wang
wangweiping@hhrrc.ac.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: 10 August 2021
Accepted: 04 October 2021
Published: 16 November 2021

Citation:
Chu C, Fan M, Song C, Li N, Zhang C, Fu S, Wang W and Yang Z (2021) Unveiling Endophytic Bacterial Community Structures of Different Rice Cultivars Grown in a Cadmium-Contaminated Paddy Field. Front. Microbiol. 12:756327. doi: 10.3389/fmicb.2021.756327

Endophytic bacteria play potentially important roles in the processes of plant adaptation to the environment. Understanding the composition and dynamics of endophytic bacterial communities under heavy metal (HM) stress can reveal their impacts on host development and stress tolerance. In this study, we investigated root endophytic bacterial communities of different rice cultivars grown in a cadmium (Cd)-contaminated paddy field. These rice cultivars are classified into low (RBQ, 728B, and NX1B) and high (BB and S95B) levels of Cd-accumulating capacity. Our metagenomic analysis targeting 16S rRNA gene sequence data reveals that *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Spirochaetes* are predominant root endophytic bacterial phyla of the five rice cultivars that we studied. Principal coordinate analysis shows that the developmental stage of rice governs a larger source of variation in the bacterial communities compared to that of any specific rice cultivar or of the root Cd content. Endophytic bacterial communities during the reproductive stage of rice form a more highly interconnected network and exhibit higher operational taxonomic unit numbers, diversities, and abundance than those during the vegetative stage. Forty-five genera are significantly correlated with Cd content in rice root, notably including positive-correlating *Geobacter* and *Haliangium*; and negative-correlating *Pseudomonas* and *Streptacidiphilus*. Furthermore, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States analysis shows that functional pathways, such as biosynthesis of siderophore and type II polyketide products, are significantly enhanced during the reproductive stage compared to those during the vegetative stage under Cd stress. The isolated endophytic bacteria from the Cd-contaminated rice roots display high Cd resistance and multiple traits that may promote plant growth, suggesting their potential application in alleviating HM stress on plants. This study describes in detail for the first time the assemblage of the bacterial endophytomes of rice roots under Cd stress and may provide insights into the interactions among endophytes, plants, and HM contamination.

Keywords: rice, endophytic bacteria, community structure, co-occurrence network, developmental stage, cadmium contamination, plant growth-promoting bacteria

INTRODUCTION

Pollution of soil with heavy metal (HM) is widespread due to the rapid development of industry and extensive application of chemical fertilizers and pesticides (Huang and Jin, 2008; Atafar et al., 2010). HMs have harmful effects on crops and soil microorganisms and weaken the function of soil ecosystems (Kossoff et al., 2014; Guo et al., 2020). Therefore, remediation of HM-contaminated soil is a challenging task which holds great promise to improve the soil environment and to ensure safer food production.

Traditional methods for remediation of HM-contaminated soil rely heavily on physical and chemical approaches that are costly and non-sustainable. In recent years, biological remediation methods which take advantage of the metal-microbe interaction that occurs in plant hosts have been exploited (Etesami, 2018; Yin et al., 2019). Compared to bulk soil microbiomes, plant-associated microbiomes, such as endophytic and rhizospheric bacteria, have shown promise for HM remediation (Afzal et al., 2019). Endophytes are microorganisms living in the plant interior without causing disease. Beneficial endophytes not only promote plant growth but also help to remediate environmental contaminants (Etesami, 2018). Under metal stress, endophytic bacteria assist in phytoextraction and phytoremediation by increasing the biomass of HM-hyperaccumulating plants and enhancing the metal uptake by plants *via* acidifying the rhizosphere environment, thus enhancing the availability of metals (Rajkumar et al., 2012; Ma et al., 2016; Luo et al., 2019). In recent times, some HM-tolerant endophytes have been shown to promote plant growth in HM-contaminated soils and reduce the uptake of toxic metals by plants *via* biosorption, precipitation, complexation, and enzymatic transformation – ultimately leading to safer methods for human food production (Etesami, 2018; Yin et al., 2019).

Under HM stress, plants are capable of recruiting specific endophytic populations that enable a high HM resistance and plant growth-promoting properties (Croes et al., 2013; Pereira and Castro, 2014; Montalban et al., 2016; Syranidou et al., 2018; Sun et al., 2019; Zadel et al., 2020). Furthermore, these microbes have been shown to influence HM uptake by plants (Luo et al., 2017, 2019). For example, in the well-known cadmium (Cd)/zinc (Zn) superaccumulator *Sedum alfredii*, the enrichment of *Bacteroidetes* and depletion of *Firmicutes* and *Planctomycetes* in the endosphere of HP (hyperaccumulating) genotype may be responsible for the metal hyper-content in its shoots (Luo et al., 2017). Subsequent studies have shown that the abundance of *Streptomycetaceae*, *Nocardioidaceae*, and *Pseudonocardiaceae* is strongly correlated with an increased shoot biomass and total Cd/Zn accumulation in *S. alfredii* (Luo et al., 2019). An inoculation of *S. alfredii* with a synthetic bacterial community resulted in a significant improvement in plant biomass, root morphology, and Cd/Zn accumulation (Luo et al., 2019).

Compared to other crops, rice can easily incorporate more Cd and is the major source of dietary Cd intake in populations that rely principally on rice (Hu et al., 2016; Wang et al., 2019). Several studies have shown that bacteria inhabiting the rhizoplane and rhizosphere of rice can have a great influence on rice growth and uptake of HMs (Hu et al., 2015, 2019; Hou et al., 2018). However, few studies analyzing the endophytic bacteria under HM conditions in rice have been carried out. Zhou et al. (2020) showed that inoculation of rice endophytic *Stenotrophomonas* sp. R5-5 can change the relative abundance of some Cd-resistant bacteria in rice plants and downregulate the expression of rice Cd transporters, thereby reducing the accumulation of Cd in rice. An arsenic (As)-resistant facultative endophytic bacterium, *Serratia* sp. F2, can lower As accumulation in rice grain *via* increasing As adsorption by Fe plaque on the root surface (Cheng et al., 2020). These studies demonstrate that specific HM-tolerant microbes can significantly influence HM accumulation in rice. However, little is known about the composition and function of the microbiomes that colonize the endosphere of rice roots under HM stress. To better understand the ecological roles of endophytes with their host plants, it is crucial to investigate and characterize endophytic populations in rice grown in a HM-contaminated environment.

Here, we present a detailed investigation of the root bacterial endophytomes of rice grown in a Cd-contaminated paddy field using low- and high-Cd-accumulating cultivars. We employed bacterial 16S ribosomal RNA gene amplicon sequencing to (1) analyze the composition and dynamic changes of endophytic bacteria communities at two developmental stages of rice (2) decipher the keystone taxa that define the bacterial population structure (3) identify bacterial biomarkers that are uniquely associated with each individual rice cultivar, and (4) analyze the endophytic bacterial taxa correlated with Cd content. In addition, we identified metabolic pathways and functional genes enriched in root endophytic communities under Cd stress. Finally, we isolated and characterized bacterial endophytes from the Cd-contaminated rice roots as potential bio-inoculants to enhance crop productivity and mitigate HM stress.

MATERIALS AND METHODS

Rice Seeds

Rice (*Oryza sativa* L.) seeds were provided by the Hunan Hybrid Rice Research Center (HHRRC), China. RBQ (Nipponbare), as a model plant, is a japonica cultivar. 728B, NX1B, BB, and S95B are indica cultivars bred by HHRRC. After years of cultivation, we observed that when soil-available Cd is 0.45–2.3 mg/kg, RBQ, 728B, and NX1B accumulate 0.08–0.48 mg Cd/kg grains, while BB and S95B accumulate 0.37–0.91 mg Cd/kg grains. Because these two groups of cultivars accumulate significantly different amounts of Cd in grains, we classify RBQ, 728B, and NX1B as low-accumulating (LA) cultivars and BB and S95B as high-accumulating (HA) cultivars. The entire development phase for all five cultivars is 100 to 112 days.

Rice Cultivation and Sampling

From June to October 2018, five rice cultivars (RBQ, 728B, NX1B, BB, and S95B) were cultivated in a Cd-contaminated

paddy field (27°58′N, 113°28′E) in Liling, Hunan province, China. This region is characterized by a humid subtropical monsoon climate with the following annual means: temperature, 18°C; sunshine, 1,500–1,910 h; rainfall, 1,300–1,600 mm; and frost-free period, 288 days. The paddy field is contaminated by Cd due to a historic Zn-Pb ore mine in Liling. The soil of the experimental area has the following characteristics: pH 5.4; electrical conductivity, 198.7 μs/cm; water content, 51.2%; organic content, 3.2%; organic carbon content, 13.7 g/kg; total nitrogen content, 2.8 g/kg; available phosphorus, 14.2 mg/kg; available potassium, 181.9 mg/kg; NH_4^+–N, 220.6 mg/kg; and NO_3^-–N, 0.4 mg/kg. Cd, chromium, manganese, copper, and lead are 0.9, 69.0, 53.4, 28.8, and 52.1 mg/kg, respectively. Among them, Cd content is 0.9 mg/kg, which greatly exceeds the risk screening value (0.3 mg/kg) defined by China Risk for soil contamination on agricultural land (Trial; GB15618-2018).

The field experiment employed a randomized block design with five rice cultivars. Before use, the plot was fully ploughed and divided into 15 zones (200 × 60 cm) with an interval of 40 cm between each zone. There were three columns in one zone, 10 holes in one column, and two seedlings in each hole. There were three replicates (zones) for each cultivar. Non-polluted water was used for irrigation. Water and fertilizer management was carried out according to the local customs.

Thirty days (vegetative stage) and 60 days (reproductive stage) after transplantation, rice root samples were collected. For each cultivar, five individuals were sampled according to diagonal sampling method in each zone, and the roots were mixed. The root samples were transported back to the laboratory under low temperature. Root samples were divided into two parts: one part was washed and air-dried, frozen in liquid nitrogen, and stored at −80°C for later DNA extraction and 16S rDNA amplification, while another part was stored at 4°C until isolation of endophytic bacteria. Root samples for the five rice cultivars at the vegetative stage were referred to as RBQ1, 728B1, NX1B1, BB1, and S95B1 and at the reproductive stage as RBQ2, 728B2, NX1B2, BB2, and S95B2.

Determination of Cd Content in Roots

Roots were washed, deactivated at 105°C for 30 min, and dried at 55°C for 48 h and then 0.5 g roots were pulverized in an agate mortar. The Cd content of root samples was determined using inductively coupled plasma mass spectrometry (ICP-MS) by Advanced Standards Technical Services Co., Ltd (Beijing). There were three replicates for each sample.

DNA Extraction, PCR Amplification of 16S rDNA, and Sequencing

0.5 g sample of rice roots from each cultivar was washed and surface-sterilized in triplicate, as described previously (Moronta-Barrios et al., 2018). There were three replicates for each cultivar. The effect of sterilization was verified by plating the last wash solution (100 μl) on LB plates before proceeding with DNA extraction.

Endophytic bacterial DNA was extracted using the FastDNA® Spin Kit for Soil (MP-Bio) according to the manufacturer's protocols. DNA quantity and purity were measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States). As described previously (Bulgarelli et al., 2015), the V5-V7 hypervariable regions of the bacterial 16S rRNA genes were amplified with primers 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′) *via* thermocycler PCR system (GeneAmp 9700, ABI, United States). PCR reactions were conducted using the following settings: 3 min initiation at 95°C, 27 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 45 s elongation at 72°C, and a final extension at 72°C for 10 min. PCR reactions were performed in triplicate with a 20 μl mixture containing 4 μl of 5× FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μm), 0.4 μl of FastPfu Polymerase, and 1 μl (10 ng/μl) of template DNA. Resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) before being quantified using the QuantiFluor™-ST (Promega, United States) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform (Illumina, San Diego, United States) according to the manufacturer's protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Processing of Sequencing Data

All raw Illumina Fastq files were demultiplexed and quality-filtered using Trimmomatic and merged using FLASH (Magoc and Salzberg, 2011). Operational taxonomic unit numbers (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1),[1] and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was identified using the RDP Classifier algorithm[2] against the Silva (SSU128) 16S rRNA database using a 70% confidence threshold.

Bioinformatics and Statistical Analyses

Rarefaction curves, composition, and alpha diversity analyses were performed on https://cloud.majorbio.com/.Principal coordinates analysis (PCoA) was performed on weighted UniFrac distance matrices (Lozupone and Knight, 2005) using the "amplicon" package (Liu et al., 2021) in R (v3.6.1). Permutational multivariate analysis of variance (PERMANOVA) was conducted using the adonis function of Vegan in R. Co-occurrence network analyses were carried out using the "igraph" package (Csardi and Nepusz, 2006) in R, and networks were visualized using Gephi (v0.9.2; Bastian et al., 2009). Linear discriminant analysis effect size (LEfSe) was applied to the OTU table using Galaxy.[3] Venn diagrams and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) were constructed *via* ImageGP.[4]

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

[3]http://huttenhower.sph.harvard.edu/galaxy/

[4]http://www.ehbio.com/ImageGP/index.php/Home/Index/index.html

SPSS v25.0 was used to perform paired sample *t*-tests, one-way ANOVA, and Spearman's rank correlations. A heat map of the relative abundance of bacterial taxa or pathways was constructed in TBTools v1.089 (Chen et al., 2020).

Isolation and Molecular Identification of Bacterial Isolates of Rice Roots

Endophytic bacteria of root were isolated as described by Zhou et al. (2020) with some modifications. 0.5 g of the surface-sterilized roots was triturated aseptically in distilled water and inoculated into 30 ml of R2A (Massa et al., 1998), TSB (tryptone 1.5%; soya peptone 0.5%; NaCl 0.5%; pH 7.4), or 1/2 LB broth (tryptone 0.5%; yeast extract 0.25%; NaCl 0.25%; pH 7.4), respectively. After incubation at 30°C for 24 h, bacterial suspension was diluted appropriately. Aliquots of 100 µl resuspension were spread on corresponding solid medium and incubated at 30°C until all colonies appeared. The colonies with distinctive morphological characteristics were picked and purified.

The isolates were identified by 16S rDNA sequencing. The gene was amplified using universal primers 27f (5′-AGAGTTT GATCCTGGCTCA-3′) and 1492r (5′-GGTTACCTTGTTACGA CTT-3′; Weisburg et al., 1991) and sequenced using an ABI 3730 Genetic Analyzer by RuiBiotech (Beijing). The sequences were used to perform BLASTN program against the 16S database of type strains at EzTaxon server (Kim and Chun, 2014).

Characterization of Cd Tolerance and Plant Growth-Promoting Potential of Bacterial Isolates

We used the broth microdilution method to test the minimum inhibitory concentration (MIC) of Cd upon bacterial strains (Wiegand et al., 2008). A 96-well microtiter plate was taken from its sterile packing, and 50 µl of each LB + $CdCl_2$ dilution was added into the respective well (1st–10th), 100 µl of LB broth in the control well (11th), and 50 µl in the growth control well (12th). The bacterial suspension was adjusted to 1×10^8 colony-forming unit/ml (equal to McFarland 0.5 standard) and then diluted 100 times. Each well containing the LB + $CdCl_2$ solution (1st–10th) and the growth control well (12th) was inoculated with 50 µl of the bacterial suspension, respectively. The final concentrations of $CdCl_2$ in the 1st–10th wells were 5, 10, 20, 40, 80, 160, 320, 640, 1,280, and 2,560 µm, respectively. The microtiter plate was incubated at 37°C for 16–20 h. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye. Each sample was repeated three times.

The PGP traits of bacterial isolates were quantitatively analyzed as follows: Indole-3-acetic acid (IAA) production was estimated using Salkowski reagent (Glickmann and Dessaux, 1995); siderophore production was determined using the Chrome Azurol S agar method (Schwyn and Neilands, 1987); phosphate solubilization estimations were carried out *via* the molybdenum blue method (Murphy and Riley, 1962), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was measured as described by Penrose and Glick (2003).

RESULTS

Cd Accumulation in Roots of Five Cultivars at Two Stages

ICP-MS was used to determine Cd content in root samples collected at the vegetative and reproductive stage (**Figure 1A**). At the vegetative stage, RBQ accumulates more Cd than other cultivars. This phenomenon is more pronounced at the reproductive stage, when RBQ has a significantly higher Cd content, followed by LA cultivars (728B and NX1B) and HA cultivars (BB and S95B). It appears that the LA cultivars accumulate more Cd in roots than those of HA cultivars.

Diversity and Abundance of Endophytic Bacterial Communities

A total of 1,236,157 16S rDNA sequences were obtained from root samples and were assigned to 2,261 OTUs based on 97% sequence similarity. Rarefaction curves of all samples tend to approach a saturation plateau, indicating that the sequencing accurately captures the endophytic communities of each rice cultivar (**Supplementary Figure 1**). The OTUs of rice cultivar RBQ, 728B, NX1B, BB, and S95B are 376, 309, 356, 448, and 359, respectively, at the vegetative stage and 686, 674, 691, 540, and 514, respectively, at the reproductive stage.

According to the taxonomic affiliations of the OTUs, there are 37 phyla (97.30% classified), 78 classes (92.30% classified), 175 orders (91.43% classified), 329 families (89.36% classified), and 614 genera (87.30% classified) in rice root endophytic communities. Among all rice cultivars, the dominant phyla include *Proteobacteria* (81.08%), *Firmicutes* (9.61%), *Actinobacteria* (5.16%), *Acidobacteria* (1.21%), *Bacteroidetes* (0.70%), and *Spirochaetes* (0.68%). The dominant classes include *Gammaproteobacteria* (56.13%), *Betaproteobacteria* (13.78%), *Clostridia* (8.15%), *Alphaproteobacteria* (7.76%), *Actinobacteria* (5.16%), *Deltaproteobacteria* (3.40%), *Bacilli* (1.43%), and *Acidobacteria* (1.21%; **Figure 1C**). The dominant genera include *Pseudomonas* (55.12%), *Ralstonia* (4.11%), *Burkholderia-Paraburkholderia* (3.90%), *Bradyrhizobium* (2.34%), *ClostridiumSensu_Stricto_1* (1.43%), *Sideroxydans* (1.37%), *Kineosporia* (1.43%), *Anaeromyxobacter* (1.23%), and *Bacillus* (1.10%; **Figure 1D**).

Shifts of Bacterial Community Structures During Two Developmental Stage of Rice

PCoA was used to compare the bacterial community composition at the OTU level at two stages. PCoA1 and PCoA2 explain 43.87 and 8.81%, respectively, of the observed variation (**Figure 1B**). The samples from the vegetative stage and reproductive stage separate clearly into two groups, indicating that the developmental stage governs a larger source of variation. PERMANOVA based on weighted UniFrac distances confirms that the developmental stage significantly influences the composition of bacterial communities (14%, $p < 0.01$). The rice

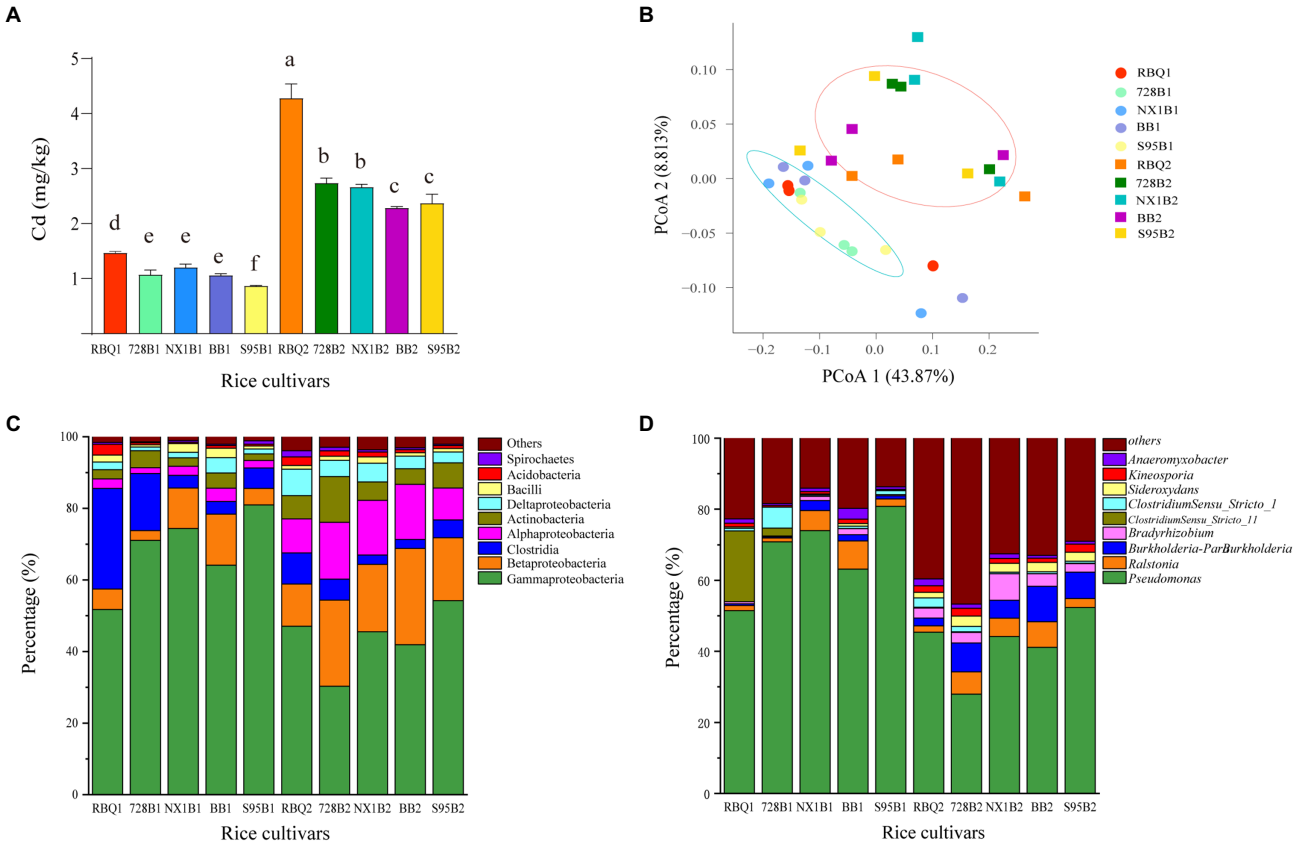


FIGURE 1 | Cd content in roots of five rice cultivars at the vegetative and reproductive stages **(A)**; Principal coordinate analysis (PCoA) based on weighted UniFrac distance matrices at observed operational taxonomic unit (OTU) level **(B)**; relative abundances of the dominant classes **(C)** and genera **(D)**; hereafter, the cultivar name followed by 1 represents the vegetative stage and by 2 represents the reproductive stage. The different lowercase letters indicate significant differences among cultivars at $p < 0.05$.

TABLE 1 | Permutational multivariate analysis of variance (PERMANOVA) results using weighted UniFrac distance matrices show effects of rice developmental stage, cultivar, and root Cd content on the bacterial community compositions.

Factor	% Explained	F. Model	R^2	value of p
Cultivar	15.07	1.32	0.15	0.25
Stage	14.04	4.90	0.14	0.011*
Cd	2.35	0.82	0.02	0.493
Cultivar: Stage	7.72	0.67	0.08	0.771
Cultivar: Cd	12.15	1.06	0.12	0.432
Stage: Cd	5.52	1.93	0.06	0.144
Cultivar: Stage: Cd	14.52	1.27	0.15	0.259
Residuals	28.64		0.29	
Total	100.00		1.00	

* *denotes significant differences at* $p < 0.05$.

cultivars do not differ significantly in bacterial compositions ($p = 0.25$). The Cd content explains 2% of the observed variance, but not significantly different ($p = 0.493$; **Table 1**).

In order to demonstrate the composition shifts of the endophytic bacterial communities at two developmental stages, two-sample *t*-tests were used to compare α-diversity indices. The results shows that observed OTUs, Shannon index, ACE index, and Chao1 at the reproductive stage are significantly higher than those at the vegetative stage ($p < 0.01$), while the Simpson index at the reproductive stage is significantly lower than that at the vegetative stage ($p < 0.01$), indicating that the endophytic bacterial community has much higher richness and diversity at the reproductive stage compared to the vegetative stage (**Supplementary Table 1**). ANOVA shows that α-diversity indices among different cultivars are not statistically different (**Figure 2, Supplementary Table 2**).

The relative abundances of dominant endophytic bacterial taxa were further compared between two developmental stages. In comparison with the vegetative stage, the relative abundance of *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria,* and *Actinomycetes* at the reproductive stage are significantly higher ($p < 0.05$), whereas the relative abundance of *Gammaproteobacteria* is significantly lower ($p < 0.01$). The relative abundance of *Clostridia*, *Bacilli*, and *Acidobacteria* does not show significant changes (**Supplementary Table 3**). At the genus level, the relative abundance of *Burkholderia-Paraburkholderia*, *Bradyrhizobium*, *Sideroxydans*, and *Kineosporia* is significantly higher ($p < 0.01$), whereas the abundance of *Pseudomonas* is significantly lower ($p < 0.01$). The relative abundance of *Ralstonia*, *Clostridium_Sensu_Stricto_1*, *Clostridium_Sensu_Stricto_11*, *Anaeromyxobacter*, and *Bacillus* does not show significant changes (**Supplementary Table 4**).

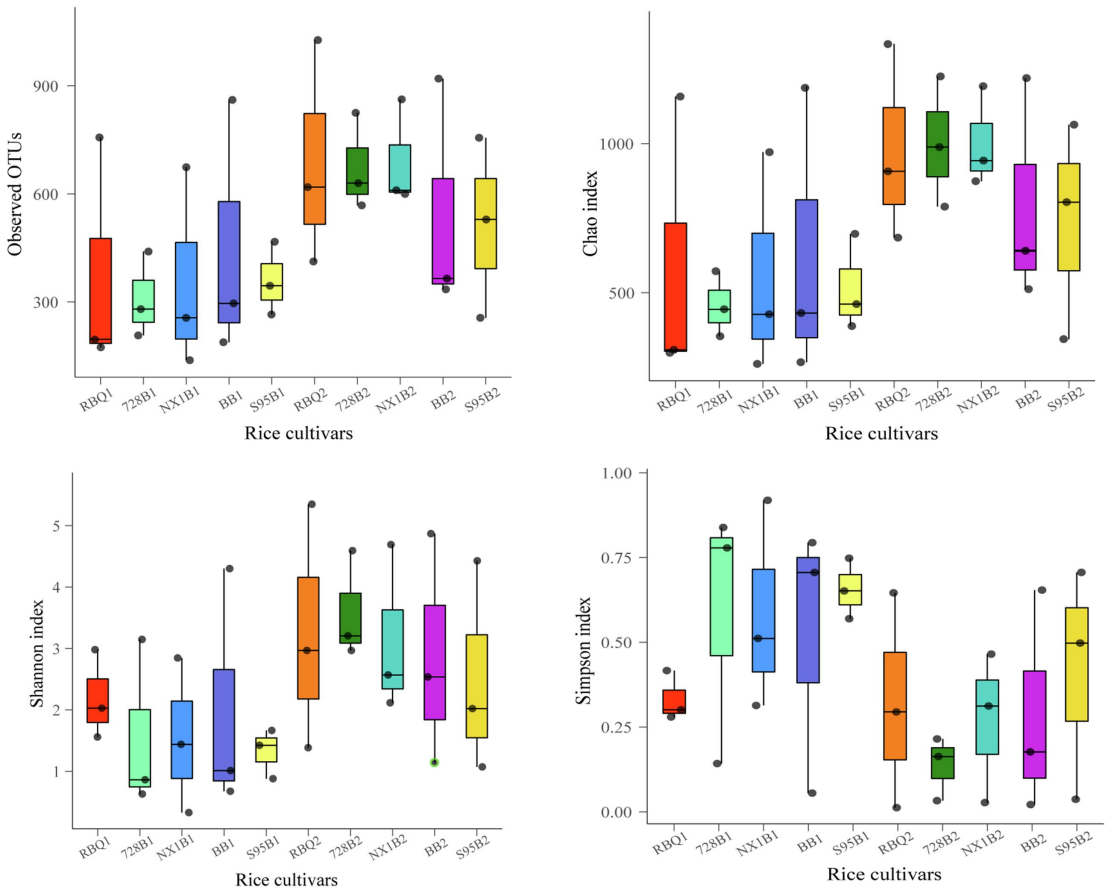


FIGURE 2 | Box plots of α-diversity indices show observed OTUs, Shannon index, ACE index, and Chao1 of five rice cultivars at the vegetative and reproductive stages. The lines at the top, bottom, and middle of the box correspond to the 75th, 25th, and 50th percentiles (median), respectively. The ends of whiskers correspond to the minimum and maximum value.

Co-occurrence Network Analysis of the Bacterial Community

Co-occurrence bacterial network analysis shows different connectivity patterns at the two developmental stages. In general, the number of correlations is substantially larger at the reproductive stage than at the vegetative stage. At the vegetative stage, the network has 266 nodes with 4,277 edges, among which 4,215 are positively correlated and 62 are negatively correlated, and the average degree of the network is 32.16. At the reproductive stage, the network has 425 nodes with 11,659 edges, among which 10,763 are positively correlated, and 896 are negatively correlated, and the average degree of the network is 54.87 (**Figure 3, Supplementary Table 5**). These findings indicate a higher complexity of the bacterial networks at the reproductive stage than at the vegetative stage.

The taxonomy of the most connected nodes varies between the vegetative and reproductive stages. At the vegetative stage, *Actinobacteria* accounts for 17.36% of the total degree of connection, followed by *Betaproteobacteria* (16.79%), *Deltaproteobacteria* (14.37%), *Alphaproteobacteria* (11.34%), and *Clostridia* (9.84%). At the reproductive stage, *Betaproteobacteria* accounts for 23.89% of the total degree of connection, followed by *Deltaproteobacteria* (22.35%), *Actinobacteria* (9.16%), *Alphaproteobacteria* (7.15%), and *Acidobacteria* (6.38%; **Supplementary Table 6**).

According to degree centrality, we classified the most central 20% as key nodes (Xian et al., 2020). The relative abundance of the key nodes at each developmental stage varies greatly, ranging from 0.005% to 1%. Several of the highly connected taxa, such as *Anaeromyxobacter*, *Geobacter*, *Haliangium*, *kineosporia,* and *Streptomyces* at the vegetative stage and *Ralstonia*, *Haliangium*, *Sideroxydans*, *Geobacter*, *Anaeromyxobacter, Bradyrhizobium* and *Micromonospora* at the reproductive stage, appear to be the keystone bacterial taxa (**Supplementary Figure 2**).

Cultivar-Associated Bacterial Biomarkers and Specific OTUs

In order to detect the bacterial taxonomic biomarkers of each cultivar, OTUs with relative abundance greater than 0.05% were compared between samples using LEfSe [linear discriminant analysis (LDA) effect size]. OTUs with the highest LDA (Wilcoxon

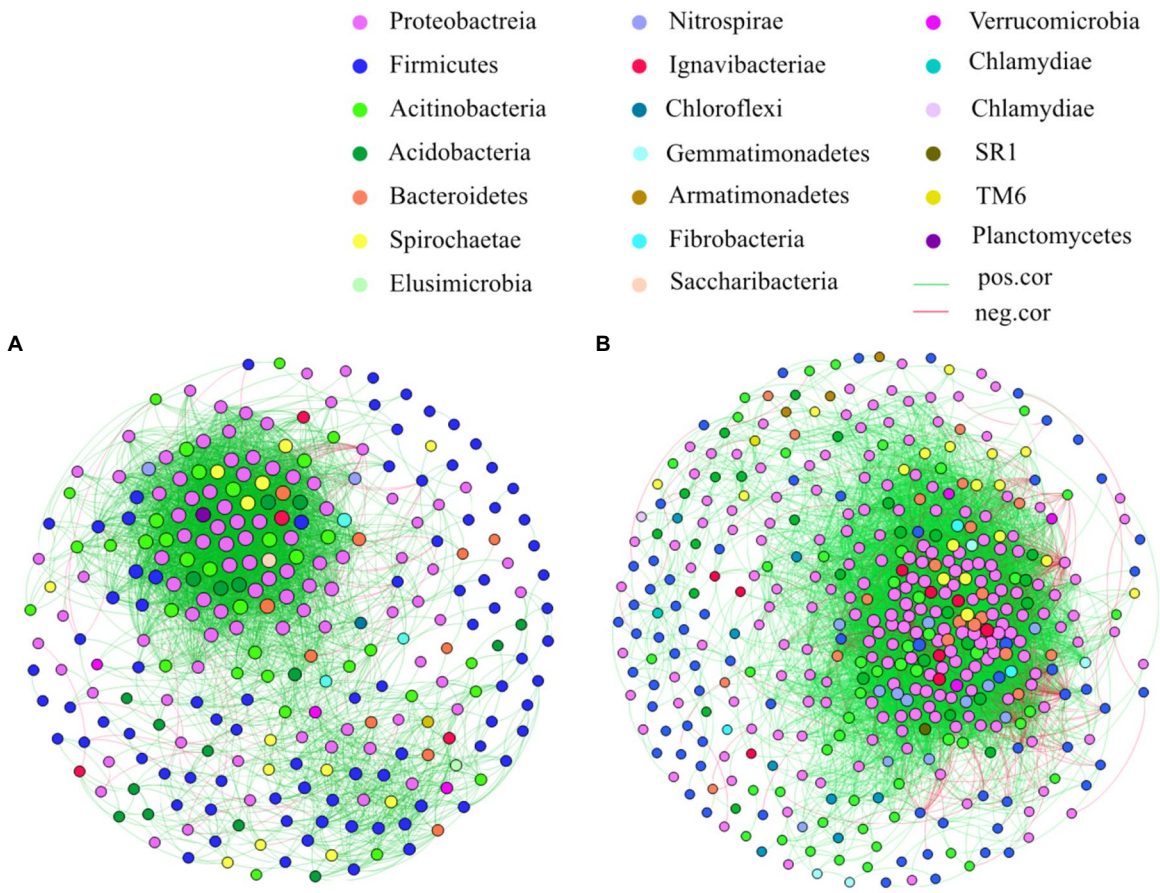


FIGURE 3 | Co-occurrence network diagrams of samples at the vegetative **(A)** and reproductive **(B)** stages. Connections stand for Spearman's correlation with $r > 0.75$ and statistical significance at $p < 0.01$. Nodes are colored by taxonomy, and the size of each node is proportional to the number of connections (i.e., degree). Green and red lines represent co-occurrence and mutual exclusion, respectively. Details regarding the co-occurrence network are reported in **Supplementary Tables 5, 6**.

$p < 0.05$, LDA score > 3) from each cultivar are shown in **Figure 4**. At the vegetative stage, only cultivar 728B and BB have differentially abundant clades, such as *Micrococcaceae* and *Arthrobacter* in BB. However, at the reproductive stage, all rice cultivars show differentially abundant clades, such as *Ruminiclostridium_1* and *Nocardia* in RBQ; *Methylcystiaceae* and *Hyphomicrobiaceae* in 728B; *Chloroflexi* in NX1B; *Sphingomonadaceae* and *Sphingomonas* in BB; and *Mycobacteriaceae* and *Mycobacterium* in S95B (**Figures 4A,B**).

The Venn diagram shows a cluster of shared OTUs and the specific OTUs for each cultivar. At the vegetative stage, there are 12 shared OTUs (77.8%) among different cultivars, mainly including *Pseudomonas* (OTU_538, 68.01%), *Ralstonia* (OTU_1351, 3.64%), and *Clostridium_Sensu_Stricto_1* (OTU_83, 1.53%). Specific OTUs of RBQ, 728B, NX1B, BB, and S95B at the vegetative stage account for 7.71, 5.66, 1.09, 2.81, and 1.19% of the bacterial communities, respectively (**Figure 4C**).

At the reproductive stage, there are 47 shared OTUs (77.60%) among different cultivars, mainly including *Pseudomonas* (OTU_538, 42.17%), *Ralstonia* (OTU_1351, 4.52%), *Burkholderia-Paraburkholderia* (OTU_1265, 3.31%; OTU_1568, 2.77%), *Sideroxydans* (OTU_2233, 1.88%), and *Bradyrhizobium* (OTU_1319, 1.88%; OTU_389, 1.71%). Specific OTUs of RBQ, 728B, NX1B, BB, and S95B at the reproductive stage account for 3.64, 7.62, 1.99, 1.33, and 1.05% of the bacterial communities, respectively (**Figure 4D**).

Bacterial Taxa Correlated With Cd Contamination

Spearman correlation analysis shows that 45 genera (7.3% of observed genera) are significantly correlated with Cd content (Spearman's $r > 0.75$, $p < 0.01$, **Supplementary Table 7**). Among them, forty-one genera are positively correlated with Cd content, such as *Geobacter*, *Haliangium*, *Uliginosibacterium*, *Ideonella*, *Micromonospora,* and *Geothrix*. Four genera are negatively correlated with Cd content, including *Pseudomonas*, *Arthrobacter*, *Streptacidiphilus*, and *Prolixibacter*. Linear regressions between the abundances of the representative bacterial genera and the Cd content confirm the abundance of unique bacteria taxa correlated with the content of Cd in roots (**Figure 5**).

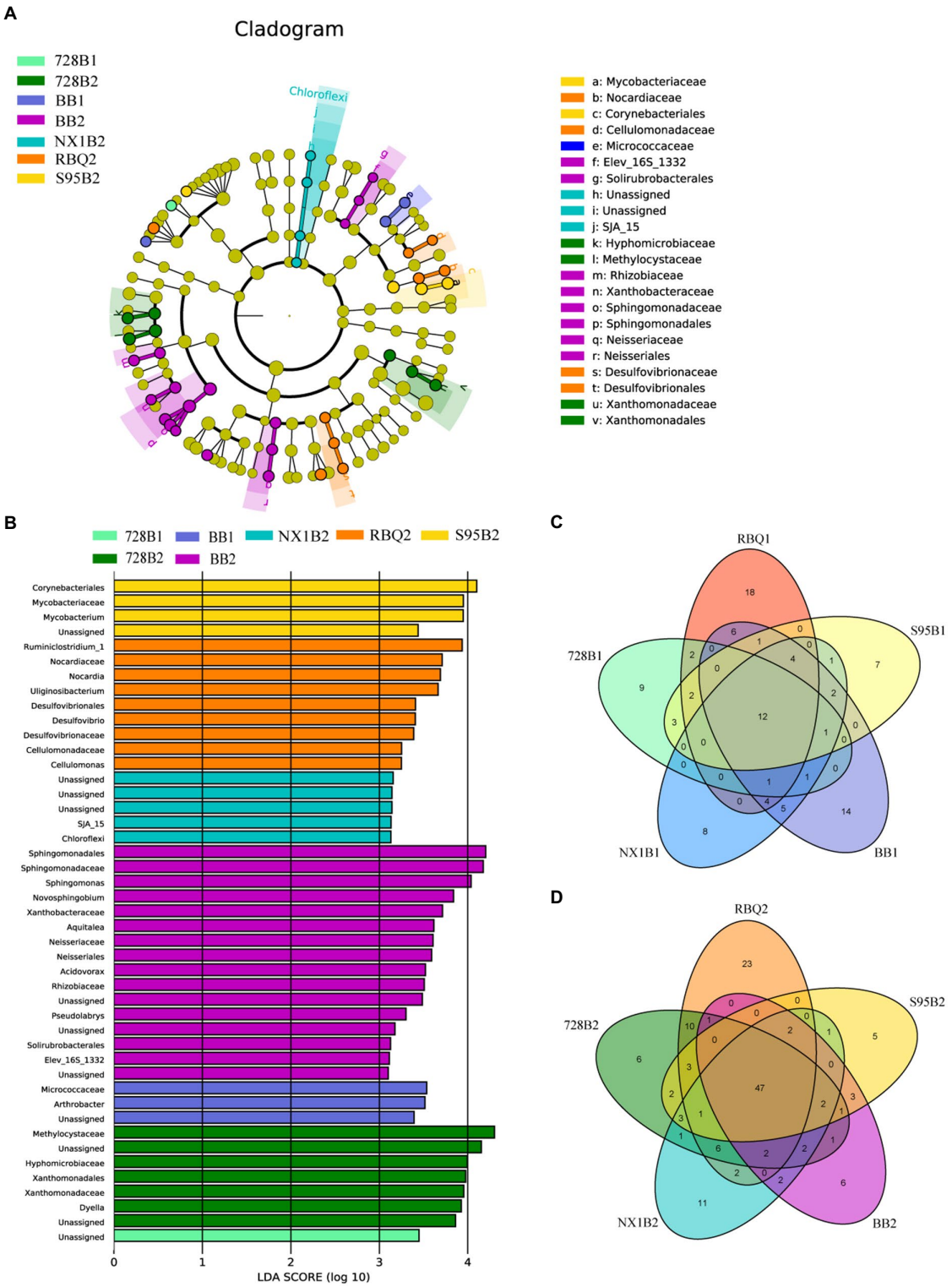


FIGURE 4 | (A–B) Taxonomic cladograms of linear discriminant analysis effect size (LEfSe) analysis of rice cultivars at two developmental stages, depicting OTUs with absolute linear discriminant analysis (LDA) scores larger than 3. Venn diagrams show shared and specific OTUs among five rice cultivars at the vegetative **(C)** and reproductive **(D)** stages.

Prediction of Functional Profiles of the Bacterial Community

A total of 297 functional pathways were analyzed for endophytic bacterial communities. KEGG Orthology (KO)-based PcoA analysis reveals functional differences of five rice cultivars across two stages (**Supplementary Figure 3**). From the vegetative to the reproductive stage, a total of 42 metabolic pathways (representing 14% of predicted functions) are significantly changed ($p < 0.05$), including bacterial motility proteins, butanoate metabolism, propanoate metabolism, bacterial chemotaxis, lipid biosynthesis proteins, fatty acid metabolism, glyoxylate and dicarboxylate metabolism, cysteine and methionine metabolism, and the biosynthesis of type II polyketide products (**Figure 6**).

Grime's Competitor/Stress-tolerator/Ruderal (CSR) theory proposes that organisms in a community face a three-way resource trade-off: competition with neighbors for resources (Competitive traits); survival in underproductive environments (Stress-tolerant traits); and survival in highly disturbed environments (Ruderal traits; Grime, 1977; Grime and Pierce, 2012). According to CSR theory and the detailed criteria for functional traits described in Wood et al. (2018), six functional pathways are classified as C traits, 11 as S traits, and seven as R traits in this study (**Supplementary Table 8**). Comparison of CSR traits between the two developmental stages reveals that the relative abundances of C traits (biosynthesis of siderophore and type II polyketide products) and S traits (Porphyrin and chlorophyll metabolism and Proteasome) are significantly increased at the reproductive stage. Most metabolic pathways in the R traits are also enhanced, especially carbon fixation pathways, citrate cycle (TCA cycle), and oxidative phosphorylation.

Cd Tolerance and PGP Potential of Cultivable Bacteria

Forty-three strains isolated from Cd-contaminated rice roots are ascribed to 12 genera in five classes (**Supplementary Table 9**). Among them, *Bacillus* comprises the largest number of taxa (53.5%), followed by *Klebsiella* (11.6%), *Paenibacillus* (7%), and *Herbaspirillum* (7%).

We tested the Cd tolerance and PGP traits of some representative isolates (**Figure 7, Supplementary Table 10**). The minimum inhibitory concentrations (MICs) of bacterial isolates to Cd are between 10 and 1,280 μm, among which *Bacillus* sp. TB3 and

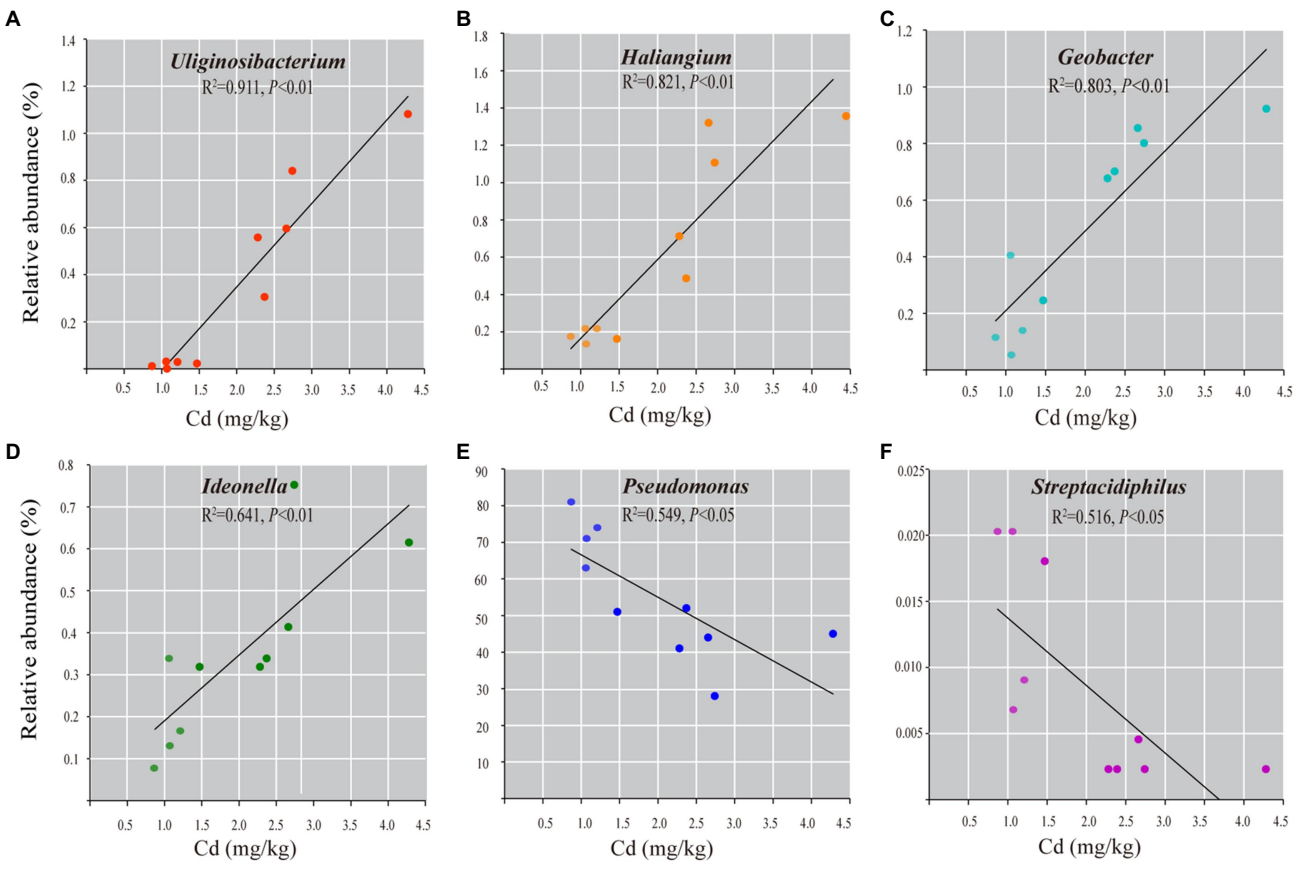


FIGURE 5 | The correlation analysis of the representative bacterial genera with Cd content in roots of rice grown in Cd-contaminated soil. Linear regression analyses show the relationships between the relative abundance of the genera of *Geobacter* **(A)**, *Uliginosibacterium* **(B)**, *Haliangium* **(C)**, *Ideonella* **(D)**, *Pseudomonas* **(E)**, and *Streptacidiphilus* **(F)** and Cd content (mg/kg dry weight). Only statistically significant correlations at $p < 0.01$ are shown.

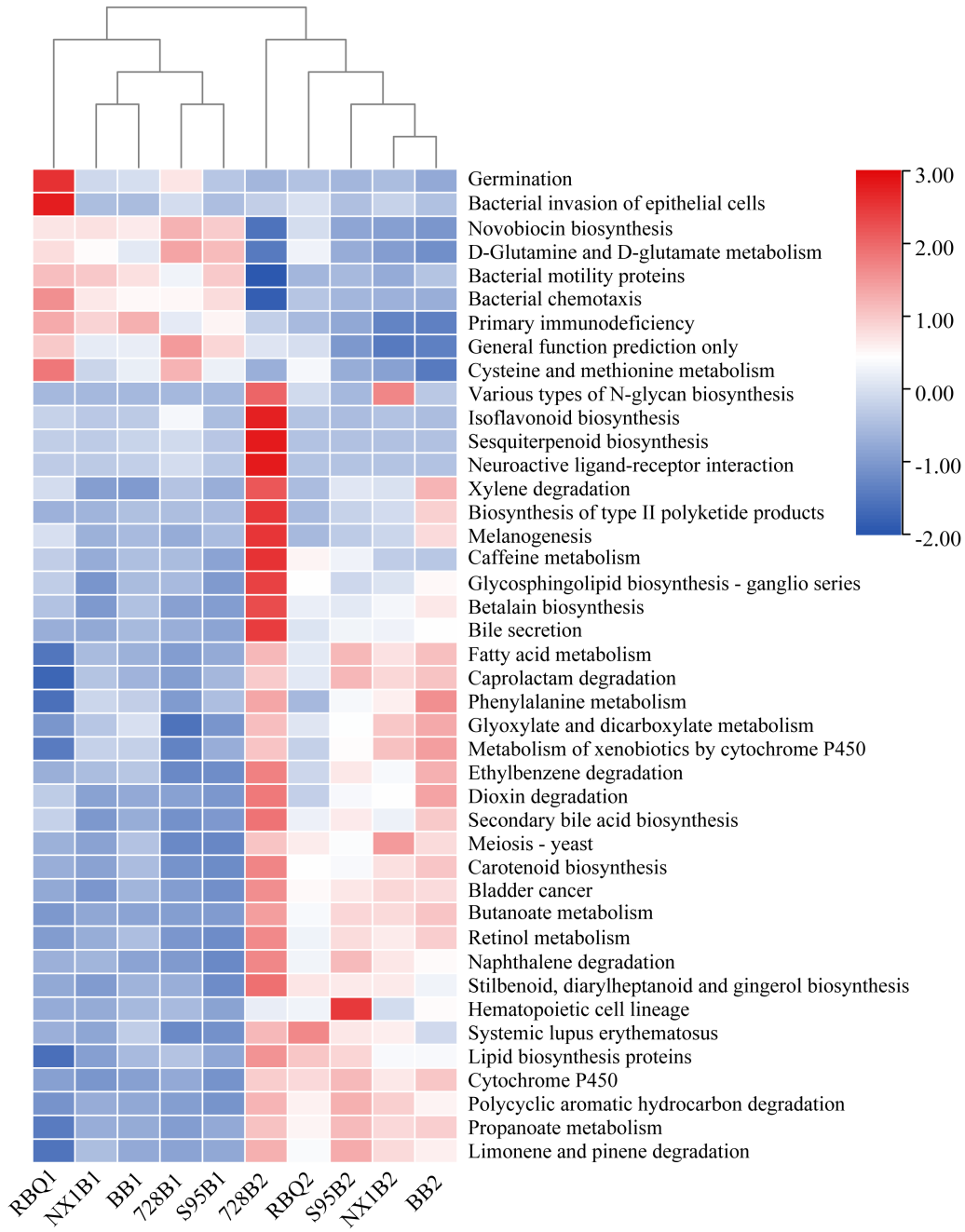


FIGURE 6 | Predicted gene functional categories of the endophytic microbiome of five rice cultivars at the vegetative and reproductive stages. The heat map is drawn for a total of 42 metabolic pathways which were significantly changed from the vegetative to reproductive stage.

TS1 show the highest tolerance (1,280 μm). As for PGP traits, *Klebsiella* sp. RBB3 shows the highest IAA production (52.84 μg/ml). *Pseudomonas* sp. 4-N2 shows the highest siderophore production (96.85 mg/l) and phosphate solubilization ability (41.46 μg/ml). The activity of ACC deaminase is highest for *Bacillus* sp. TB1 (12.84 U/mg), whereas some isolates are negative for the assay. Most of the endophytic bacteria isolated from the Cd-contaminated roots show traits of a higher Cd resistance and PGP potential. The endophytic bacteria with those traits can be exploited as potential bioinoculants to enhance crop productivity and mitigate HM stress.

DISCUSSION

High-throughput sequencing studies have revealed a high diversity of endophytic bacteria in rice roots (Sun et al., 2008;

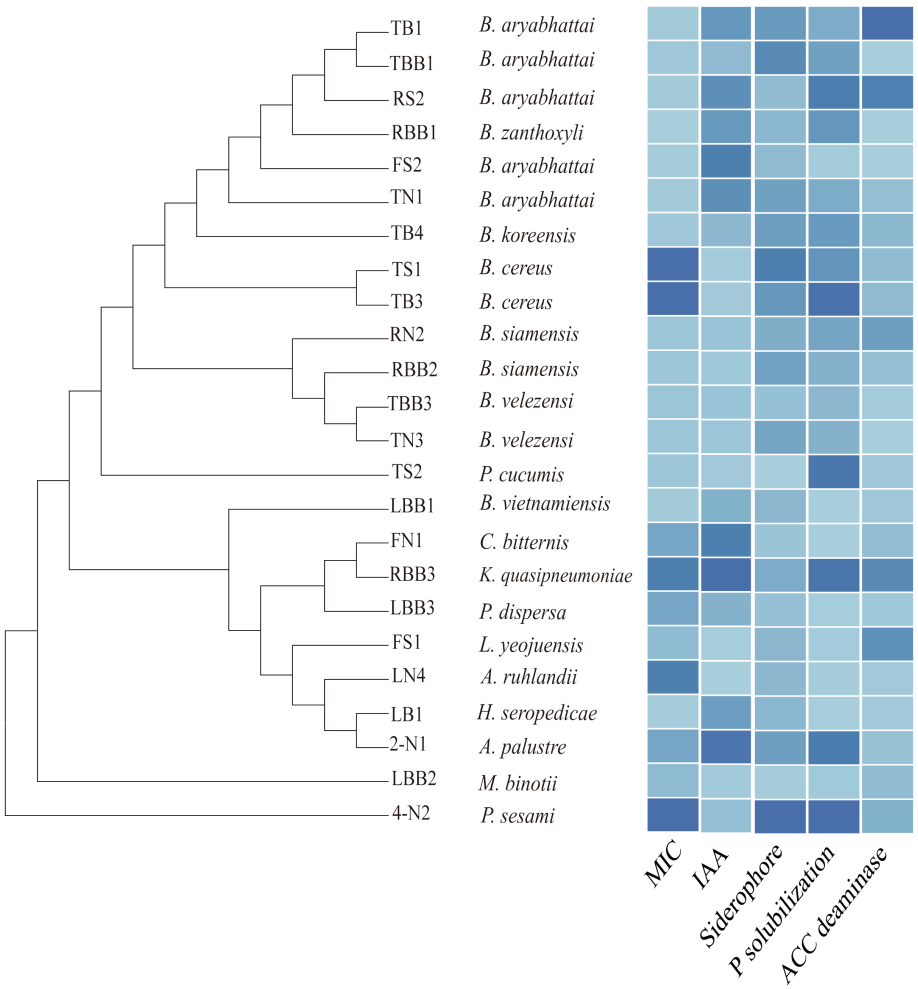


FIGURE 7 | Phylogenetic tree of the representative bacterial isolates and their minimum inhibitory concentrations (MICs) to Cd and plant growth-promoting (PGP) traits, including production of indole-3-acetic acid (IAA), siderophore, phosphate solubilization, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activities. The color gradient from dark to light represents the value from high to low.

Edwards et al., 2015; Ding et al., 2019; Walitang et al., 2019). In this study, by using five cultivars grown in a natural Cd-contaminated paddy field, we observed *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Spirochaetes* are the most dominant phyla, and *Pseudomonas*, *Ralstonia*, *Burkholderia-Paraburkholderia*, *Bradyrhizobium*, *ClostridiumSensu_Stricto_1*, *Sideroxydans*, *Kineosporia*, *Anaeromyxobacter*, and *Bacillus* are the most dominant genera.

The endophytic microbiota has been found to be beneficial to plant growth, nutrition uptake, and resistance to biotic and abiotic stresses (Castrillo et al., 2017; Kim and Lee, 2020). In this study, we found that *Proteobacteria* makes up the largest fraction of the bacterial communities, which include gamma, alpha, beta, and delta subclasses. In *Alphaproteobacteria*, *Bradyrhizobium* and *Sphingomonas* are two representatives reported to be nitrogen fixers (Knief et al., 2012; Sessitsch et al., 2012; Hardoim et al., 2015). In *Betaproteobacteria*, *Burkholderia-Paraburkholderia* shows multiple phytobeneficial traits (Madhaiyan et al., 2008; King et al., 2019). *Pseudomonas*, affiliated to the *Gammaproteobacteria*, is the most abundant genus, which has been detected in various rice tissues, including root, stem, leaves, and seeds (Walitang et al., 2019). It has been reported that *Pseudomonas* can synthesize PGP substance (such as phytohormone) to facilitate plant growth or produce antibiotics to increase resistance to pathogens (Weller et al., 2002; Sivasakthi et al., 2013; Han et al., 2015). We found that *Deltaproteobacteria* is another dominant bacterial population in rice root. Compared with the root-associated bacteria of other crops, rice root is significantly enriched for *Deltaproteobacteria* (Ding et al., 2019). One possible explanation is that the flooded nature of rice cultivation could favor the growth of anaerobic microorganisms in the *DeltaProteobacteria*, such as *Geobacter* and *Anaeromyxobacter*, which are involved in the reduction of Fe (III) and S (Ding et al., 2015; Sun et al., 2015).

Endophytic microbiota change with the growth and developmental stages of host plants (Santoyo et al., 2016). In this study, the alpha diversity indices (Observed OTUs, Shannon, ACE and Chao 1) of bacterial communities at the reproductive stage are significantly higher than that at the vegetative stage, indicating a more mature community is established at the reproductive stage. Other studies also reported that rice root microbiota varies dramatically during the vegetative stage and becomes stabilized during the reproductive stage until rice ripening (Edwards et al., 2018; Zhang et al., 2018). Xun et al. (2021) found that bacterial communities with a higher phylogenetic diversity are more stable and more resistant to environmental disturbance. Our co-occurrence network analysis further reveals that the endophytic bacterial community has established a complex symbiotic relationship during the reproductive stage. It is worth noting that although *Pseudomonas* and *Burkholderia-Paraburkholderia* are the most dominant genera, they are not in the top 20% key nodes of the network. In contrast, *Sideroxydans*, *Ralstonia*, *Haliangium*, *Bradyrhizobium*, *Micromonospora*, *Anaeromyxobacter,* and *Geobacter* are the key nodes of the network. Among them, some species have been reported to participate in nitrogen fixation or CO_2 fixation (Knief et al., 2012; Federico et al., 2015), phosphate solubilizing (Carro et al., 2018), and the iron cycle (Weiss et al., 2007; Ding et al., 2015). We suggest that the fundamental metabolic functions (especially nitrogen and carbon metabolism, phosphorus metabolism, and iron cycle) are the key engines for the assemblage of rice endophytic bacterial communities, which is in agreement with what has been found in soil microbiome (Xun et al., 2021).

Several studies have demonstrated that HM hyper-accumulating or tolerant plants harbor endophytic populations with a high HM resistance, indicating that an environmental stress influences the endophytic microbiomes (Luo et al., 2019; Zadel et al., 2020). As the Cd content in roots is significantly different among LA and HA cultivars, we further analyzed whether specific bacterial taxa may be affected by Cd contamination. Spearman correlation analysis shows that 45 genera are significantly correlated with Cd content. Some genera, which are positively correlated to Cd content, were also reported in other HM-polluted environments. For example, *Geobacter* and *Geothrix* are the two dominant genera in the uranium mine sediment (Li et al., 2018). Recent studies revealed that *Geobacter*, as a Fe (III)-reducing bacterium, plays an important role in controlling the mobility of As in the rice rhizosphere (Dai et al., 2020; Li et al., 2021). The negatively correlated genera, such as *Pseudomonas* and *Arthrobacter*, have been reported as the most represented genera in HM-contaminated sites (Pires et al., 2017). The inoculation of seeds with *Pseudomonas* or *Arthrobacter* has been shown to facilitate the root development of *Brassica napus* L. under the Zn/Cd stress (Croes et al., 2013; Montalban et al., 2016). In this study, *Pseudomonas* and *Arthrobacter* are enriched in roots with a low Cd content, suggesting their potential applications in alleviating HM stress in host plants.

In order to understand the characteristics of metabolic pathways of rice root endophyte under HM stress, PICRUSt and CSR theory (Grime, 1977; Grime and Pierce, 2012) were used to conduct a preliminary analysis of the functional characteristics of the endophytic bacterial communities. Wood et al. (2018) reveal that a bacterium's competitive attributes are critical for its ability to occupy and proliferate in a Cd-contaminated rhizosphere. In this study, we observe that the bacterial biosynthesis of type II polyketide products and siderophore (C traits) is enriched at the reproductive stage, indicating that endophytic bacteria might promote host plant resistance to pathogens by synthesizing polyketide antibiotics and contribute to Fe uptake by siderophore production. Siderophores may also participate in chelating HM ions and thus reduce the toxicity of Cd to host plants (Etesami, 2018). Among S traits, porphyrin metabolism and proteasome function increase significantly. Mn-porphyrins are potent antioxidants, scavenging O^{2-} radicals (Miriyala et al., 2012), and proteasomes are involved in protein degradation and the recycling of amino acids (Becker and Darwin, 2017), suggesting that these two pathways may be involved in alleviating HM-induced oxidative stress and removal of damaged proteins. As for R traits, the enhancement of carbon fixation pathways, citrate cycle (TCA cycle), and oxidative phosphorylation is favorable for endophytes to utilize nutrients for proliferation and occupying ecological niches.

From the above studies based on 16S rRNA gene amplicon sequencing and bioinformatic analysis, we profiled the root endophytic bacterial populations under Cd contamination. Based on these studies, we decided to isolate beneficial endophytic strains with potential applications in HM remediation. Plant growth-promoting bacteria (PGPB) are known to play a vital role in enhancing crop productivity and plant resistance to HM stresses (Etesami, 2018; Guo et al., 2020). Some genera affiliated to *Bacillus*, *Neorhizobium*, *Delftia*, *Pseudomonas*, *Cupriavidus*, and *Stenotrophomonas* have been reported to enhance rice growth and reduce the accumulation of HM in grains (Siripornadulsil and Siripornadulsil, 2013; Lin et al., 2016; Li et al., 2017; Liu et al., 2018; Treesubsuntorn et al., 2018). In this study, we isolated cadmium-resistant endophytic bacteria from 12 genera, including *Bacillus*, *Paenibacillus*, *Klebsiella*, *Herbaspirillum*, and *Pseudomonas*. Most of them show multiple PGP activities, such as phosphate solubilization, production of phytohormones (e.g., IAA), siderophores, and ACC deaminase. These activities suggest their beneficial roles in improving rice growth under stress conditions. Further studies are necessary to understand the highly complex interactions between rice and its associated microbe and to exploit the endophytic PGPB for remediation of HM contamination in paddy fields.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SRR11528738-SRR11528760 and MZ923694-MZ923717.

AUTHOR CONTRIBUTIONS

MF, NL, and CZ carried out the samples preparation and bacterial gDNA extraction. CC, MF, and CS performed bioinformatics and statistics analyses. CC and SF analyzed the Cd resistance and PGP traits of the isolates. CC and MF wrote the manuscript draft under the supervision of WW and ZY. All authors contributed to the article and approved the submitted version.

FUNDING

This research was financially supported by the Agricultural Science-Technology Innovation Fund of Hunan Province, China (grant no. 2018QN03) and the Science and Technology Program of Changsha, China (grant no. kq1801033).

ACKNOWLEDGMENTS

We especially thank Drs. Larry Bowman, Zhihong Jane Yang, Phillip Patten for their assistance with English language and grammatical editing. We also thank Dr. Xiaohui Li for his helpful advice on statistical analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.756327/full#supplementary-material

REFERENCES

Afzal, I., Shinwari, Z. K., Sikandar, S., and Shahzad, S. (2019). Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. *Microbiol. Res.* 221, 36–49. doi: 10.1016/j.micres.2019.02.001

Atafar, Z., Mesdaghinia, A., Nouri, J., Homaee, M., Yunesian, M., Ahmadimoghaddam, M., et al. (2010). Effect of fertilizer application on soil heavy metal concentration. *Environ. Monit. Assess.* 160, 83–89. doi: 10.1007/s10661-008-0659-x

Bastian, M., Heymann, S., and Jacomy, M. (2009). Gephi an open source software for exploring and manipulating networks. *Third International AAAI Conference on Weblogs and Social Media* 8, 361–362.

Becker, S. H., and Darwin, K. H. (2017). Bacterial proteasomes: mechanistic and functional insights. *Microbiol. Mol. Biol. Rev.* 81:e00036-16. doi: 10.1128/mmbr.00036-16

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Carro, L., Nouioui, I., Sangal, V., Meier-Kolthoff, J. P., Trujillo, M. E., Montero-Calasanz, M. D. C., et al. (2018). Genome-based classification of micromonosporae with a focus on their biotechnological and ecological potential. *Sci. Rep.* 8:525. doi: 10.1038/s41598-017-17392-0

Castrillo, G., Teixeira, P., Paredes, S. H., Law, T. F., Lorenzo, L. d., Feltcher, M. E., et al. (2017). Root microbiota drive direct integration of phosphate stress and immunity. *Nature* 543, 513–518. doi: 10.1038/nature21417

Chen, C., Chen, H., Zhang, Y., Thomas, H. R., Frank, M. H., He, Y., et al. (2020). TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* 13, 1194–1202. doi: 10.1016/j.molp.2020.06.009

Cheng, C., Nie, Z., He, L., and Sheng, X. (2020). Rice-derived facultative endophytic Serratia liquefaciens F2 decreases rice grain arsenic accumulation in arsenic-polluted soil. *Environ. Pollut.* 259:113832. doi: 10.1016/j.envpol.2019.113832

Croes, S., Weyens, N., Janssen, J., Vercampt, H., Colpaert, J. V., Carleer, R., et al. (2013). Bacterial communities associated with Brassica napus L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. *Microb. Biotechnol.* 6, 371–384. doi: 10.1111/1751-7915.12057

Csardi, G., and Nepusz, T. (2006). The igraph software package for complex network research. *Interjournal Complex Systems* 1695, 1–9.

Dai, J., Tang, Z., Jiang, N., Kopittke, P. M., Zhao, F., and Wang, P. (2020). Increased arsenic mobilization in the rice rhizosphere is mediated by iron-reducing bacteria. *Environ. Pollut.* 263:114561. doi: 10.1016/j.envpol.2020.114561

Ding, L., Cui, H., Nie, S., Long, X., Duan, G., and Zhu, Y. (2019). Microbiomes inhabiting rice roots and rhizosphere. *FEMS Microbiol. Ecol.* 95:fiz040. doi: 10.1093/femsec/fiz040

Ding, L., Su, J., Xu, H., Jia, Z., and Zhu, Y. (2015). Long-term nitrogen fertilization of paddy soil shifts iron-reducing microbial community revealed by RNA-(13)C-acetate probing coupled with pyrosequencing. *ISME J.* 9, 721–734. doi: 10.1038/ismej.2014.159

Edwards, J., Johnson, C., Santos-Medellin, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U. S. A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Edwards, J. A., Santos-Medellín, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., et al. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLoS Biol.* 16:e2003862. doi: 10.1371/journal.pbio.2003862

Etesami, H. (2018). Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects. *Ecotoxicol. Environ. Saf.* 147, 175–191. doi: 10.1016/j.ecoenv.2017.08.032

Federico, R., Eugenia, J. O., Ludo, D., and Roberto, D. P. (2015). Microbial fixation of CO_2 in water bodies and in drylands to combat climate change, soil loss and desertification. *New Biotechnol.* 32, 109–120. doi: 10.1016/j.nbt.2013.12.002

Glickmann, E., and Dessaux, Y. (1995). A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* 61, 793–796. doi: 10.1128/aem.61.2.793-796.1995

Grime, J. P. (1977). Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *Am. Nat.* 111, 1169–1194. doi: 10.1086/283244

Grime, J. P., and Pierce, S. (2012). *The Evolutionary Strategies that Shape Ecosystems*. Oxford: Wiley-Blackwell.

Guo, J., Muhammad, H., Lv, X., Wei, T., Ren, X., Jia, H., et al. (2020). Prospects and applications of plant growth promoting rhizobacteria to mitigate soil metal contamination: A review. *Chemosphere* 246:125823. doi: 10.1016/j.chemosphere.2020.125823

Han, Y., Wang, R., Yang, Z., Zhan, Y., Ma, Y., Ping, S., et al. (2015). 1-aminocyclopropane-1-carboxylate deaminase from pseudomonas stutzeri A1501 facilitates the growth of rice in the presence of salt or heavy metals. *J. Microbiol. Biotechnol.* 25, 1119–1128. doi: 10.4014/jmb.1412.12053

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., et al. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi: 10.1128/MMBR.00050-14

Hou, D., Wang, R., Gao, X., Wang, K., Lin, Z., Ge, J., et al. (2018). Cultivar-specific response of bacterial community to cadmium contamination in the rhizosphere of rice (*Oryza sativa* L.). *Environ. Pollut.* 241, 63–73. doi: 10.1016/j.envpol.2018.04.121

Hu, Y., Cheng, H., and Tao, S. (2016). The challenges and solutions for cadmium-contaminated rice in China: A critical review. *Environ. Int.* 92, 515–532. doi: 10.1016/j.envint.2016.04.042

Hu, M., Li, F., Liu, C., and Wu, W. (2015). The diversity and abundance of As(III) oxidizers on root iron plaque is critical for arsenic bioavailability to rice. *Sci. Rep.* 5:13611. doi: 10.1038/srep13611

Hu, M., Sun, W., Krumins, V., and Li, F. (2019). Arsenic contamination influences microbial community structure and putative arsenic metabolism gene abundance in iron plaque on paddy rice root. *Sci. Total Environ.* 649, 405–412. doi: 10.1016/j.scitotenv.2018.08.388

Huang, S., and Jin, J. (2008). Status of heavy metals in agricultural soils as affected by different patterns of land use. *Environ. Monit. Assess.* 139, 317–327. doi: 10.1007/s10661-007-9838-4

Kim, M., and Chun, J. (2014). "16S rRNA gene-based identification of bacteria and archaea using the EzTaxon server," in *New Approaches to Prokaryotic Systematics. Vol. 41*. eds. M. Goodfellow, I. Sutcliffe and J. Chun (Elsevier), 61–74.

Kim, H., and Lee, Y. (2020). The rice microbiome: A model platform for crop holobiome. *Phytobiomes J.* 4, 5–18. doi: 10.1094/PBIOMES-07-19-0035-RVW

King, E., Wallner, A., Rimbault, I., Barrachina, C., Klonowska, A., Moulin, L., et al. (2019). Monitoring of rice transcriptional responses to contrasted colonizing patterns of phytobeneficial Burkholderia s.l. reveals a temporal shift in JA systemic response. *Front. Plant Sci.* 10:1141. doi: 10.3389/fpls.2019.01141

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., et al. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6, 1378–1390. doi: 10.1038/ismej.2011.192

Kossoff, D., Dubbin, W. E., Alfredsson, M., Edwards, S. J., Macklin, M. G., and Hudson-Edwards, K. A. (2014). Mine tailings dams: characteristics, failure, environmental impacts, and remediation. *Appl. Geochem.* 51, 229–245. doi: 10.1016/j.apgeochem.2014.09.010

Li, Y., Pang, H., He, L., Wang, Q., and Sheng, X. (2017). Cd immobilization and reduced tissue cd accumulation of rice (*Oryza sativa* wuyun-23) in the presence of heavy metal-resistant bacteria. *Ecotoxicol. Environ. Saf.* 138, 56–63. doi: 10.1016/j.ecoenv.2016.12.024

Li, B., Wu, W., Watson, D. B., Cardenas, E., Chao, Y., Phillips, D. H., et al. (2018). Bacterial community shift and coexisting/coexcluding patterns revealed by network analysis in a uranium-contaminated site after bioreduction followed by reoxidation. *Appl. Environ. Microbiol.* 84:e02885-17. doi: 10.1128/aem.02885-17

Li, B., Xu, R., Sun, X., Han, F., Xiao, E., Chen, L., et al. (2021). Microbiome-environment interactions in antimony-contaminated rice paddies and the correlation of core microbiome with arsenic and antimony contamination. *Chemosphere* 263:128227. doi: 10.1016/j.chemosphere.2020.128227

Lin, X., Mou, R., Cao, Z., Xu, P., Wu, X., Zhu, Z., et al. (2016). Characterization of cadmium-resistant bacteria and their potential for reducing accumulation of cadmium in rice grains. *Sci. Total Environ.* 569, 97–104. doi: 10.1016/j.scitotenv.2016.06.121

Liu, Y., Qin, Y., Chen, T., Lu, M., Qian, X., Guo, X., et al. (2021). A practical guide to amplicon and metagenomic analysis of microbiome data. *Protein Cell* 12, 315–330. doi: 10.1007/s13238-020-00724-8

Liu, Y., Tie, B., Li, Y., Lei, M., Wei, X., Liu, X., et al. (2018). Inoculation of soil with cadmium-resistant bacterium Delftia sp. B9 reduces cadmium accumulation in rice (*Oryza sativa* L.) grains. *Ecotoxicol. Environ. Saf.* 163, 223–229. doi: 10.1016/j.ecoenv.2018.07.081

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Luo, J., Tao, Q., Jupa, R., Liu, Y., Wu, K., Song, Y., et al. (2019). Role of vertical transmission of shoot endophytes in root-associated microbiome assembly and heavy metal hyperaccumulation in sedum alfredii. *Environ. Sci. Technol.* 53, 6954–6963. doi: 10.1021/acs.est.9b01093

Luo, J., Tao, Q., Wu, K., Li, J., Qian, J., Liang, Y., et al. (2017). Structural and functional variability in root-associated bacterial microbiomes of cd/Zn hyperaccumulator *sedum alfredii*. *Appl. Microbiol. Biotechnol.* 101, 7961–7976. doi: 10.1007/s00253-017-8469-0

Ma, Y., Rajkumar, M., Zhang, C., and Freitas, H. (2016). Beneficial role of bacterial endophytes in heavy metal phytoremediation. *J. Environ. Manag.* 174, 14–25. doi: 10.1016/j.jenvman.2016.02.047

Madhaiyan, M., Poonguzhali, S., Kwon, S. W., Song, M. H., and Sa, T. (2008). Molecular characterization of Burkholderia strains isolated from rice cultivars (*Oryza sativa* L.) for species identification and phylogenetic grouping. *J. Microbiol. Biotechnol.* 18, 1005–1010.

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Massa, S., Caruso, M., Trovatelli, F., and Tosques, M. (1998). Comparison of plate count agar and R2A medium for enumeration of heterotrophic bacteria in natural mineral water. *World J. Microb. Biot.* 14, 727–730. doi: 10.1023/A:1008893627877

Miriyala, S., Spasojevic, I., Tovmasyan, A., Salvemini, D., Vujaskovic, Z., St Clair, D., et al. (2012). Manganese superoxide dismutase, MnSOD and its mimics. *Biochim. Biophys. Acta* 1822, 794–814. doi: 10.1016/j.bbadis.2011.12.002

Montalban, B., Croes, S., Weyens, N., Lobo, M. C., Perez-Sanz, A., and Vangronsveld, J. (2016). Characterization of bacterial communities associated with Brassica napus L. growing on a Zn-contaminated soil and their effects on root growth. *Int. J. Phytoremediation* 18, 985–993. doi: 10.1080/15226514.2016.1183566

Moronta-Barrios, F., Gionechetti, F., Pallavicini, A., Marys, E., and Venturi, V. (2018). Bacterial microbiota of rice roots: 16S-based taxonomic profiling of endophytic and rhizospheric diversity, endophytes isolation and simplified endophytic community. *Microorganisms* 6:14. doi: 10.3390/microorganisms6010014

Murphy, J., and Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 27, 31–36. doi: 10.1016/S0003-2670(00)88444-5

Penrose, D. M., and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant.* 118, 10–15. doi: 10.1034/j.1399-3054.2003.00086.x

Pereira, S. I. A., and Castro, P. M. L. (2014). Diversity and characterization of culturable bacterial endophytes from *Zea mays* and their potential as plant growth-promoting agents in metal-degraded soils. *Environ. Sci. Pollut. Res. Int.* 21, 14110–14123. doi: 10.1007/s11356-014-3309-6

Pires, C., Franco, A. R., Pereira, S. I. A., Henriques, I., Correia, A., Magan, N., et al. (2017). Metal(loid)-contaminated soils as a source of culturable heterotrophic aerobic bacteria for remediation applications. *Geomicrobiol J.* 34, 760–768. doi: 10.1080/01490451.2016.1261968

Rajkumar, M., Sandhya, S., Prasad, M. N. V., and Freitas, H. (2012). Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnol. Adv.* 30, 1562–1574. doi: 10.1016/j.biotechadv.2012.04.011

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant-Microbe Interact.* 25, 28–36. doi: 10.1094/MPMI-08-11-0204

Siripornadulsil, S., and Siripornadulsil, W. (2013). Cadmium-tolerant bacteria reduce the uptake of cadmium in rice: potential for microbial bioremediation. *Ecotoxicol. Environ. Saf.* 94, 94–103. doi: 10.1016/j.ecoenv.2013.05.002

Sivasakthi, S., Kanchana, D., Usharani, G., and Saranraj, P. (2013). Production of plant growth promoting substance by *Pseudomonas fluorescens* and *Bacillus subtilis* isolates. *Int. J. Microbiol. Res.* 4, 227–233. doi: 10.5829/idosi.ijmr.2013.4.3.75171

Sun, L., Qiu, F., Zhang, X., Dai, X., Dong, X., and Song, W. (2008). Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microb. Ecol.* 55, 415–424. doi: 10.1007/s00248-007-9287-1

Sun, M., Xiao, T., Ning, Z., Xiao, E., and Sun, W. (2015). Microbial community analysis in rice paddy soils irrigated by acid mine drainage contaminated water. *Appl. Microbiol. Biotechnol.* 99, 2911–2922. doi: 10.1007/s00253-014-6194-5

Sun, W., Xiong, Z., Chu, L., Li, W., Soares, M. A., White, J. F., et al. (2019). Bacterial communities of three plant species from Pb-Zn contaminated sites and plant-growth promotional benefits of endophytic *microbacterium* sp. (strain BXGe71). *J. Hazard. Mater.* 370, 225–231. doi: 10.1016/j.jhazmat.2018.02.003

Syranidou, E., Thijs, S., Avramidou, M., Weyens, N., Venieri, D., Pintelon, I., et al. (2018). Responses of the endophytic bacterial communities of *Juncus acutus* to pollution with metals, emerging organic pollutants and to bioaugmentation with indigenous strains. *Front. Plant Sci.* 9:1526. doi: 10.3389/fpls.2018.01526

Treesubsuntorn, C., Dhurakit, P., Khaksar, G., and Thiravetyan, P. (2018). Effect of microorganisms on reducing cadmium uptake and toxicity in rice (*Oryza sativa* L.). *Environ. Sci. Pollut. Res. Int.* 25, 25690–25701. doi: 10.1007/s11356-017-9058-6

Walitang, D., Samaddar, S., Choudhury, A., Chatterjee, P., Ahmed, S., and Sa, T. (2019). "Diversity and plant growth-promoting potential of bacterial endophytes in rice," in *Plant Growth Promoting Rhizobacteria (PGPR): Prospects for Sustainable Agriculture*. ed. R. Z. Sayyed (Singapore: Springer), 1–17.

Wang, P., Chen, H., Kopittke, P., and Zhao, F. (2019). Cadmium contamination in agricultural soils of China and the impact on food safety. *Environ. Pollut.* 249, 1038–1048. doi: 10.1016/j.envpol.2019.03.063

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991

Weiss, J. V., Rentz, J. A., Plaia, T., Neubauer, S. C., Merrill-Floyd, M., Lilburn, T., et al. (2007). Characterization of neutrophilic Fe(II)-oxidizing bacteria isolated from the rhizosphere of wetland plants and description of Ferritrophicum radicicola gen. Nov. sp. nov., and Sideroxydans paludicola sp. nov. *Geomicrobiol J.* 24, 559–570. doi: 10.1080/01490450701670152

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi: 10.1146/annurev.phyto.40.030402.110010

Wiegand, I., Hilpert, K., and Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175. doi: 10.1038/nprot.2007.521

Wood, J. L., Tang, C., and Franks, A. E. (2018). Competitive traits are more important than stress-tolerance traits in a cadmium-contaminated rhizosphere: A role for trait theory in microbial ecology. *Front. Microbiol.* 9:121. doi: 10.3389/fmicb.2018.00121

Xian, W., Salam, N., Li, M., Zhou, E., Yin, Y., Liu, Z., et al. (2020). Network-directed efficient isolation of previously uncultivated Chloroflexi and related bacteria in hot spring microbial mats. *NPJ Biofilms Microbiomes* 6:20. doi: 10.1038/s41522-020-0131-4

Xun, W., Liu, Y., Li, W., Ren, Y., Xiong, W., Xu, Z., et al. (2021). Specialized metabolic functions of keystone taxa sustain soil microbiome stability. *Microbiome* 9:35. doi: 10.1186/s40168-020-00985-9

Yin, K., Wang, Q., Lv, M., and Chen, L. (2019). Microorganism remediation strategies towards heavy metals. *Chem. Eng. J.* 360, 1553–1563. doi: 10.1016/j.cej.2018.10.226

Zadel, U., Nesme, J., Michalke, B., Vestergaard, G., Płaza, G. A., Schröder, P., et al. (2020). Changes induced by heavy metals in the plant-associated microbiome of Miscanthus x giganteus. *Sci. Total Environ.* 711:134433. doi: 10.1016/j.scitotenv.2019.134433

Zhang, J., Zhang, N., Liu, Y., Zhang, X., Hu, B., Qin, Y., et al. (2018). Root microbiota shift in rice correlates with resident time in the field and developmental stage. *Sci. China Life Sci.* 61, 613–621. doi: 10.1007/s11427-018-9284-4

Zhou, J., Li, P., Meng, D., Gu, Y., Zheng, Z., Yin, H., et al. (2020). Isolation, characterization and inoculation of cd tolerant rice endophytes and their impacts on rice under cd contaminated environment. *Environ. Pollut.* 260:113990. doi: 10.1016/j.envpol.2020.113990

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 December 2021
doi: 10.3389/fmicb.2021.765269

Differentiation and Variability in the Rhizosphere and Endosphere Microbiomes of Healthy and Diseased Cotton (*Gossypium* sp.)

Yingwu Shi[1,2,3,4], Hongmei Yang[1,2,3], Ming Chu[1,2,3], Xinxiang Niu[3,4], Ning Wang[1,2], Qing Lin[1,2], Kai Lou[1,2], Changgeng Zuo[1,2,5], Jingyi Wang[1,2,5], Qiang Zou[1,2,5] and Yumeng Zhang[1,2,5]*

[1] Institute of Microbiology, Xinjiang Academy of Agricultural Sciences, Urumqi, China, [2] Xinjiang Laboratory of Special Environmental Microbiology, Urumqi, China, [3] Key Laboratory of Agricultural Environment in Northwest Oasis of Ministry of Agriculture and Countryside, Urumqi, China, [4] Institute of Soil, Fertilizer, and Agricultural Water Conservation, Xinjiang Academy of Agricultural Sciences, Urumqi, China, [5] College of Life Sciences and Technology, Xinjiang University, Urumqi, China

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
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***Correspondence:**
Yingwu Shi
syw1973@126.com;
shiyingwu@xaas.ac.cn
orcid.org/0000-0003-2511-2386

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *26 August 2021*
Accepted: *22 October 2021*
Published: *06 December 2021*

Citation:
Shi Y, Yang H, Chu M, Niu X, Wang N, Lin Q, Lou K, Zuo C, Wang J, Zou Q and Zhang Y (2021) Differentiation and Variability in the Rhizosphere and Endosphere Microbiomes of Healthy and Diseased Cotton (Gossypium sp.). Front. Microbiol. 12:765269. doi: 10.3389/fmicb.2021.765269

The plant microbiome is a key determinant of health and productivity. However, it is still difficult to understand the structural composition of the bacterial and fungal microbiomes of diseased and healthy plants, especially the spatial dynamics and phylogenies of endophytic and rhizosphere microbial communities. We studied the differentiation and variability in the rhizosphere and endosphere microbiomes of healthy and diseased cotton from north and south of the Tianshan Mountains using the methods of PCR-based high-throughput sequencing and real-time quantitative PCR. The endophytic and rhizosphere bacterial abundances in the diseased plants were greater than those of healthy plants. The numbers of endophytic and rhizosphere fungi associated with diseased plants were greater than those associated healthy plants ($p < 0.05$). Endophytic and rhizosphere bacteria did not share common OTUs. The dominant rhizosphere bacteria were Proteobacteria (29.70%), Acidobacteria (23.14%), Gemmatimonadetes (15.17%), Actinobacteria (8.31%), Chloroflexi (7.99%), and Bacteroidetes (5.15%). The dominant rhizosphere fungi were Ascomycota (83.52%), Mortierellomycota (7.67%), Basidiomycota (2.13%), Chytridiomycota (0.39%), and Olpidiomycota (0.08%). The distribution of dominant bacteria in different cotton rhizosphere soils and roots differed, with the dominant bacteria *Pseudomonas* (15.54%) and *Pantoea* (9.19%), and the dominant fungi *Alternaria* (16.15%) and *Cephalotrichum* (9.10%) being present in the greatest numbers. At sampling points in different ecological regions, the total numbers of cotton endophytic and rhizosphere microbiome OTUs from southern to northern Xinjiang showed an increasing trend. There were significant differences in the composition and diversity of rhizosphere microbes and endophytes during the entire cotton growth period and in representative ecological regions ($p < 0.01$), whereas rhizosphere microbes and endophytes showed no significant differences among the four growth periods and in representative ecological regions. RB41, H16, Nitrospira, and Sphingomonas play important roles in the microbial ecology

of cotton rhizosphere soil. Pseudomonas accounted for a large proportion of the microbes in the cotton rhizosphere soil. This study provides an in-depth understanding of the complex microbial composition and diversity associated with cotton north and south of the Tianshan Mountains.

Keywords: endophytic microbiome, rhizosphere microbiome, healthy and diseased, cotton, diversity, Illumina MiSeq

INTRODUCTION

Cotton is an important natural fiber crop worldwide. It grows well at moderate temperatures and high light exposure, and it is suitable for growing in dry areas, such as Xinjiang, China. Endophytic and rhizosphere microbes promote plant growth, improve plant resistance to external adverse environmental conditions as well as diseases and insect pests (Bainard et al., 2016; Beckers et al., 2017; Aghdam and Brown, 2021).

Verticillium wilt is a soil-borne disease that infects cotton vascular bundles. The main pathogen is *Verticillium dahliae* Kleb., which can infect cotton (Tjamos et al., 2000; Zhu et al., 2007). It has characteristics of a long survival time in the soil and a complex infection process, making it difficult to control (Tjamos et al., 2000; Zhu et al., 2007).

The community and quantity dynamics of endophyte in cotton have been studied. Misaghi and Donndelinger (1990) isolated six endophytic bacteria from two cotton varieties, and the dominant genus was *Erwinia*. McInroy and Kloepper (1995) isolated 32 genera of endophytic bacteria from cotton rhizomes and analyzed the dynamics of endophytic bacteria in cotton. Lu et al. (1991) found that cotton vascular bacteria are mainly *Bacillus* spp., followed by *Xanthomonas* spp., *Erwinia* spp., and *Chromobacterium* spp. Xia et al. (1996, 1997) used cotton endophytic bacteria to induce cotton's systemic resistance to *Verticillium* wilt. Li et al. (2009) found that cotton variety, growth period, and organs affect the number of endophytic bacteria in cotton, and endophytic bacteria generally form the dominant population with a high diversity level. Zhou et al. (2012) studied the synergistic control of cotton *Verticillium* wilt by antagonistic endophytes at different cotton growth stages.

The relationships between rhizosphere microorganisms and host plants are very complicated (Egamberdieva et al., 2008). Environmental factors affect the compositions of plant microbial communities (Xu et al., 2014). Rhizosphere microorganisms having biological control potential inhabit the surfaces of plant roots. They also avoid pathogen resistance after the use of chemicals (Varo et al., 2016). The occurrence of plant diseases is closely correlated with the community structure of rhizosphere microbes.

There are many discussions about plant endophytes originating from rhizosphere microorganisms (Hallmann et al., 1997; Cocking, 2003). However, the relationship between endophytes and rhizosphere microorganisms is still unclear. Current studies have shown that the microbiome of cotton changes with space and time and affects availability of soil nitrogen, thereby affecting the growth and reproduction of plant (Zhang et al., 2011; Tian and Zhang, 2017; Moronta-Barrios et al., 2018; Lu et al., 2020; Putrie et al., 2020). Therefore, the changes in microbiome may affect healthy of cotton.

In this study, we evaluate microbiome niche differentiation of microbial communities associated with the rhizosphere soil and the root endosphere of field grown cotton using pyrosequencing in cotton fields from seven typical ecological regions in Xinjiang, China. It was studied to clarify the relationships between rhizosphere microorganisms and endophytes, reveal the spatiotemporal dynamics of rhizosphere microorganisms and endophytes, and clarify the difference between rhizosphere microorganisms and endophytes on diseased and healthy cotton plants.

MATERIALS AND METHODS

Sampling Area Description

This study was mainly carried out on the northern slope and southern foot of the Tianshan Mountains, including seven typical ecological cotton regions, Shihezi, Wusu, Jinghe, Hami, Korla, Alar, and Tumshuk (**Supplementary Table 1**). Xinjiang is in an arid to semi-arid area, with little rainfall all year round, an arid climate, and abundant light and heat resources.

Collection of Rhizosphere Soil and Plant Samples

Randomly samples of good growth and uniform size were collected at the cotton seedling, bud, flowering stage, and flocculent stages. Each sample consisted of three randomly selected plants. Samples collected included roots and rhizosphere soil (0–3 mm from the root surface) (Zhang and Kong, 2014). All samples were placed in aseptic bags which placed ice straightway and transported back to our lab. The plant materials were surface-sterilized according to Shi et al. (2021b) method.

DNA Extraction, and 16S/ITS rRNA Amplification and Sequencing

For the total DNA extraction, and the PCR amplification and sequencing, of the cotton root tissue and rhizosphere soil samples, an EZ-10 Spin Column Plant Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) and MOBIO Soil DNA Extraction Kit (Qiagen) were used, respectively (Shi et al., 2020, 2021b). DNA samples of sufficient quality were sent to Shanghai Meiji Biomedical Technology Co., Ltd. for PCR amplification and high-throughput sequencing. The

bacterial 16S rRNA universal primer pairs 799F/1115 and 338f/806r were selected to amplify the 16S rRNA sequences of endophytic and rhizospheric bacteria (Shi et al., 2020, 2021b). The fungal ITS rRNA universal primer pairs ITS1F/ITS2 and ITS1F/ITS4 were selected to amplify the ITS rRNA sequences of endophytic and rhizospheric fungi (Shi et al., 2021a). High-throughput sequencing was performed using Illumina's MiSeq sequencing platform.

Data Processing and Bioinformatics Analysis

After the sequencing was completed, the Fast Length Adjustment of Short reads software for sequence splicing was used in accordance with the overlap between PE reads, and then effective sequences were obtained after Tag filtering and Tag de-chimerism. The UPARSE software was used to perform OTU clustering and species diversity analyses on the obtained effective sequences on the basis of a 97% similarity level (Tian and Zhang, 2017; Lu et al., 2020; Zhu et al., 2020). Mothur and Qiime software packages were used to calculate the biodiversity index of the microbiome of cotton based on the OTU classification level. This included the ACE, Chao, Shannon, and Simpson indices (Lopez-Garcia et al., 2018). Through hierarchical cluster and principal component analyses of cotton organ samples, the similarities and differences in microbiome community compositions among different organ samples were explored.

RESULTS

Variability in the Community Diversity of Cotton Rhizosphere and Endophytic Bacteria and Fungi

The microbiome abundance levels in the rhizosphere soil and root tissues of diseased plants are greater than those of healthy plants, but the fungal abundance in the root tissues of diseased plants was less than that of healthy plants (**Figures 1A,B**). At the OTU and species levels, there are significant differences between endophytic fungi and rhizosphere fungi in the diseased and healthy cotton plants, while the differences in bacteria are not significant.

The q-PCR results showed that there were significant differences in the numbers of endophytic and rhizosphere microbiomes from healthy and diseased cotton (**Figures 2A–D**). The number of endophytic bacteria in diseased plants was higher than in healthy plants, while the rhizosphere bacteria of healthy cotton plants are higher than diseased plants ($p > 0.05$). The number of endophytic and rhizosphere fungi from diseased plants was higher than from healthy plants ($p > 0.05$).

As shown in **Figures 3A,B**, endophytic and rhizospheric bacteria from diseased and healthy cotton do not represent the same OTUs, whereas endophytic and rhizospheric fungi are from seven identical OTUs. As shown in **Table 1**, at the OTU level, there are significant differences in the numbers of the rhizosphere and endophytic microbe OTUs from diseased and healthy cotton ($p < 0.05$; **Tables 2**, **3**).

Differences in the Composition of Cotton Rhizosphere and Endophytic Microbiome

At the phylum level, there were 14 bacterial phyla present in the two communities, 4 endobacteria and 14 rhizobacteria. Proteobacteria represent the most abundant phyla (60.6% of the total readings), followed by Acidobacteria (11.6%), Gemmatimonadetes (7.6%), Actinobacteria (6.9%), Chloroflexi (4.0%), Bacteroidetes (2.9%), Nitrospirae (1.8%), Firmicutes (1.4%), and Planctomycetes (1.0%). Proteobacteria are composed of alpha-, beta-, and gamma-proteobacteria (**Figure 4A**). There were no common OTUs between the endobacteria and rhizobacteria. The dominant rhizosphere flora were Proteobacteria (29.70%), Acidobacteria (23.14%), Gemmatimonadetes (15.17%), Actinobacteria (8.31%), Chloroflexi (7.99%), Bacteroidetes (5.15%), Nitrospirae (3.67%), Planctomycetes (2.07%), and Verrucomicrobia (1.16%). Among root endophytic bacteria, Proteobacteria (91.41%), Actinobacteria (5.49%), Firmicutes (2.10%), and Bacteroidetes (0.67%) represented the most commonly observed OTUs (**Figure 4A**).

There were six fungal phyla in two communities, six phyla of rhizosphere fungi, and four phyla of endophytic fungi. Ascomycota was the most abundant (88.66% of the total reading), followed by Mortierellomycota (4.28%), Basidiomycota (2.26%). Ascomycota is composed of Sordariomycetes, Dothideomycetes, and Leotiomycetes (**Figure 4B**). The dominant rhizosphere flora were Ascomycota (83.52%), Mortierellomycota (7.67%), Basidiomycota (2.13%). Among root endophytic fungi, Ascomycota (93.80%), Basidiomycota (2.38%), Mortierellomycota (0.90%), and represent the most commonly observed OTUs (**Figure 4B**).

Consistent with these observations, the nonparametric analyses of variance of the 16S and ITS data confirmed the dissimilarities of the rhizosphere and endophytic microbial communities (Pr = 0.001, $R^2 = 1.0000$; Pr = 0.001, $R^2 = 0.9798$).

In general, the distributions of dominant bacteria in different cotton rhizosphere soils and roots (average relative abundance > 0.1%) were different, with the bacteria *Pseudomonas* (15.54%) and *Pantoea* (9.19%), and fungi *Alternaria* (16.15%) and *Cephalotrichum* (9.10%) existing in the largest numbers (**Figures 4A,B**).

Spatiotemporal Dynamics of Bacterial and Fungal Communities in Cotton Rhizospheres and Roots

The developmental stage explained 91.98% of the rhizosphere and endophytic bacteria using the weighted UniFrac distance, but explained only 47.05% of the rhizosphere and endophytic bacteria using the Bray-Curtis distance (**Figures 5A,B**). In addition, the developmental stage explained 90.30% of the rhizosphere and endophytic fungi using the weighted UniFrac distance, but explained only 24.21% of the rhizosphere and endophytic fungi using the Bray-Curtis distance (**Figures 5C,D**). As the developmental stage changed, the total number of endophytic bacterial OTUs in cotton first increased and then decreased, and the total

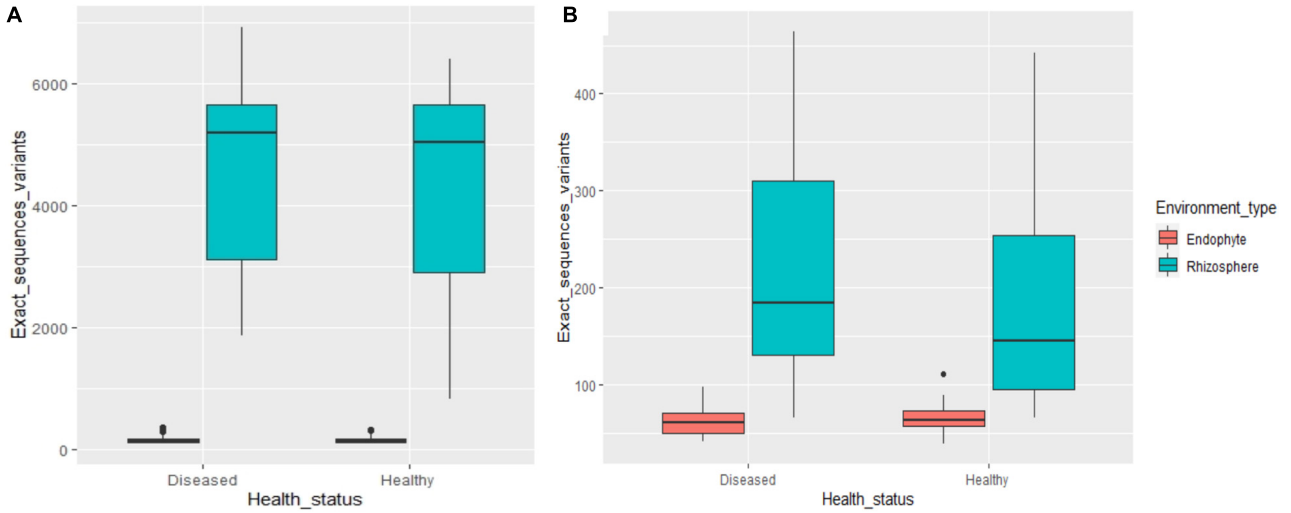


FIGURE 1 | The number of OTUs of rhizosphere and endophytic bacteria **(A)** and fungi **(B)** in healthy and diseased cotton ($P < 0.05$).

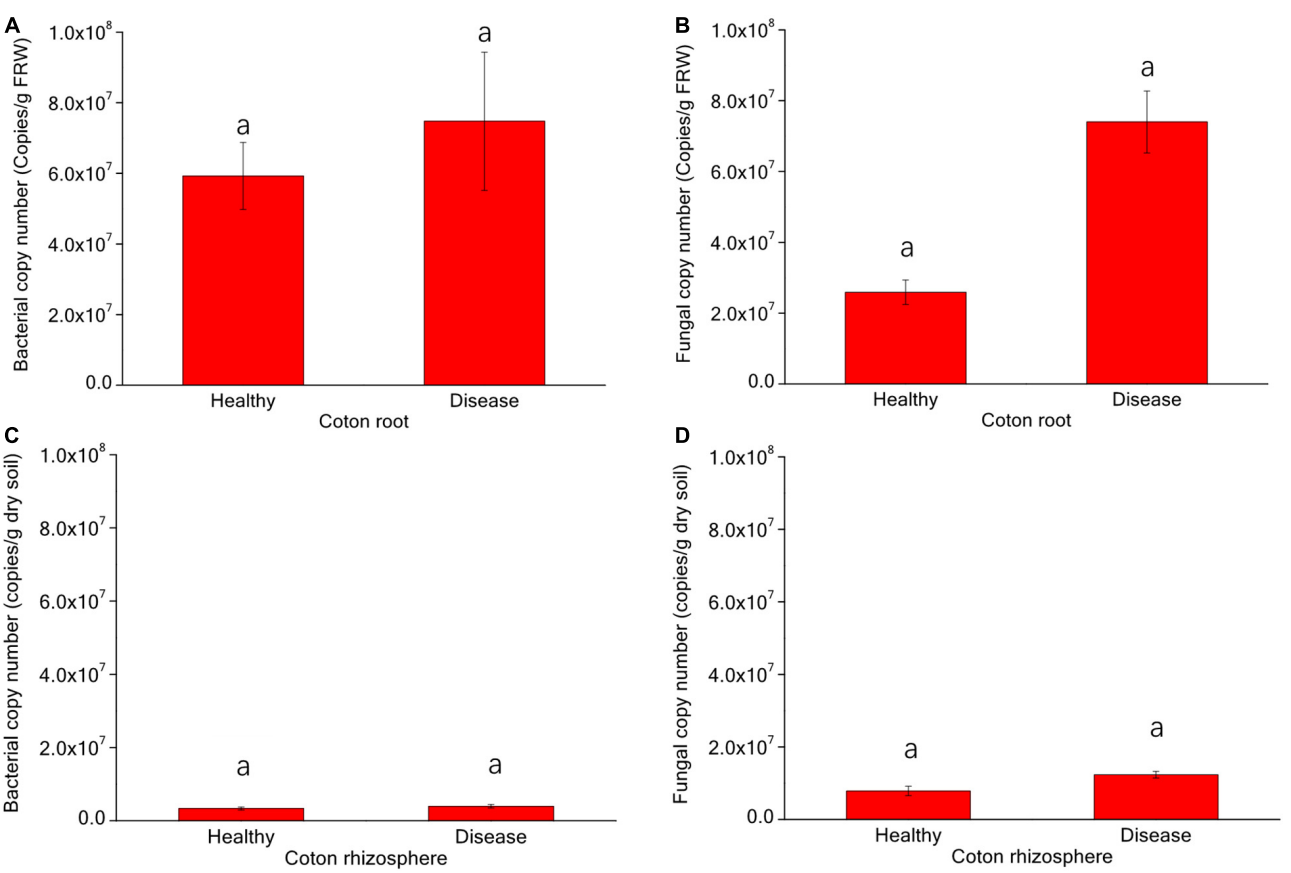


FIGURE 2 | Variation of numbers (**A** endophytic bacteria; **B** endophytic fungi; **C** rhizosphere bacteria; and **D** rhizosphere fungi) of rhizosphere and endophytic bacteria and fungi in healthy and diseased cotton ($P > 0.05$).

number of OTUs in the rhizosphere decreased. The total number of endophytic fungal OTUs decreased and then increased, and the total number of rhizospheric fungal OTUs increased (**Supplementary Figures 1A,B**). With a change in the developmental stage, the total number of endophytic microbiomes OTUs in diseased and healthy cotton plants

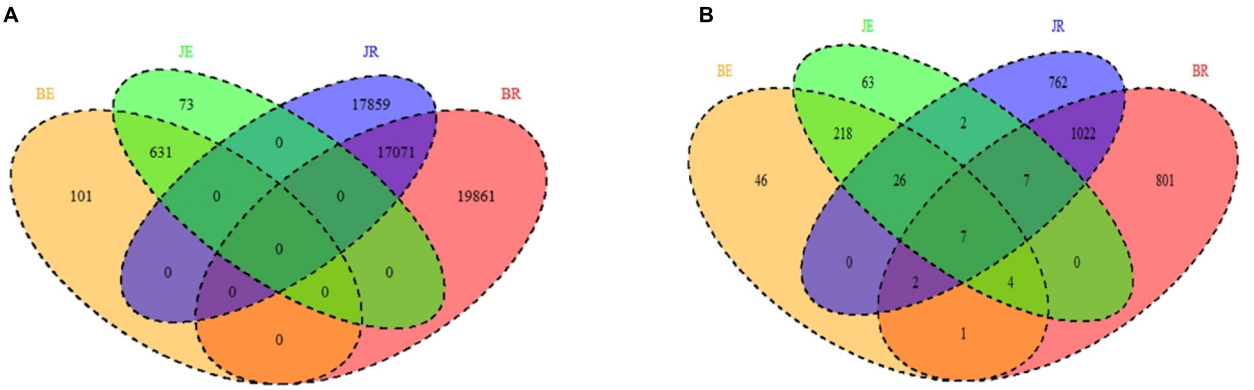


FIGURE 3 | Venn diagram showing the unique and shared OTUs (3% distance level) (**A** bacteria; **B** fungi) in the rhizosphere soil and root of cotton by different niche and pathological statuses (endophytic diseased, endophytic healthy, rhizosphere diseased, and rhizosphere healthy, respectively). BE, JE, BR, and JR represent endophytic diseased, endophytic healthy, rhizosphere diseased, and rhizosphere healthy, respectively.

TABLE 1 | Analysis of similarity (ANOSIM) of the difference at Phylum and OUT among samples associated with *Gossypium* sp.

Phylogenetic level	Phylum		OTU	
	R	*P*	*R*	*P*
Bacteria	0.35	0.001**	0.192601	0.001**
Fungi	0.24	0.001**	0.14221	0.001**

*Plant compartment effects on the bacterial community structures were calculated using ANOSIM (analysis of similarities) with the Spearman rank correlation method in Primer 7 (10,000 permutations). Plant compartments (rhizosphere soil, root, stem, and leaf) were a priori defined groups at two phylogenetic levels: phylum level and OTU level. Significance levels: *P ≤ 0.01; **P ≤ 0.001; ***P ≤ 0.0001. R, ANOSIM test statistic. Graphical results of ANOSIM are displayed in Additional file.*

decreased, while the total number of OTUs in the rhizosphere of diseased and healthy cotton plants first decreased and then increased (**Supplementary Figures 2A,B**).

During the entire cotton growth period and in representative ecological regions, there were significant differences in the numbers of rhizospheric and endophytic pathogens as assessed by qPCR ($p < 0.01$; **Supplementary Figures 3A,B**). There were significant differences in the community compositions of cotton rhizosphere bacteria and endophytic fungi at different developmental stages (**Supplementary Figures 3A,B**). An analysis revealed that the relative abundances of microorganisms on diseased and healthy plants were quite different, and they were mainly distributed in the three bacterial phyla, Proteobacteria (60.27 and 58.81%, respectively), Acidobacteria (11.89 and 11.80%, respectively), and Gemmatimonadetes (6.25 and 7.03%, respectively), and two fungal genera, Verticillium (5.43 and 2.98%, respectively) and Acremonium (1.03 and 2.44%, respectively) (**Supplementary Figures 4A,B**).

At sampling points in different ecological regions, the total numbers of rhizosphere and endophytic bacteria OTUs in cotton from southern to northern Xinjiang showed an increasing trend (**Supplementary Figures 5A,B**). The total number of rhizosphere and endophytic fungi OTU is basically the same at sampling points. At sampling points in different ecological regions, only the number of endophytic fungi in healthy plants is reduced, the rest of the number of rhizosphere and endophytic microbiomes increasing in diseased and healthy cotton plants from southern to northern Xinjiang (**Supplementary Figures 5A,B**).

Networks and Phylogeny in the Rhizosphere and Endophytic Microbiomes of Diseased and Healthy Cotton Plants

As shown in **Figure 6A**, 44 nodes representing genera RB41, H16, Nitrospira, Sphingomonas Steroidobacter, Aeromonas, Lysobacter, and Erwinia are closely related to other bacterial genera, and their degrees of connection are the highest among all the nodes. Lysobacter, which are dominant in relative abundance in the rhizosphere and internal environment, occupy important positions in the microbial ecology of cotton rhizosphere soil and have very important impacts on other bacterial groups. In addition, as shown in **Figure 6A**, Erwinia accounts for a large proportion of the microbes in the cotton rhizosphere soil, which is consistent with the bacterial community composition (**Figure 6A**). In **Figure 6A**, RB41 and norank_c__Acidobacteria belong to Acidobacteria, H16, unclassified_f__Enterobacteriaceae and Sphingomonas belong to Proteobacteria, and Nitrospira belongs to Nitrospirae, with a higher total relative abundance. RB41, H16, Nitrospira, and Sphingomonas also play important roles in the composition of the rhizosphere soil bacterial community in cotton fields.

As shown in **Figure 6B**, the two nodes representing the genera *Alternaria*, *Cephalotrichum*, *Pseudogymnoascus*, *Mortierella*, *Chaetomium*, *Pseudeurotium*, *unclassified_k__Fungi*, *Cladosporium*, and *Verticillium* are closely related to other true genera, and their degrees of connection are the highest at all the nodes. RB41, H16, Nitrospira, norank_c__Acidobacteria, unclassified_f__Enterobacteriaceae, norank_f__OM1_clade, and Sphingomonas, which are dominant in relative abundance in the rhizosphere and internal environments and have nitrogen

TABLE 2 | Adonis analysis of the difference among bacteria in samples associated with *Gossypium* sp.

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group_factor$BJRE	3	9.110279	3.03676	8.587604	0.192601	0.001
Residuals	108	38.1911	0.353621	–	0.807399	–
Total	111	47.30138	–	–	1	–

The larger the value of R2 (the ratio of group variance to total variance), the more significant the differences were among the organs and habitats. $P < 0.05$ indicates a high reliability of the test.

TABLE 3 | Adonis analysis of the difference among samples fungi in associated with *Gossypium* sp.

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
group_factor$BJRE	3	7.064223	2.354741	5.968319	0.14221	0.001
Residuals	108	42.61033	0.39454	–	0.85779	–
Total	111	49.67456	–	–	1	–

transformation abilities, occupy important positions in the microbial ecology of cotton rhizosphere soil and have a very important impacts on other bacterial groups.

An indicator species analysis was used to identify the sensitive OTUs associated with each healthy group, and their positions in the soil microbial community were shown by constructing a co-occurrence network (**Figures 6C,D**). Three sensitive OTUs correlated with cotton plant health were defined as the cornerstones of the network. The most detailed classifications were the bacteria *Pseudomonas*, *Bacillus*, and *Serratia*, as well as the fungi *Alternaria*, *Cladosporium*, *Cephalotrichum*, *Acremonium*, *Chaetomium*, *Stemphylium*, *Premium*, and *Stachybotrys*. In addition, we visualized two modules showing a high interactivity in the network, with the largest module being regarded as the central module.

As shown in **Figure 7**, rhizosphere and endophytic bacteria are phylogenetically significantly different, whereas rhizospheric and endophytic fungi are phylogenetically similar. Among the bacteria, *Proteobacteria* and *Actinobacteria* are the dominant genera, among endophytic fungi, *Alternaria* is the dominant genus, and among rhizospheric fungi, *Pseudogymnoascus*, *Mortierella*, *Pseudeurotium*, and *Cephalotrichum* are the dominant genera.

DISCUSSION

The Microbiome Differentiation Between the Cotton Rhizosphere and the Endogenous Environment

In this study, cotton rhizosphere and endophytic microbiomes were significantly different at the OTU level, unsimilar to previous reports (Beckers et al., 2017; Qiao et al., 2017; Tian and Zhang, 2017; Huang, 2018). Endophytes are almost ubiquitous in different plants. Their original sources are thought to be the epiphytic flora of plant rhizospheres or root surfaces, leaf surfaces, internal seed tissues, and tissue-cultured seedlings. Some studies believe that rhizosphere soil is the primary colonization source of endophytes. Endophytes exist in the roots, stems, branches, and leaves of plants, and they also colonize the xylem and between cells. There are limited reports of endophytes colonizing cells (Dong et al., 2003).

Cheng (2018) found that the tomato microbiome is mainly composed of three groups: Proteobacteria, Actinobacteria, and Firmicutes. Among them, Proteobacteria is the main dominant member of the tomato root microbiome, accounting for 34.15 and 39.18% of the total number of tomato rhizosphere and root endophytic bacteria, respectively. The structures and compositions of the endophytic and rhizosphere bacteria of tomato root are different, and this is mainly manifested by the high enrichment of *Pseudomonas* and *Bacillus* in rhizosphere endophytes. Some common plant probiotics, such as *Bacillus* and *Pseudomonas*, account for small proportions of rhizosphere bacteria, whereas *Pseudomonas* is the main dominant group of endophytes. This was similar to our findings.

The microbial communities in the two microenvironments, cotton root tissue and rhizosphere soil, are significantly different, indicating that the functions of the two microenvironments differ. Most of the endophytic fungi in healthy and diseased cotton plants belong to imperfect fungi and Ascomycota. The number of *Verticillium* fungi increased in the middle and late stages of cotton growth, indicating that *V. dahliae* infection affects these stages.

Soil Is the Decisive Factor for the Cotton Rhizosphere Microbial Community

Various factors, such as soil pH, water content, electrical conductivity, and nutrient contents, including alkali-hydrolyzable nitrogen and available phosphorus, are related to changes in soil bacterial community structures (Preem et al., 2012; Yang et al., 2018). Liu et al. (2021) showed that the physical and chemical properties of soil have significant impacts on bacterial community diversity levels and population structures, whereas field diseases have no significant impacts on soil bacterial community diversity levels and population structures.

Here, the physical and chemical properties of the soil were responsible for the significant differences in the bacterial community structures in the rhizosphere soil between healthy

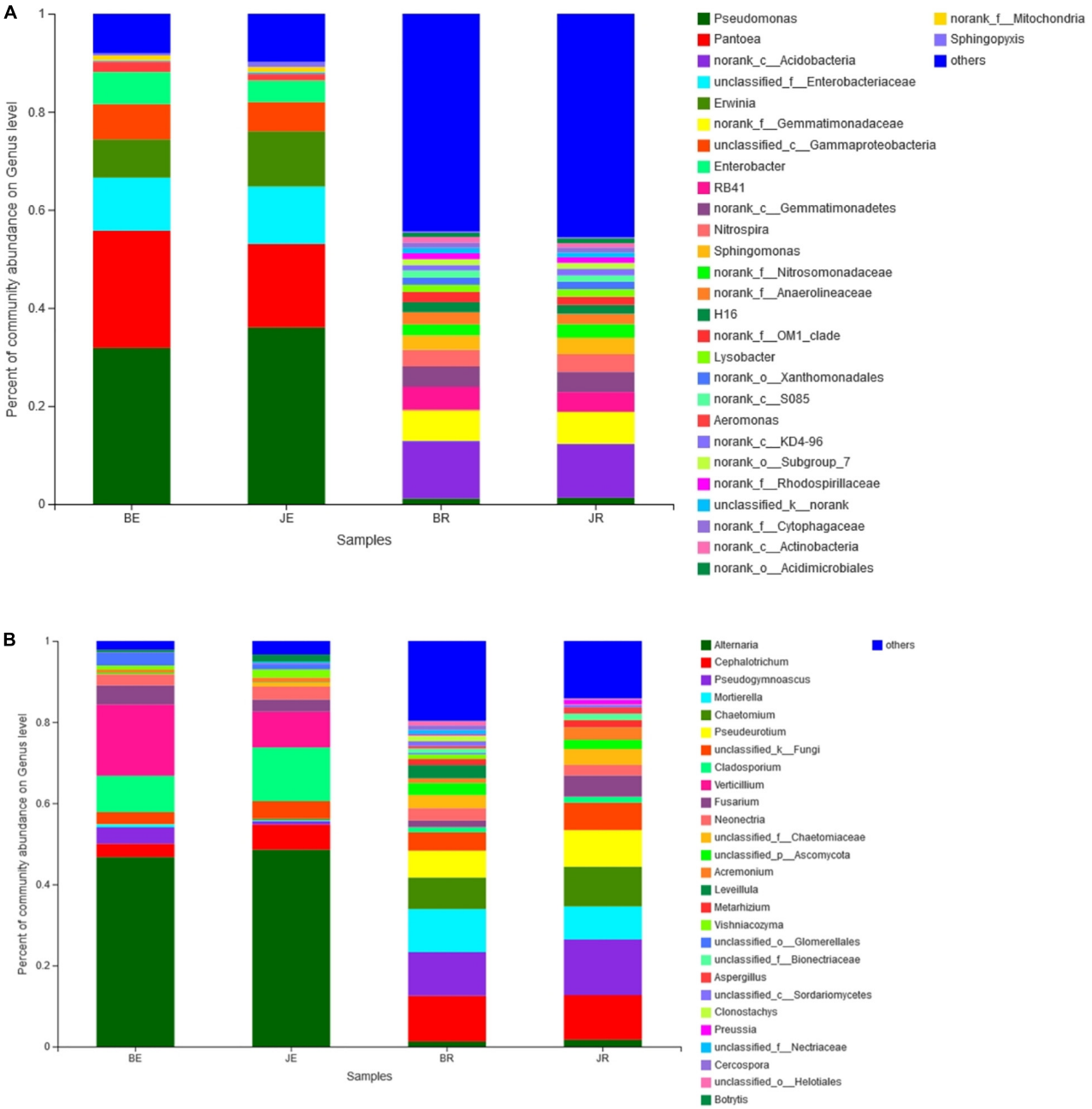


FIGURE 4 | Bacteria **(A)** and Fungal **(B)** composition of the different communities at the genera level. Relative read abundance of different bacterial phyla within the different communities. Phyla, classes, and genera making up less than 1% of total composition in all three libraries were classified as "others".

and diseased cotton plants. Soil pH and total salt content were the main factors affecting the bacterial community diversity and population structure. The pH value was significantly positively correlated with bacterial community diversity, whereas total salt was significantly negatively correlated with bacterial community diversity. These conclusions are consistent with those of previous studies, and total nitrogen, total phosphorus, and organic matter were not significantly correlated with bacterial community diversity. Marschner et al. (2001) also found that nitrogen fertilizer has no significant effects on soil bacterial community structures. Thus, the conclusions of this study are consistent with those of previous studies.

Based on previous studies, soil physical and chemical properties, soil types, and soil moisture are the main factors affecting soil bacterial communities (Kuramae et al., 2012; Qiao et al., 2017). There are some controversies about the influence of plant species, crop rotation, and other factors on the structures of soil microbial communities. The reduction in soil-borne diseases

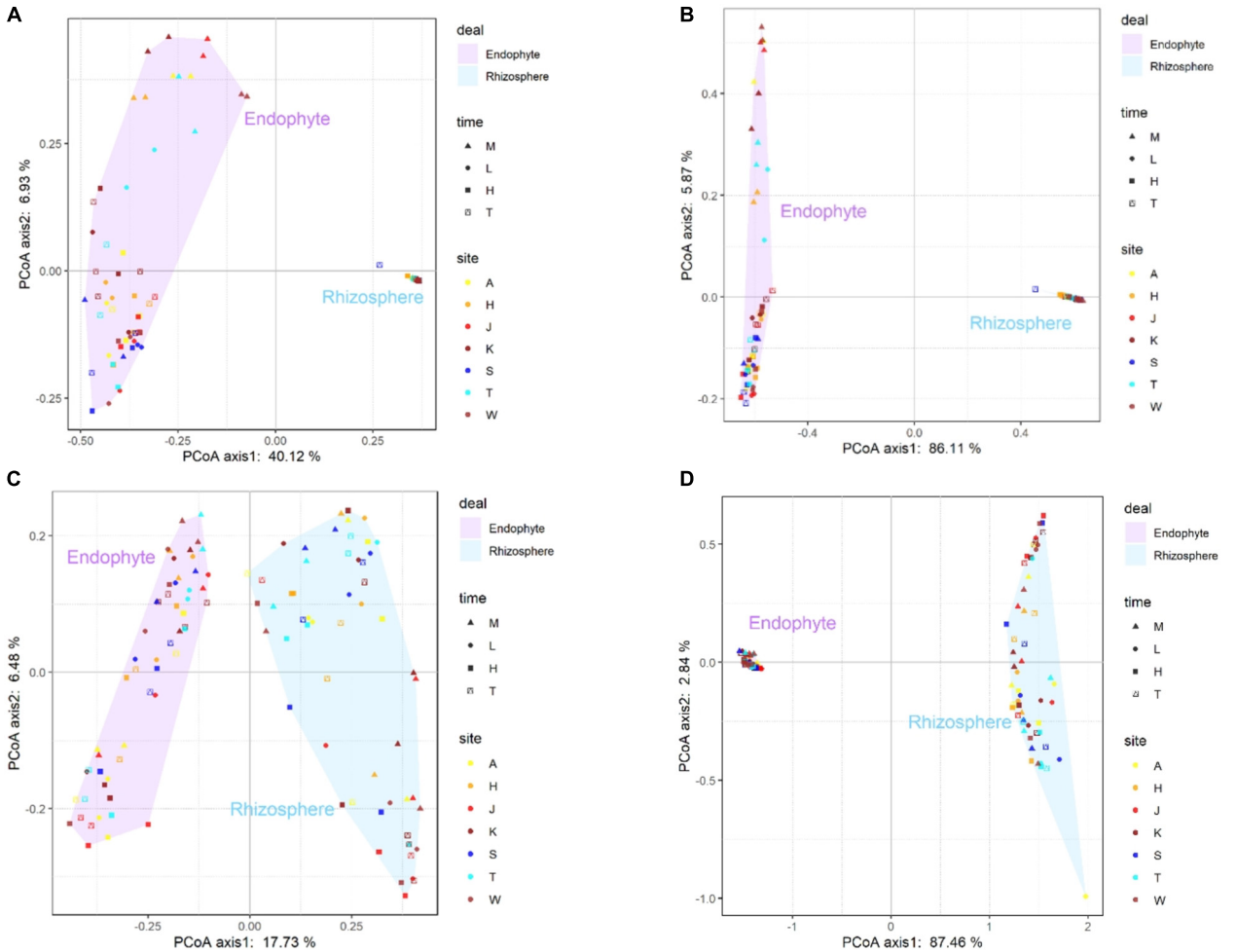


FIGURE 5 | The principal-coordinate analysis (PCoA) plots were based on Bray-Curtis **(A)** and weighted-unfrac **(B)** dissimilarity of bacterial **(A,B)** and fungal **(C,D)** communities in sampling site (A, K, T, H, S, W, and J represent the Alaer, Kuerle, Tumushuke, Hami, Shihezi, Wusu, and Jinghe, respectively) and growing season (M, L, H, and T represent seedling, bud, flowering, and boll opening stages, respectively) Differentiation in the rhizosphere and endosphere microbiomes was significantly (PERMANOVA $p < 0.05$).

is correlated with the decrease in pathogenic bacteria in the soil, the decrease in the pathogenicity of pathogenic bacteria or the increase in the beneficial microorganism's population (Bailey and Lazarovits, 2003; Bonanomi et al., 2010).

At the genus level, the abundances of the beneficial bacteria *Lysobacter* and *Pseudomonas* in the soil of severely diseased fields in Aksu were 3.67 and 3.15%, respectively, which were significantly greater than those in the non-diseased fields (2.35 and 0.18%, respectively). However, the abundances of *Bacillus* and *Streptomyces* were not significantly different between the severely diseased fields in Aksu, Shihezi, and Korla and the control fields (Liu et al., 2021). Differential marker analyses also found that *Pseudomonadales* were significantly enriched in the seriously diseased fields in Aksu, whereas *Actinobacteria* were significantly enriched in the severely diseased fields in Shihezi. A correlation analysis showed that the number of OTUs and the alpha-diversity index of cotton soil bacteria are positively correlated with soil pH and total potassium content, and negatively correlated with total salt content, carbon–nitrogen ratio, and organic matter, total nitrogen, and total phosphorus contents, as well as cotton field diseases (Liu et al., 2021). There is no obvious correlation between the degrees, and the soil pH value and total salt content are the main factors affecting the diversity of soil bacteria (Liu et al., 2021).

Microbial Communities in the Cotton Rhizosphere and Endogenous Environment During Different Growth Periods

Most of the endophytic bacteria in healthy and diseased cotton plants belonged to the phylum Proteobacteria, and the diversity of the community structure was closely correlated with the growth and development of cotton (Zhang et al., 2011). Healthy cotton plants were more abundant than diseased cotton plants, indicating that the invasion of *V. dahliae* affected the endophytes.

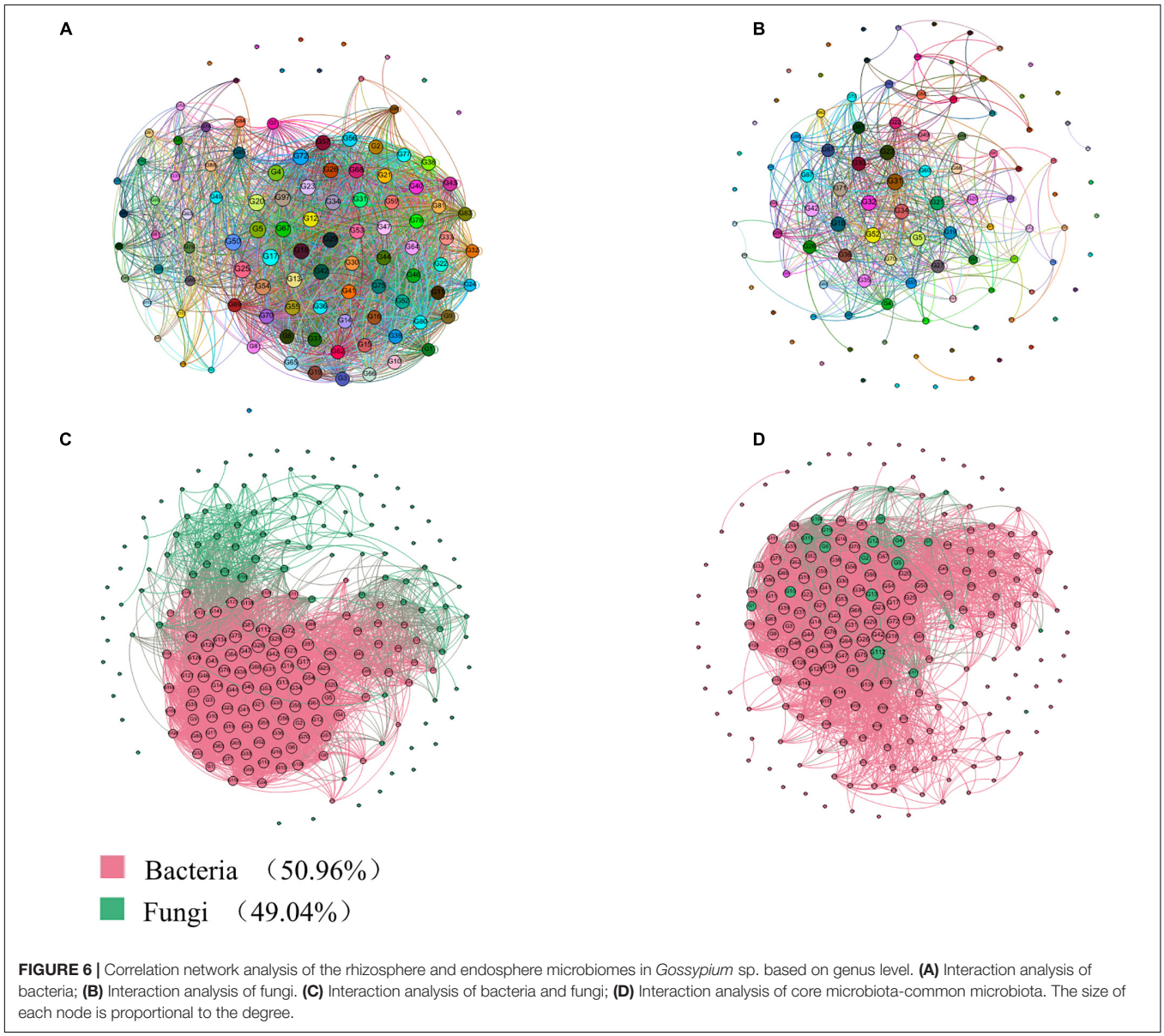


FIGURE 6 | Correlation network analysis of the rhizosphere and endosphere microbiomes in *Gossypium* sp. based on genus level. **(A)** Interaction analysis of bacteria; **(B)** Interaction analysis of fungi. **(C)** Interaction analysis of bacteria and fungi; **(D)** Interaction analysis of core microbiota-common microbiota. The size of each node is proportional to the degree.

The number of endophytic bacteria in healthy cotton plants during the same period was greater than in diseased cotton plants (Zhang et al., 2011).

The results of the study showed that most of the endophytic fungi in healthy cotton plants and diseased cotton plants belonged to imperfect fungi and Ascomycota. In the middle and late stages of cotton growth, the number of Verticillium fungi increased, indicating that the infection of *Verticillium dahliae* can affect the diversity of endophytic fungal community structure in the middle and late stages of diseased cotton plants. Research by Liu et al. (2016) showed that *V. dahliae* infections reduce the diversity of endophytic bacteria in cotton plants and increase the diversity of the endophytic fungal community.

In addition, the abundance of rhizosphere microorganisms also plays an important role in maintaining plant health. This study found that the 16S rRNA gene copy number associated with the healthy plants was significantly higher than that of the diseased plants, and the 16S rRNA gene copy number of the rhizosphere bacteria was positively correlated with the health of the plants, which is consistent with previous studies.

The copy number of the 16S rRNA gene of rhizosphere bacteria on healthy cotton is significantly higher than that on the diseased plants. *Verticillium* wilt is always accompanied by a substantial increase in the copy number of the rhizosphere fungal ITS region. The severity of the disease is correlated with the fungal ITS region, and the ratio of ITS copy number to bacterial 16S rRNA gene copy number is significantly positively correlated with disease occurrence ($p < 0.001$). The bacterial community in the rhizosphere soil has an inhibitory effect on the occurrence of cotton *Verticillium* wilt, whereas the fungal community of the rhizosphere soil was conducive to the occurrence of *Verticillium* wilt.

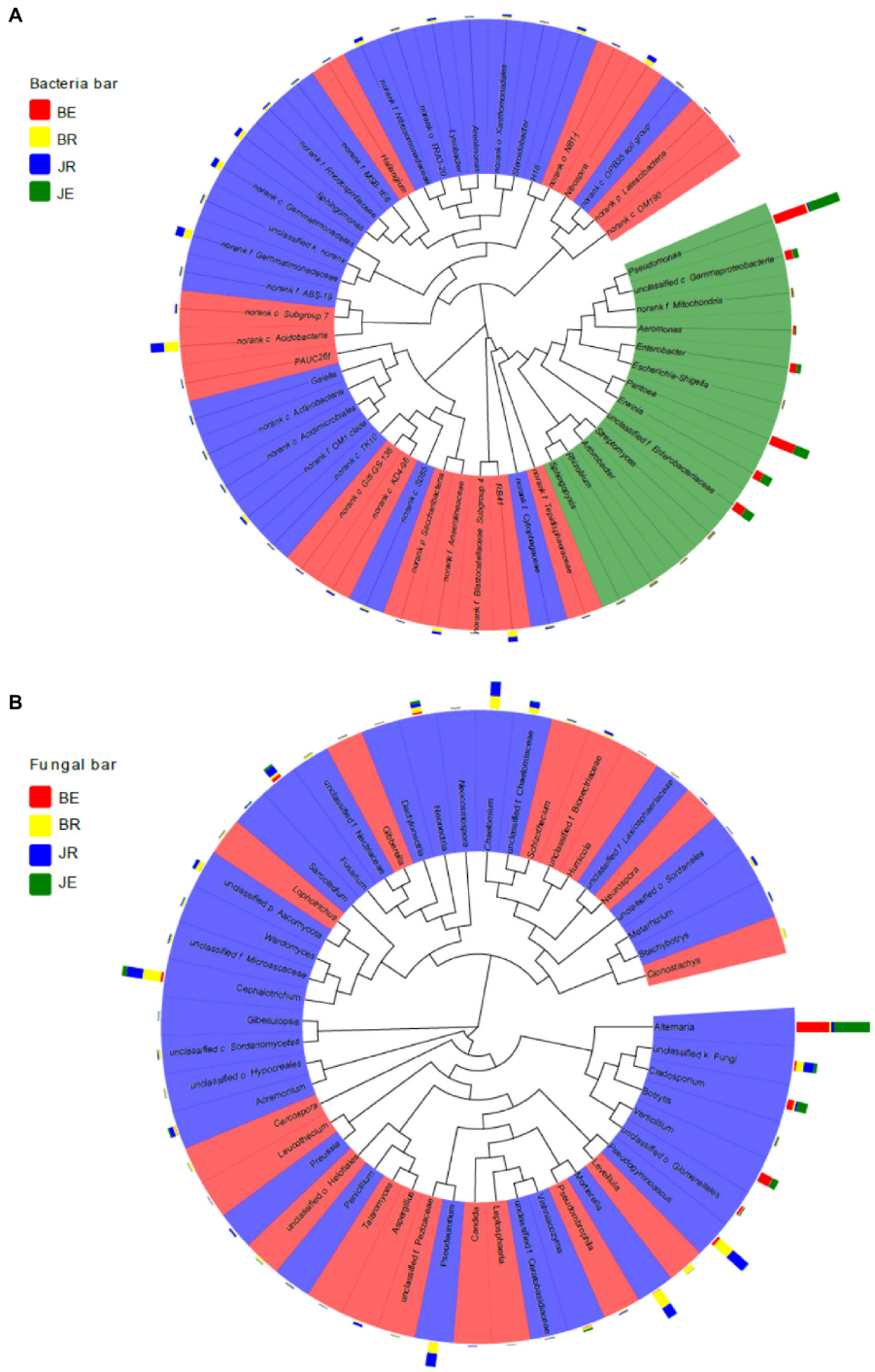


FIGURE 7 | A Phylogenetic tree of the top 50 bacterial **(A)** and fungal **(B)** genera and their distributions in the rhizosphere soils and root of cotton with different pathological statuses. The background color of the taxonomic designation represent its classification at the genus level. The bar plot exhibits the relative abundance of the genus in the BE, JE, BR, and JR samples. BE, JE, BR, and JR, respectively, represent endophytic diseased, endophytic healthy, rhizosphere diseased, rhizosphere healthy, respectively.

This study overcame the limitations of traditional culture methods and provides a fast, reliable and reproducible method for analyzing changes in rhizosphere microbial abundance. Using this technology, through the absolute quantification of the rhizosphere bacteria and fungi between adjacent plants and the detection of the pathogenic microsclerotia, it is clear that the severity of *Verticillium* wilt in cotton increases significantly with the increase in the number of microsclerotia in the soil ($p < 0.05$).

Distributions of Microorganisms in Plant Microenvironments

The endophyte microbiome is obviously different from the rhizosphere microbiome, which is the result of the plant niche effect. However, endophytes are closely related to rhizosphere microorganisms.

The cumulative relative abundance of OTUs belonging to modules in soil was significantly ($p < 0.01$) higher than that in roots. In addition, different modules contained different bacterial and fungal phyla (**Figure 2C**), indicating that different ecological groups have specific microbial lineages.

Drivers of Microbiome Niche Differentiation

The driving factors behind the differences in microbial niches are the differences in root exudates and internal plant environments (Beckers et al., 2017). A 16S amplicon analysis of the bacterial community structures in different niches, including rhizosphere soil, roots, stems, and leaves, was performed. Compared with that of endophytes, the structure of the rhizosphere microbiome is more stable. Not only do the differences between the rhizosphere and roots need to be further analyzed, but also the endophytes in the stems and leaves. Each plant also has an independent ecological niche, and the core bacteria in different niches of poplars have been identified (Beckers et al., 2017). Understanding the complex host–microbe interactions of cotton provides a theoretical basis for explaining prokaryotic- and eukaryotic-related phytoremediation applications, sustainable agriculture, and secondary metabolite production.

CONCLUSION

The dominant rhizospheric microflora were Proteobacteria, Acidobacteria, Gemmatimonadetes, Actinobacteria, Chloroflexi, Bacteroidetes, and Ascomycota, Mortierellomycota, Basidiomycota. The abundance of endophytic and rhizosphere bacteria and fungi in diseased cotton plants is higher than that in healthy plants. Endophytic and rhizospheric bacteria did not share common OTUs. The distribution of dominant bacteria in different cotton rhizosphere soils and roots differed, with the dominant bacteria Pseudomonas and Pantoea, and the dominant fungi Alternaria and Cephalotrichum being present in the greatest numbers. At sampling points in different ecological regions, the total numbers of cotton endophytic and rhizosphere microbiome OTUs from southern to northern Xinjiang showed an increasing trend. There were significant differences in the composition and diversity of rhizosphere microbes and endophytes during the entire cotton growth period and in representative ecological regions, whereas rhizosphere microbes and endophytes showed no significant differences among the four growth periods and in representative ecological regions. Pseudomonas accounted for a large proportion of the microbes in the cotton rhizosphere soil. RB41, H16, Nitrospira, Sphingomonas also play important roles in the rhizosphere soil.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA762464.

AUTHOR CONTRIBUTIONS

YS designed the work and wrote the manuscript. HY and MC collected the data. XN, NW, QL, KL, CZ, JW, QZ, and YZ edited the manuscript. All authors approved the final version of this manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (Project Nos. 41471220 and 31860024), the Nation Key Research Program of China (No. 2017YFD0200608-05), the National Natural Science Foundation of Xinjiang Uygur Autonomous Region (Project No. 2018D01A44), Project of Renovation Capacity Building for the Young Sci-Tech Talents Sponsored by Xinjiang Academy of Agricultural Sciences (xjnkq-2019014), China Postdoctoral Science Foundation Funded the Sixtieth Batch (No. 2016M602953XB), and the Xinjiang Uygur Autonomous Region Outstanding Young Talent Training Program (No. qn2005yx023).

ACKNOWLEDGMENTS

We sincerely thank all the students and staff in the Institute of Microbiology, Xinjiang Academy of Agricultural Sciences for their assistance in field work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.765269/full#supplementary-material

REFERENCES

Aghdam, S. A., and Brown, A. M. V. (2021). Deep learning approaches for natural product discovery from plant endophytic microbiomes. *Environ. Microbiome* 16, 1–20. doi: 10.1186/s40793-021-00375-0

Bailey, K. L., and Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. *Soil Tillage Res.* 72, 169–180. doi: 10.1016/S0167-1987(03)00086-2

Bainard, L. D., Hamel, C., and Gan, Y. T. (2016). Edaphic properties override the influence of crops on the composition of the soil bacterial community in a semiarid agroecosystem. *Appl. Soil Ecol.* 105, 160–168. doi: 10.1016/j.apsoil.2016.03.013

Beckers, B., De Beeck, M. O., Weyens, N., Boerjan, W., and Vangronsveld, J. (2017). Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown poplar trees. *Microbiome* 5, 1–17. doi: 10.1186/s40168-017-0241-2

Bonanomi, G., Antignani, V., Capodilupo, M., and Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. *Soil Biol. Biochem.* 42, 136–144. doi: 10.1016/j.soilbio.2009.10.012

Cheng, Z. Q. (2018). *Community Structure, Diversity, Succession And Function Of The Tomato Rhizobacteria And Endophytes*. Fuzhou: Fujian Normal University.

Cocking, E. C. (2003). Endophytic colonization of plant roots by nitrogenfixing bacteria. *Plant Soil* 252, 169–175.15. doi: 10.1023/A:1024106605806

Dong, Y., Iniguez, A. L., and Triplett, E. W. (2003). Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342. *Plant Soil* 257, 49–59. doi: 10.1023/A:1026242814060

Egamberdieva, D., Kamilova, F., Validov, S., Gafurova, L., Kucharova, Z., and Lugtenberg, B. (2008). High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. *Environ. Microbiol.* 10, 1–9. doi: 10.1111/j.1462-2920.2007.01424.x

Hallmann, J., QuadtHallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43, 895–914. doi: 10.1139/m97-131

Huang, Y. H. (2018). Comparison of rhizosphere and endophytic microbial communities of Chinese leek through high-throughput 16S rRNA gene Illumina sequencing. *J. Integr. Agric.* 17, 359–367. doi: 10.1016/S2095-3119(17)61731-3

Kuramae, E. E., Yergeau, E., Wong, L. C., Pijl, A. S., van Veen, J. A., and Kowalchuk, G. A. (2012). Soil characteristics more strongly influence soil bacterial communities than land-use type. *FEMS Microbiol. Ecol.* 79, 12–24. doi: 10.1111/j.1574-6941.2011.01192.x

Li, C. H., Deng, Y. Y., Zhao, M. W., Tang, C. M., Li, S. P., and Lv, H. W. (2009). Population dynamics and antagonism toward *Fusarium oxysporium* f. sp. Vasinfectum. and *Verticillium dahliae* Kleb of endophytic bacteria from cotton. *Acta Microbiol. Sin.* 49, 1196–1202.

Liu, H. Y., Zhang, R. F., Wang, W., Yang, H. L., and Yao, J. (2021). Characteristics of soil bacterial community structure in cotton fields with different incidence of *Verticillium* wilt in Xinjiang. *Ecol. Environ. Sci.* 30, 72–80.

Liu, Z., Li, Y., Sun, Y., Zhang, X. K., Chen, B., and Wang, Q. (2016). Analysing endophytic fungi communities in cotton roots infected with *Verticillium*. *Acat Agric. Boreali Occidentalis Sin.* 25, 42–47.

Lopez-Garcia, A., Pineda-Quiroga, C., Atxaerandio, R., Perez, A., Hernandez, I., García-Rodríguez, A., et al. (2018). Comparison of Mothur and QIIME for the analysis of rumen microbiota composition based on 16S rRNA amplicon sequences. *Front. Microbiol.* 9:3010. doi: 10.3389/fmicb.2018.03010

Lu, S. Y., Chen, B., and Cai, Z. X. (1991). Microflora in cotton vascular system and its function of disease prevention . *Chinese J. Microecol.* 3:52.

Lu, Y., Zhang, E., Hong, M., Yin, X., Cai, H., Yuan, L., et al. (2020). Analysis of endophytic and rhizosphere bacterial diversity and function in the endangered plant *Paeonia ludlowii*. *Arch. Microbiol.* 202, 1717–1728. doi: 10.1007/s00203-020-01882-3

Marschner, P., Yang, C. H., Lieberei, R., and Crowley, D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol. Biochem.* 33, 1437–1445. doi: 10.1016/S0038-0717(01)00052-9

McInroy, J. A., and Kloepper, J. W. (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Can. J. Microbiol.* 41, 895–901. doi: 10.1139/m95-123

Misaghi, I. J., and Donndelinger, C. R. (1990). Endophytic bacteria in symptom-free cotton plants. *Phytopathology* 80, 808–811. doi: 10.1094/Phyto-80-808

Moronta-Barrios, F., Gionechetti, F., Pallavicini, A., Marys, E., and Venturi, V. (2018). Bacterial microbiota of rice roots: 16S-based taxonomic profiling of endophytic and rhizospheric diversity, endophytes isolation and simplified endophytic community. *Microorganisms* 6:14. doi: 10.3390/microorganisms6010014

Preem, J. K., Truu, J., Truu, M., Mander, U., Oopkaup, K., Lohmus, K., et al. (2012). Bacterial community structure and its relationship to soil physicochemical characteristics in alder stands with different management histories. *Ecol. Eng.* 49, 10–17. doi: 10.1016/j.ecoleng.2012.08.034

Putrie, R. F. W., Aryantha, I. N. P., Iriawati, I., and Antonius, S. (2020). Diversity of endophytic and rhizosphere bacteria from pineapple (*Ananas comosus*) plant in semi-arid ecosystem. *Biodivers. J. Biol. Divers.* 21, 3084–3093. doi: 10.13057/biodiv/d210728

Qiao, Q., Wang, F., Zhang, J., Chen, Y., Zhang, C., Liu, G., et al. (2017). The variation in the rhizosphere microbiome of cotton with soil type, genotype and developmental stage. *Sci. Rep.* 7, 1–10. doi: 10.1038/s41598-017-04213-7

Shi, Y., Yang, H., Chu, M., Niu, X., Huo, X., Gao, Y., et al. (2021b). Endophytic bacterial communities and spatiotemporal variations in cotton roots in Xinjiang, China. *Can. J. Microbiol.* 67, 506–517. doi: 10.1139/cjm-2020-0249

Shi, Y., Yang, H., Chu, M., Niu, X., Huo, X., Gao, Y., et al. (2021a). Diversity and spatiotemporal dynamics of fungal communities in the rhizosphere soil of cotton in the arid region of Northwest China. *Microb. Ecol.* 82, 87–99. doi: 10.1007/s00248-020-01646-y

Shi, Y., Yang, H., Chu, M., Niu, X., Huo, X., Gao, Y., et al. (2020). Diversity and space–time dynamics of the bacterial communities in cotton (*Gossypium hirsutum*) rhizosphere soil. *Can. J. Microbiol.* 66, 228–242. doi: 10.1139/cjm-2019-0196

Tian, X. Y., and Zhang, C. S. (2017). Illumina-based analysis of endophytic and rhizosphere bacterial diversity of the coastal halophyte *Messerschmidia sibirica*. *Front. Microbiol.* 8:2288. doi: 10.3389/fmicb.2017.02288

Tjamos, E. C., Rowe, R. C., Heale, J. B., and Fravel, D. R. (2000). *Advances In Verticillium Research And Disease Management*. St. Paul, MN: APS Press, 357.

Varo, A., Raya-Ortega, M. C., and Trapero, A. (2016). Selection and evaluation of micro-organisms for biocontrol of *Verticillium dahliae* in olive. *J. Appl. Microbiol.* 121, 767–777. doi: 10.1111/jam.13199

Xia, Z. J., Gu, B. K., and Wu, A. M. (1996). Studies on induced resistance of cotton plants against verticillium dahliae by endophytic and rhizosphere bacteria. *Chin. J. Biol. Control* 12, 7–10.

Xia, Z. J., Gu, B. K., Wu, A. M., and Fu, Z. Q. (1997). Isoenzymatic activities involved in induced resistance of cotton to Verticillium dahliae by Endophytic Bacteria. *Jiangsu J. Agric. Sci.* 13, 36–38.

Xu, W. J., Jin, X. D., and Yang, Q. S. (2014). Research progress on factors influencing plant rhizosphere microorganism. *J. Henan Agric. Sci.* 43, 6–12.

Yang, T., Ala, M., Zhang, Y., Wu, J., and Wang, A. (2018). Characteristics of soil moisture under different vegetation coverage in Horqin Sandy Land, northern China. *PLoS One* 13:e019880. doi: 10.1371/journal.pone.0198805

Zhang, C., and Kong, F. (2014). Isolation and identification of potassiumsolubilizing bacteria from tobacco rhizospheric soil and their effect on tobacco plants. *Appl. Soil Ecol.* 82, 18–25. doi: 10.1016/j.apsoil.2014.05.002

Zhang, Y., Du, B. H., Jin, Z. G., Li, Z. H., Song, H. N., and Ding, Y. Q. (2011). Analysis of bacterial communities in rhizosphere soil of healthy and diseased cotton (*Gossypium* sp.) at different plant growth stages. *Plant Soil* 339, 447–455. doi: 10.1007/s11104-010-0600-2

Zhou, Y., Yang, T., Yang, P., Wang, B., and Sun, Z. (2012). Antagonistic endophytic bacteria isolated from different growth stages of cotton for the control of cotton *Verticillium* wilt. *Agric. Sci. Technol. Hunan* 13, 778–783.

Zhu, B., Wu, J., Ji, Q., Wu, W., Dong, S., Yu, J., et al. (2020). Diversity of rhizosphere and endophytic fungi in *Atractylodes macrocephala* during continuous cropping. *PeerJ* 8:e8905. doi: 10.7717/peerj.8905

Zhu, L., Zhang, X., Tu, L., Zeng, F., Nie, Y., and Guo, X. (2007). Isolation and characterization of two novel dirigent-like genes highly induced in cotton (*Gossypium barbadense* and *G. hirsutum*) after infection by *Verticillium dahliae*. *Plant Pathol.* 89, 41–45.

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 December 2021
doi: 10.3389/fmicb.2021.790223

Identification, Characterization, and Evaluation of Nematophagous Fungal Species of *Arthrobotrys* and *Tolypocladium* for the Management of *Meloidogyne incognita*

*Rami Kassam[1], Jyoti Yadav[1], Gautam Chawla[1], Aditi Kundu[2], Alkesh Hada[1], Nisha Jaiswal[1], Haritha Bollinedi[3], Deeba Kamil[4], Prameela Devi[4] and Uma Rao[1]**

[1] Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India, [2] Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi, India, [3] Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India, [4] Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Laith Khalil Tawfeeq Al-Ani, Universiti Sains Malaysia, Malaysia
Kgabo Martha Pofu, University of Limpopo, South Africa

***Correspondence:**
Uma Rao
umarao@iari.res.in
orcid.org/0000-0002-1233-2921

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *06 October 2021*
Accepted: *10 November 2021*
Published: *10 December 2021*

Citation:
Kassam R, Yadav J, Chawla G, Kundu A, Hada A, Jaiswal N, Bollinedi H, Kamil D, Devi P and Rao U (2021) Identification, Characterization, and Evaluation of Nematophagous Fungal Species of Arthrobotrys and Tolypocladium for the Management of Meloidogyne incognita. Front. Microbiol. 12:790223. doi: 10.3389/fmicb.2021.790223

Root-knot nematodes belonging to the genus *Meloidogyne* are agriculturally important pests, and biocontrol strategies offer safer alternatives for their management. In the present study, two fungal species from Indian soils were identified as *Arthrobotrys thaumasia* and *Tolypocladium cylindrosporum* based on morphological characteristics and further confirmed using molecular markers. *In vitro* evaluation of *A. thaumasia* against *M. incognita* and *Caenorhabditis elegans* showed 82 and 73% parasitism, respectively, whereas *T. cylindrosporum* gave 65.2 and 57.7% parasitism, respectively. Similarly, culture filtrates of *A. thaumasia* caused 57.7 and 53.7% mortality of *M. incognita* and *C. elegans,* respectively, whereas *T. cylindrosporum* caused higher mortality of 87.3 and 64%, respectively. Besides, greenhouse evaluation of both fungi against *M. incognita* infecting tomato significantly reduced nematode disease burden reflecting parasitic success measured as the total number of galls, egg masses, eggs per egg mass, and derived nematode multiplication factor. Application of *A. thaumasia* and *T. cylindrosporum* reduced nematode multiplication factor by 80 and 95%, respectively, compared with control. General metabolite profiling of tested fungi using gas chromatography–mass spectrometry and ultra-performance liquid chromatography–quadrupole/time of flight mass spectrometry reported for the first time here showed presence of various volatile and non-volatile compounds with nematicidal activity, *viz.*, trimethyl-heptadiene, methyl-hexadecanol, dodecadienal, decane, terpendole E, dodecane, acetamido-6-anthraquinone, and hexadecanol. Also, other compounds such as undecane, dibutyl-disulfide, octadecenal, paganin, talathermophilin, dactylarin, tolypyridone A, tolypyridone B, pyridoxatin, and destruxin were identified, reported in the literature to possess antibacterial, antifungal, and insecticidal properties. This is the first report of the occurrence of both fungi from India and pioneer demonstration of *T. cylindrosporum* for root-knot nematode management.

Keywords: ***Arthrobotrys thaumasia*, *Tolypocladium cylindrosporum*, nematophagous fungi, *Meloidogyne incognita*, biocontrol, parasitism, metabolite profiling**

INTRODUCTION

Plant-parasitic nematodes (PPNs) are considered hidden enemies and pose a major threat to both agricultural and horticultural crops. They have a universal distribution and cause an estimated yield loss of US$ 173 billion every year (Elling, 2013). Amongst the top 10 economically important PPN species worldwide, root-knot nematodes (RKNs) belonging to the genus *Meloidogyne* are considered to be the most severe ones (Rady, 2018). The pre-parasitic J2s of the most important RKN species, *Meloidogyne incognita*, penetrate and infect the root tip using a hollow needle-like protrusible stylet. The stylet is used for probing the plant tissue and entering into the vascular cylinder, where it injects the esophageal gland secretions that induce the formation of specialized feeding cells known as giant cells by suppressing the plant host immune system. Eventually, the feeding J2s undergo consecutive molts to J3 (third stage juveniles) and J4 (fourth stage juveniles) stages and young females that develop into reproducing mature females that lay eggs (Poveda et al., 2020). Despite enormous damage caused by PPNs in various crops, there is still a dearth of effective and efficacious nematode management option(s).

Traditionally, management of PPNs relied on integrated cultural and physical tactics such as clean cultivation practices, crop rotation, solarization of the soil before planting, adequate fertilization, and removal of infected plants and weeds (Oka et al., 2007). Additionally, one of the most effective, economical, and environmentally safe methods to reduce yield losses from nematode diseases is to use nematode-resistant cultivars. However, commercially viable resistant varieties and/or cultivars may not be either available for all the crops or limited in numbers for only specific crops due to lack of resistant sources required for varietal development (Ploeg et al., 2019). Chemical nematicides are the mainstay due to their ability to reduce high densities of nematodes in the soil in a short period to avoid significant yield losses (Regmi and Desaeger, 2020). However, due to their high toxicity and possible environmental and health hazards, most of the chemical nematicides, fumigants, and insecticides have been withdrawn or banned from the global market (Ruiz-Suárez et al., 2015; Banakar et al., 2020; Hada et al., 2021b,c). In addition, limited label claim of some of the recently introduced synthetic chemicals such as fluensulfone, fluopyram, etc., restricts their usage in all the crops and also against different nematodes. Therefore, using novel comprehensive approaches is the need of the hour for sustainable agricultural production.

Nematode management using bio-control strategies has been known to be a safer alternative and practical approach. This is reflected by a considerable investment of venture capital in research required for developing biocontrol options (Escudero et al., 2017). One of the biocontrol approaches is to regulate nematode populations using nematophagous fungi, which have antagonistic activity against infective juveniles (Tian et al., 2007). Nematophagous fungi and/or endophytic fungi can directly attack, kill, immobilize, or repel nematodes, confuse them while finding their host, interfere with giant cell development, compete for resources, or use a combination of these options (Schouten, 2016). It can also capture, parasitize, or paralyze nematodes and act as natural enemies of plant and animal-parasitic nematodes. They are divided into four groups, i.e., endoparasitic fungi, nematode-trapping fungi (NTF), opportunistic fungi, and toxin-producing fungi (Siddiqui and Shaukat, 2004). NTFs are the unique group of soil-inhabiting fungi that can switch from the saprophytic to pathogenic lifestyle once they come in contact with nematodes as a response to nutrient depletion, and the predatory behavior adapted by them is exciting. *Arthrobotrys oligospora* Fres. 1852 (Orbiliaceae: Orbiliales) was the first recognized nematode-trapping fungus and the most abundant in the environment (Niu and Zhang, 2011). The nematode-trapping process of the network structure of *A. oligospora* demonstrated that a specialized mycelial structure traps the nematodes followed by penetration of nematode cuticle, after which it digests the body contents (Barron, 2003). Zhang et al. (2010) revealed that *A. scaphoides* isolated from soil using *Panagrellus redivivus* nematodes as bait caused three-dimensional (3D) adhesive networks that trapped nematodes within 2 days. The utilization of NTFs will be an attractive alternative for the biological control of infective larvae. The importance of physicochemical processes of NTF is of immense interest, and researchers have collectively revealed that trapping and/or immobilization are associated with upregulation of several signaling pathways, intercellular communications, production of adhesive proteins, and organic metabolites as well as nitrate assimilation (Yang et al., 2011).

Besides, Wang B.L. et al. (2018) highlighted that *Caenorhabditis elegans* was attracted toward *A. oligospora* due to three fungal metabolites, namely 2(5*H*)-furanone, furan-2-yl methanol, and furan-2-carbaldehyde. However, the compound 3-hydroxy-2-methyl-4*H*-pyran-4-one (known as maltol) displayed a significant increase in the formation of 3D traps. Likewise, a quantity of four fungal metabolites, e.g., desferriferrichrome, linoleyl alcohol, non-adecanamide, and citicoline, were found to increase when fungi switch the lifestyle to the predatory stage, and they also showed considerable nematicidal activity (Kuo et al., 2020). Metabolite profiling of 100 wild isolates of NTF in three different species, *A. oligospora*, *Arthrobotrys thaumasia*, and *Arthrobotrys musiformis*, revealed the production of thousands of metabolites belonging to various structural families such as peptide, siderophore, fatty alcohol, and fatty acid amide during their interaction with *C. elegans* as demonstrated by liquid chromatography–mass spectrometry (LC–MS) analyses.

The endophytic entomopathogenic fungus *Tolypocladium* spp. is known to survive in the soil during the absence of insects using nematodes as alternate hosts (Samson and Soares, 1984). *T. cylindrosporum* is a saprotroph and an entomopathogenic fungus studied as a biological control agent against insects of several orders but, to the best of our knowledge, so far, not known to be useful against PPNs. Nematophagous fungi serve as both predators and decomposers in the environment, and there might be regional differences in the effectiveness of different fungal isolates (Wang F.H. et al., 2017). Hence, isolation, identification, and characterization of native strains of fungi with predatory activity are crucial to identify the potential fungi along with an understanding of their ecology, biology, mode of action, and interactions to exploit them successfully against target PPNs.

In view of the importance of nematophagous fungi as biocontrol agents for nematode management, we had isolated and identified 81 fungal isolates up to generic level from 17 soil samples collected across different states of India using *C. elegans* and *M. incognita* as bait (Kassam et al., 2021). In continuation, the present study is aimed to identify the species of two important fungal isolates, *Arthrobotrys* and *Tolypocladium*, using morphological characters and molecular markers. Furthermore, the effect of nutrition, temperature, and pH on growth rate and trap formation has been studied. Besides, fungal parasitization against *M. incognita* and *C. elegans* was evaluated under *in vitro* and *in vivo* conditions. We have also profiled the volatile and non-volatile chemical compounds [volatile organic compounds (VOCs)] produced by these fungal isolates.

MATERIALS AND METHODS

Nematode Cultures

C. elegans strain N95 was maintained on a nematode growth medium with *Escherichia coli* strain OP50 lawn. A chunk of agar containing hundreds of worms was cut and transferred onto an overnight grown OP50 lawn in a fresh Petri plate. Plates were incubated at 25°C for 3 days for nematode multiplication.

The authenticated population of *M. incognita* was maintained and multiplied on tomato roots (*Solanum lycopersicum* L. cv. Pusa Ruby) in the greenhouse. Approximately 35-day-old plants were harvested, roots washed free of soil, and used for collecting fresh egg masses, which were hatched *via* modified Baermann's technique (Whitehead and Hemming, 1965) to obtain infective second-stage juveniles (J2s) required for all the experiments.

Morphological and Molecular Classification of the Fungal Isolates

In the present study, we have taken the two Indian isolates of *Arthrobotrys* and *Tolypocladium* for species identification. Pure cultures of both the fungal isolates were grown separately in potato dextrose broth (PDB) at 25°C for 1 week. Cultures of *Arthrobotrys* spp. and *Tolypocladium* spp. were observed under an Olympus BX50 compound microscope, and morphological measurements of conidia, conidiophores, and phialides (length and width) were recorded. For molecular characterization, genomic DNA was extracted from a 1-week-old mycelial mat using the CTAB method as previously described by Wang et al. (2015). Polymerase chain reaction (PCR) analyses were carried out with genomic DNA extracted from the fungal isolates to amplify the two markers; internal transcribed spacers (ITSs) and β-tubulin using universal primers, ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) as well as βt2A (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and βt2B (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′), respectively. PCR amplification was carried out as per the procedure by Glass and Donaldson (1995). The amplified products were analyzed by electrophoresis on a 1.2% (w:v) agarose gel (Sigma Aldrich, United States) and visualized using a gel documentation system (Alpha Image Analyzer, United States). PCR products were further purified using a PCR clean-up kit (Macherey-Nagel, Germany) and sequenced (Applied Biosystems, United States). The sequences of ITS and β-tubulin generated for the tested fungi were compared with previously reported sequences in the GenBank database. All the sequences were imported for alignment by setting default parameters in the ClustalW algorithm into a MEGA X software application package. To search for homologs of both genes, sequences from each isolate were subjected to the Nucleotide Basic Local Algorithm Search Tool of the National Center for Biotechnology Information.[1] The sequences were further analyzed and submitted to GenBank. Additionally, phylogenetic trees using the sequences of ITS and β-tubulin of the tested isolates were constructed in MEGA X using the maximum composite likelihood approach considering 1,000-bootstrap replications under Kimura two-parameter distance models (Kimura, 1980). For this, *Neurospora crassa* and *Cordyceps ophioglossoides* were used as out-groups (Sung et al., 2007; Swe et al., 2008; Wright et al., 2009). In addition, the identified fungal isolates were submitted to the Indian Type Culture Collection (ITCC), which is an affiliate member of the World Federation for Culture Collections and is registered with the World Data Centre for Microorganisms (registration number 430).

Effect of Nutrition, Temperatures, and pH on Growth Rate and Trap Formation of the Selected Fungi

Pure cultures of both the fungi were grown in six different media, *viz.*, potato dextrose agar (PDA), cornmeal agar (CMA), Czapek malt agar (CzMA), rose bengal agar (RBA), peptone-yeast-glucose, and synthetischer nährstoffarmer agar (SNA) (HiMedia, India) in 90-mm diameter (dm) Petri plates at 25°C to examine the differences in structure, color, growth rate, and sporulation among colonies. For this, a small piece of agar measuring around 5 × 5 mm was cut from a well-established colony and placed upside down at the center of the fresh Petri plate containing different media. Three replicates (n = 3) for each medium were used, and observations were made for all the replicates separately. The fungal growth was determined at 3-, 6-, 9-, and 12-day post-inoculation (dpi) by measuring the colony diameter. The growth rate was estimated as growth rate per day (mm/day) = [the average diameter of hyphae measured in that day - 5 (diameter of original inoculum)]/days of culture, as described earlier by Wang F.H. et al. (2017).

Furthermore, the medium that supported the maximum growth rate was used to evaluate the effect of different temperatures, *viz.*, 15, 20, 25, 30, and 35°C, by following the procedures described earlier. Additionally, a combination of the specific medium and temperature supporting maximum fungal growth was studied to evaluate the effect of different pH levels (ranging from 4 to 10). Three replicates (n = 3) were used for each treatment. The growth rate was estimated at 3, 6, 9, and 12 days after inoculation by a formula adopted from Wang F.H. et al. (2017). The mean value of the diameter of fungal hyphae

[1] https://www.ncbi.nlm.nih.gov/

was measured each day in each group and used for calculating the growth rate by deducting the original diameter of the fungal disc used for sub-culturing.

In vitro Efficacy of Fungal Filtrate on Nematode Mortality

The two selected fungi were grown on a PDB medium for 10 days at 25°C with 180 rpm in an incubator shaker. Approximately 100 surface-sterilized nematodes (both *M. incognita* and *C. elegans*) were added "separately" into 5-ml Eppendorf tubes containing 3-ml fungal filtrate (FF) of each isolate. The tubes were incubated on the shaker at 120 rpm at 25°C for 3 days. The worms were washed thrice with sterile water (SW) followed by re-incubation in SW for 24 h at 25°C for revival in the nematode movement. Then, the nematodes were examined under a stereo binocular microscope to record dead and live nematodes, and nematode mortality percentage was calculated as% mortality = (number of dead nematodes/total number of nematodes) × 100. There were three replicates ($n = 3$) for each treatment, and observations were made for all the replicates independently. Worms in the PDB medium served as the control for comparison.

In vitro Evaluation of Direct Fungal Parasitism Against *M. incognita* and *C. elegans*

Fungal parasitism against *M. incognita* J2s and *C. elegans* L3s was evaluated in water agar plates. Briefly, fungi were grown on PDA at 25°C for 6 days, and then, 5-dm discs were transferred into 2% water-agar plates containing 1% ampicillin (100 μg ml^{-1}). The plates were incubated at 25°C for 3 days, and then 100 surface-sterilized worms were added to the plates separately (Contina et al., 2017). The number of captured larvae was scored after 3 days using a light microscope (40×). Three replicates ($n = 3$) were used for each treatment and compared with control (nematodes only in water agar medium). Parasitization was calculated as previously described by Siddiqui and Shaukat (2004) as% parasitization = (number of parasitized nematodes/total number of nematodes) × 100.

Captured nematodes of both *M. incognita* J2s and *C. elegans* L3s were also examined under a scanning electron microscope. For this, worms were fixed in 2% glutaraldehyde in 0.1-M phosphate buffer (pH 7.2) overnight, followed by 2% osmium tetroxide fixation for 6 h and dehydration using a series of ethanol as described by Den Belder et al. (1993).

In vivo Evaluation of Fungi Against *M. incognita*

One-month-old healthy tomato seedlings (*S. lycopersicum* L. cv. Pusa Ruby) were transplanted into 4-inch dm pots filled with 400-g autoclaved soil, sand, and farmyard manure (50:25:25), which were kept in the greenhouse. At the same time, fungal suspensions were prepared by culturing the colonies on medium, which showed a high sporulation level, and incubated at 25°C for 10 days. Subsequently, 5 ml of sterile distilled water was added to the plate and the spores were scraped using a spatula. The mixture was placed in a small beaker and stirred for 10 min, filtered using cheesecloth, and quantified using a hemocytometer on a light microscope. Finally, the fungal suspensions were adjusted to get 1×10^6 spores/ml and used for inoculation around the root zone during transplantation. After 1 week, 800 J2s of *M. incognita* were inoculated at the rate of 2 J2s per gram of soil, and the pots were maintained in the greenhouse. Plants that received sterile distilled water served as control. Five replicates ($n = 5$) were used for each treatment, and observations were made for all the replicates individually. Plants were carefully harvested 45 dpi, and roots were washed free of soil. Plant growth parameters, *viz*., plant length, fresh weight, and dry weight were recorded. Additionally, nematode disease burden was determined as the number of galls, egg masses, and eggs per egg mass and used for deriving the nematode multiplication factor (MF) as [(total number of egg masses per plant × average number of eggs per egg mass) ÷ initial nematode inoculum level] as described previously by Hada et al. (2020, 2021a).

Characterization of Volatile and Non-volatile Molecules of the Selected Isolates

Extraction of Fungal Volatiles for Gas Chromatography–Mass Spectrometry Analysis

The selected fungal isolates were grown on PDA media (HiMedia, India) in Petri plates (90 mm diameter) for 10 days. Fungal mats along with media were taken out separately and extracted with hexane (50 ml × 3) thrice using a bath sonicator at 30-Hz amplitude for 30 min. The extracts were filtered, pooled, and passed through anhydrous sodium sulfate (20 g) to remove traces of water if any. The extracts were concentrated separately under reduced pressure in a rotary evaporator (Heidolph, Germany) and dissolved in gas chromatography–mass spectrometry (GC–MS) grade hexane for further analysis (Saha et al., 2015a).

Analysis by Gas Chromatography–Mass Spectrometry

GC–MS analysis was carried out using 7890A GC (Agilent Technologies, United States) equipped with an HP-5MS column (30 m × 0.25 mm/0.25 μm, Agilent Co., United States), which was directly connected to a triple-axis HED-EM 5975C mass spectrometer (Agilent Co., United States). The injection volume was 1 μl with flow mode in split control. The carrier gas flow was set at 1 ml min^{-1} helium. The oven temperature was initially held at 40°C for 2 min. Thereafter, the temperature was raised with a gradient of 3°C min^{-1} until the temperature reached 130°C and held for 2 min. Again, the temperature was raised with a gradient of 5°C min^{-1} up to 220°C and held for 1 min. Finally, the oven temperature was raised to 280°C with an increment of 10°C min^{-1}. The total runtime was 59 min. The MS acquisition parameters were set with the ion source temperature 175°C, electron ionization 70 eV, full scan mode (50–550 mass units), and transfer line temperature 250°C. Compounds were identified by matching their mass spectra. Volatile organic compounds (VOCs) were identified by library matching from the National Institute of Standards and Technologies Mass Spectra Library (Saha et al., 2015a).

Extraction of Fungal Metabolites for Ultra-Performance Liquid Chromatography–Quadrupole/Time of Flight-Electrospray Ionization–Mass Spectrometry Analysis

The selected fungi were again grown on PDA for 10 days. Fungal mats along with media were taken out separately and extracted with methanol (50 ml, each) thrice using a bath sonicator at 30 Hz. amplitude for 30 min. The extracts were filtered and pooled, and the solvent was evaporated under vacuum in a rotary evaporator that resulted in respective concentrates. The concentrates obtained for each fungus were dissolved in LC–MS grade methanol separately for further analysis (Saha et al., 2015b).

Analysis by Ultra-Performance Liquid Chromatography–Quadrupole/Time of Flight-Electrospray Ionization–Mass Spectrometry

The analysis was performed on ultra-performance liquid chromatography–quadrupole/time of flight mass spectrometry (QToF-MS, Synapt G2 HDMS, Waters Corporation, Manchester, United Kingdom). The QToF-MS was operated with electrospray ionization (ESI) at a nominal mass resolution of 20,000 and controlled by MassLynx 4.1 software. The data acquisition was made with the MS^Efunction in continuum mode in the range of m/z 50–1,200. The chromatographic separation was performed on an ACQUITY Ultra-Performance Liquid Chromatography (UPLC) BEH C_{18} column (2.1 × 100 mm, 1.8 μm, Waters India Pvt. Ltd., Bangalore) at 35°C. The mobile phase consisted of A phase: methanol–water (20:80) and B phase: methanol–water (80:20) with 0.1% formic acid in both the phases. A gradient program was used with 0.4 ml/min flow rate, with 0–4.0 min 100% A, 4.0–7.0 min 70% A, 7.0–12.0 min 50% A, 12–15 min 30% A, and 15–25.0 min 100% A. The injection volume was 5 μl, and the samples were maintained at 25°C throughout the analysis (Saha et al., 2015b).

Statistical Analysis

Data of laboratory experiments were analyzed in a completely randomized design. Greenhouse experiments were conducted in the randomized complete block design, and data were subjected to analysis of variance and Duncan's multiple range test at 1 and 5% level of significance using statistical package version 160 (SPSS 16.0; IBM Corp., United States). All the experiments were conducted thrice to validate the final outcome.

RESULTS

Morphological and Molecular Classification of the Fungal Isolates

Species identification has been primarily made using morphological characters along with the measurement of taxonomically useful features. The *Arthrobotrys* isolate exhibited straw white-colored colonies with raised concentric rings along with thin and hairy rings on PDA media. The length and width of conidia and conidiophores of *A. thaumasia* were approximately 24.58–60 and 10.15–22.88 μm, as well as 211–446 and 2.3–5.4 μm (n = 50), respectively. Furthermore, the shape of the conidium appeared as an inverted pear with 1–3 septa. All these characteristics matched with the reported description of the fungus *A. thaumasia* (**Supplementary Table 1** and **Figure 1A**), and we designated our isolate as *A. thaumasia* At_RK.

Similarly, in the case of *Tolypocladium* isolate, the colony appeared hairy with whitish cream as well as reverse yellow to pale on PDA media. The conidium was 2–4.3 and 1.3–1.7 μm in length and width, respectively. Likewise, the length and width of conidiophores were approximately 31–44 and 1.1–2.8 μm, respectively. The phialide length and width were 4.5–8.5 and 2–3.2 μm, respectively. Finally, conidia hyaline were smooth-walled, short cylindrical, straight and/or slightly curved, and both ends obtusely rounded. Comparison of all these characteristics matched with the reported description of *T. cylindrosporum* (**Supplementary Table 1** and **Figure 1B**), and our isolate was designated as *T. cylindrosporum* Tc_RK.

To further confirm the species identity, the fungi discussed earlier were characterized using two molecular markers, ITS and β-tubulin. Sizes of ITS and β-tubulin that could be amplified in both the isolated fungi were 580 and 370 bp, respectively. Purified PCR products were sequenced and submitted to the National Center for Biotechnology Information database (**Supplementary Table 2**). Homology search of ITS and β-tubulin sequences using BLAST program showed that *A. thaumasia* At_RK sequence was 99% (accession: KT215216.1) and 97% (accession: EU977531.1) identical to earlier reported sequences of *A. thaumasia*, respectively. Similarly, the ITS sequence of *T. cylindrosporum* Tc_RK was 99.06% (accession: NR_167967.1), identical to a previously reported sequence of *T. cylindrosporum*.

Furthermore, we constructed an evolutionary tree based on the sequences of the ITS, and β-tubulin that demonstrated *A. thaumasia* At_RK was closest to *A. thaumasia* strain CBS 376 97 (accession: KT215216.1), whereas β-tubulin sequence was close to *A. thaumasia* isolate 111 (accession: EU977531). Similarly, the ITS sequence of *T. cylindrosporum* Tc_RK was closest to *T. cylindrosporum* isolate TCDAs18R1A9 (accession: MT911434.1), whereas that of β-tubulin was close to *T. tropicale* strain IQ214 (accession: KF747166.1) and *T. tropicale* strain MX338 (accession: KF747190.1). The results of phylogenetic analysis based on the sequence of ITS and β-tubulin indicated that the isolates in the present study could be different geographical strains of *A. thaumasia* and *T. cylindrosporum* (**Supplementary Figure 1**).

Additionally, the studied fungal isolates were submitted to ITCC with strain/accession numbers: ITCC8969 for *T. cylindrosporum* Tc_RK and ITCC8970 for *A. thaumasia* At_RK (**Supplementary Table 2**).

Effect of Nutrition, Temperatures, and pH on Growth Rate and Trap Formation of the Fungal Colonies

Selected fungal isolates cultured on different nutrient media showed clear variations in the growth rates and trap formation at different time intervals. The highest growth rate of *A. thaumasia*

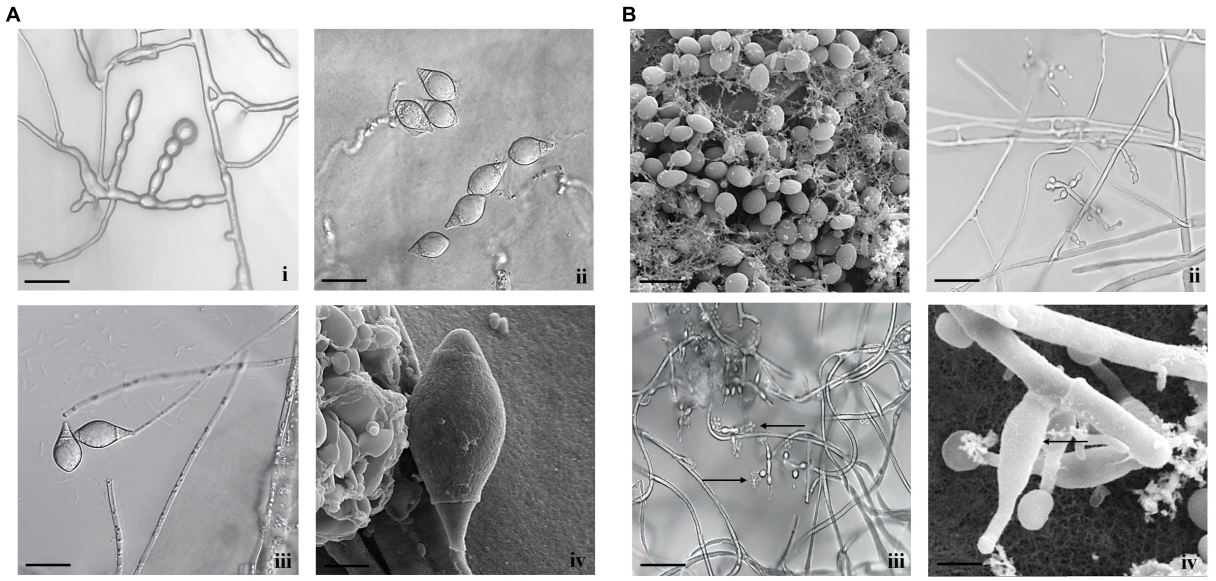


FIGURE 1 | Morphological characteristics of **(A)** *A. thaumasia* At_RK **(i)** chlamydospores, **(ii)** conidia, **(iii)** conidiophores with conidia (scale bar = 20 μm), **(iv)** conidia observed by scanning electron microscope (SEM) (scale bar = 5 μm), **(B)** *T. cylindrosporum* Tc_RK **(i)** conidia observed by SEM (scale bar = 5 μm), **(ii)** conidiophores with phialides (scale bar = 20 μm), **(iii)** conidiophores with phialides along with conidia (scale bar = 20 μm), **(iv)** phialides under SEM (scale bar = 2 μm).

was observed significantly in SNA media (2.00 mm/day), followed by CMA (1.375 mm/d) and CzMA (1.272 mm/day) compared with other tested media after 3 days (**Supplementary Figure 2A**). Likewise, at 6 dpi, the growth rates were observed to be significantly higher in SNA (1.432 mm/day), CMA (1.405 mm/day), PDA (1.405 mm/day), and CzMA (1.395 mm/day) media, respectively. Finally, the hyphae covered the entire Petri plate, and growth rates of fungus were equal in all the media after 9 dpi (0.94 mm/day) and 12 dpi (0.71 mm/day). Concurrently, sporulation was observed, and the number of spores was counted using a hemocytometer. The maximum sporulation was noticed on RBA (30.3×10^4 spore/ml) followed by CzMA medium (9.1×10^4 spore/ml) (**Supplementary Table 3**). The response to different media shows that *A. thaumasia* mycelia are very sensitive to salts and amino acids because they promote trap formation at specific nutrient combinations and inhibit it partially or completely at other combinations. There are significant differences among the different media compositions ($p < 0.01$).

In the case of *T. cylindrosporum* isolate, the growth was slow in all the tested media (**Supplementary Figure 2B**), but PDA media comparatively showed a considerable growth rate (0.64 mm/day) at 3 and 6 dpi. Subsequently, the maximum growth rate was observed in the PDA media (0.525 mm/day) and lowest in CMA media (0.328 mm/d) after 9 and 12 dpi. Consequently, the fungal sporulation was also observed in all the tested media, and the maximum sporulation was obtained on SNA media (9006.3×10^4 spore/ml) followed by PDA (7203.3×10^4 spore/ml) and CMA media (3427.2×10^4 spore/ml) (**Supplementary Table 3** and **Supplementary Figure 3A**).

Furthermore, *A. thaumasia* grown on SNA media that provided a maximum growth rate was used to evaluate the effect of different temperatures. The data indicate that the growth rate of *A. thaumasia* was significantly higher at 30, 25, 20, and 15°C (0.293, 0.634, 0.61, and 0.523 mm/day) after 3, 6, 9, and 12 dpi, respectively. In the case of *T. cylindrosporum* that grew on PDA media, it exhibited the quickest growth rate at 20°C after 3 and 6 dpi (0.525 and 0.465 mm/day, respectively), and the highest growth was noticed after 9 and 12 dpi (0.563 and 0.475 mm/day, respectively) as compared with other treatments ($p < 0.01$). The fungus could not grow at 35°C after 3 and 6 dpi but could grow in the range from 15 to 30°C (**Supplementary Figure 3B**).

Additionally, a combination of the specific media and temperature supporting maximum fungal growth was studied to evaluate the effect of different pH levels. For this, an isolate of *A. thaumasia* was cultured on SNA media at 30°C. The result showed that the radial growth rate at pH 6–9 (8.575 and 8.475 mm/day) was significantly faster than other tested pH after 3 days. The highest growth under different pH recorded was at pH 9 > pH 6 > pH 7 = pH 8 > pH 10 > pH 5 > pH 4. Similarly, a culture of *T. cylindrosporum* grown on PDA medium at 20°C caused the highest growth rate at pH 6 (1.55–5.15 mm/day) followed by pH 9 and 10, and minimal growth was recorded at pH 4 (0.525–1.975 mm/day) (**Supplementary Figure 3C**).

In vitro Evaluation of Fungal Filtrate of the Tested Isolates on Nematode Mortality

The FF of the selected isolates were found to be effective against both *M. incognita* J2s and *C. elegans* L3s compared with control in the PDB medium. *T. cylindrosporum* Tc_RK and *A. thaumasia* At_RK caused 87.3 ± 6.02 and 57.7 ± 3.5% mortality of *M. incognita* J2s, respectively. Likewise, FF of *T. cylindrosporum*

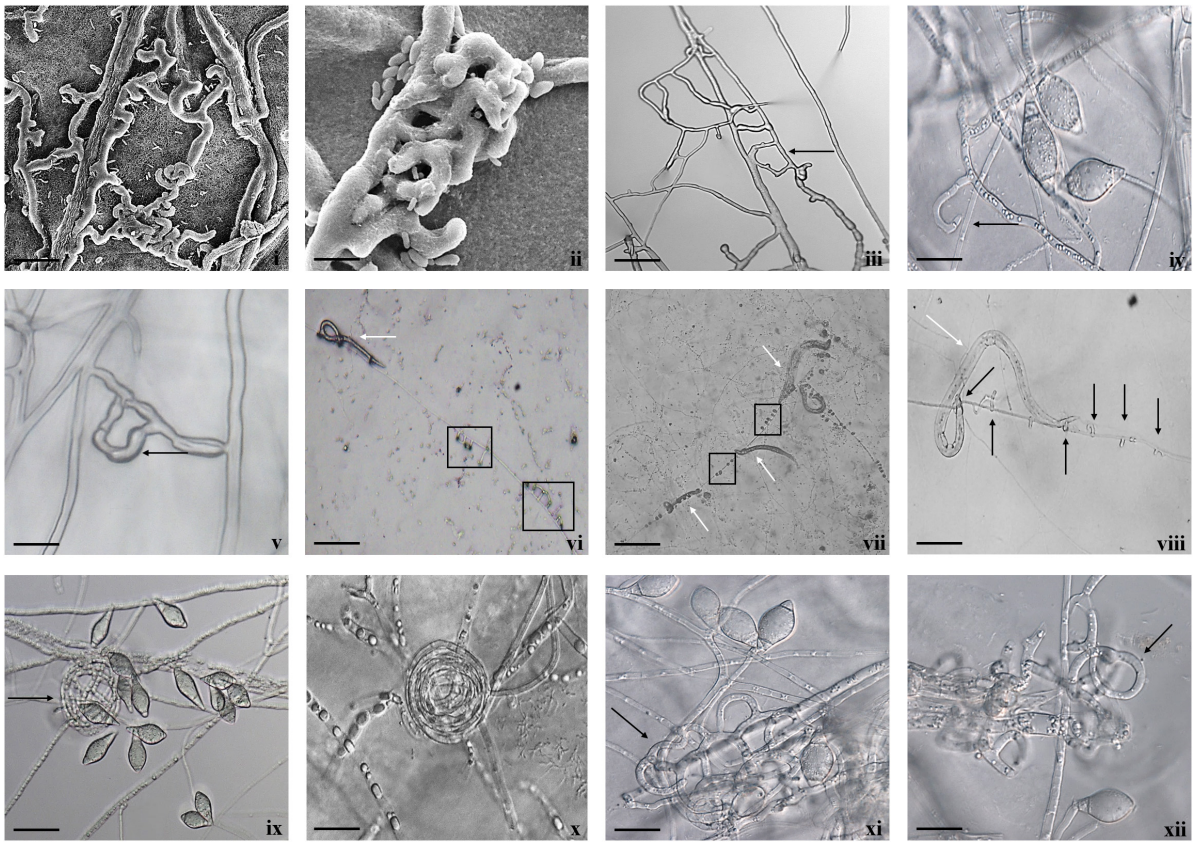


FIGURE 2 | Diversity of trapping structures of *A. thaumasia* At_RK. **(i,ii)** Scanning electron micrographs showing process of trap formation of adhesive two-dimensional (2D) network (scale bar = 20, 5 μm), **(iii)** adhesive 2D (scale bar = 50 μm), **(iv,v)** vegetative hyphae along with an initial branch forming a loop (black arrow) by turning around to meet a peg formed on parent hypha (scale bar = 50, 20 μm), **(vi–viii)** non-constricting rings (black arrows), capturing nematodes (white arrows), **(ix,x)** non-constricting rings (scale bar = 20 μm), **(xi,xii)** 3D adhesive network (scale bar = 20 μm).

Tc_RK and *A. thaumasia* At_RK caused 64 ± 3.6 and 53.7 ± 2.3% mortality in *C. elegans* L3s (**Supplementary Table 4**). Both *M. incognita* and *C. elegans* worms exhibited normal behavior in the PDB control without any mortality.

In vitro Evaluation of Direct Fungal Parasitism Against *M. incognita* and *C. elegans*

Fungal parasitism against *M. incognita* J2s and *C. elegans* L3s was evaluated on water agar plates under *in vitro* conditions. The tested fungal isolates were found to be effective against both *M. incognita* and *C. elegans* after 3 days compared with control in the water agar plates (**Supplementary Table 4**). Results showed that *A. thaumasia* At_RK formed different types of traps to hunt the nematodes, *viz.*, 2D adhesive network, 3D adhesive network, and non-constricting rings (**Figure 2**). It caused approximately 82 ± 3.6% parasitism of *M. incognita* J2s. During parasitization, the fungal mycelium penetrated the nematode cuticle, developed inside its body, consumed the body contents, then ruptured the cuticle, and subsequently grew out of the body (**Figure 3**). The fungus mostly penetrated the nematode body along the lateral lines in both the nematodes (**Figures 3**, **4**). Similarly, the percentage of *C. elegans* parasitized by *A. thaumasia* At_RK was approximately 73 ± 4.5%. It captured *C. elegans*, penetrated the cuticle and grew inside the body, and then ruptured the cuticle (**Figure 4**). It was noticed that it colonized approximately five to six *M. incognita* J2s but only one to two *C. elegans* L3s.

Direct parasitism of *T. cylindrosporum* Tc_RK against both the nematodes was not as clear as in *A. thaumasia*. The capture and colonization of *M. incognita* by *T. cylindrosporum* were 65.2 ± 3.1% after 3 dpi and 57.7 ± 3.6% in *C. elegans* (**Supplementary Table 4**). The attachment of fungal spores on *M. incognita* cuticle was observed as a first step of the parasitism process (**Figure 5**). The fungal parasitization and spore attachment on *C. elegans* were significantly less compared with *M. incognita*, but consumption of nematode contents was detected quite well, as shown in **Figure 6**.

In vivo Evaluation of Selected Fungi Against *M. incognita*

Fungal spore suspension having 1 × 10^6 spores/ml was inoculated in the vicinity of tomato roots during transplantation. The selected fungal isolates significantly increased plant growth parameters in plant length, weight, and dry weight when

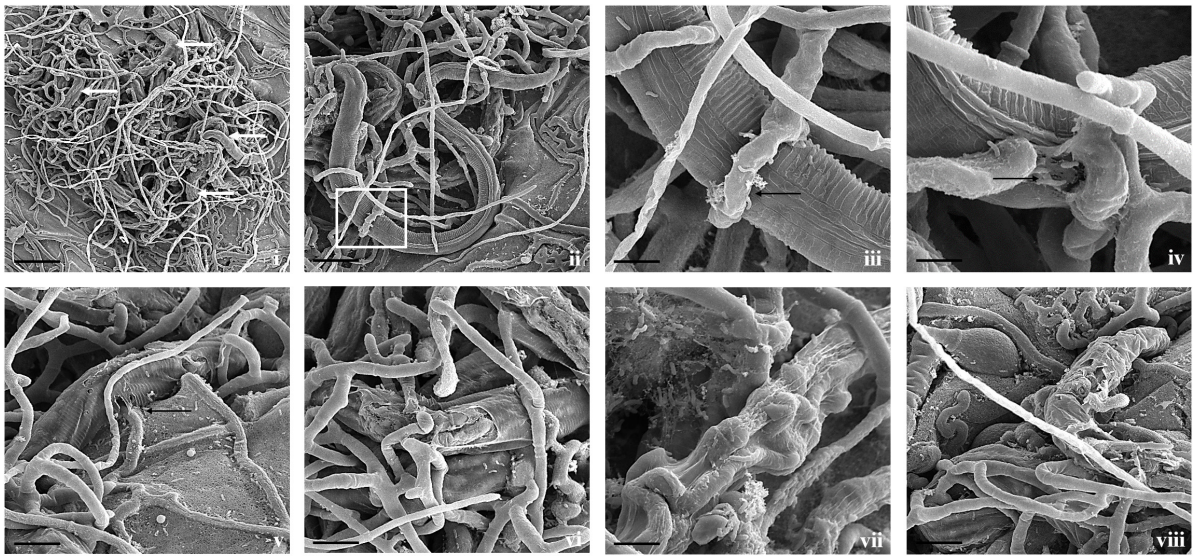


FIGURE 3 | Scanning electron micrographs of interaction between *A. thaumasia* At_RK and *M. incognita*. **(i,ii)** Fungal colonization against many nematodes (white arrows), larvae trapped in fungal networks at various points on nematode body (scale bar = 50, 20 μm), **(iii)** magnification of area highlighted (panel ii**)** showing pressure caused by modified hyphae on nematode cuticle (scale bar = 5 μm), **(iv)** hyphal penetration site (black arrows) on nematodes cuticle along lateral lines (scale bar = 5 μm), **(v,vi)** fungus rupturing nematode cuticle (scale bar = 10 μm), **(vii,viii)** consumption of nematode body contents by fungus, surface of *M. incognita* is irregular indicating internal growth of hyphae (scale bar = 5, 10 μm).

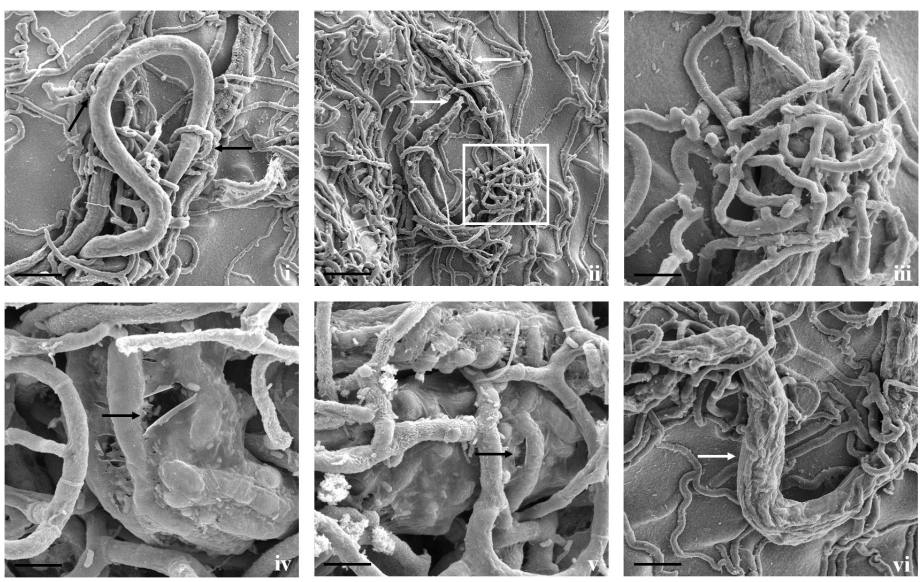


FIGURE 4 | Scanning electron micrographs of interaction between *A. thaumasia* At_RK and *C. elegans*. **(i)** Larva of *C. elegans* captured after 24 h of interaction (scale bar = 20 μm), **(ii)** fungal colonization outside nematode body, traps adhered to cephalic and tail regions of *C. elegans* 3 days after predation (scale bar = 50 μm), **(iii)** magnification of area highlighted (panel ii) showing nematode captured by fungal adhesive trap (scale bar = 10 μm), **(iv,v)** fungus rupturing nematode cuticle (black arrows) (scale bar = 10 μm), **(vi)** fungus growing inside nematode body (white arrows) (scale bar = 20 μm).

compared with the control plants treated with only nematodes and SW. The maximum plant length (68.2 cm) and weight (14.92 g) were observed in the *T. cylindrosporum* Tc_RK-treated samples. Also, plants treated with *A. thaumasia* At_RK showed an average length of 66.8 cm, whereas the average weight was 13.4 g (**Table 1**). Interestingly, *A. thaumasia* At_RK-treated plants showed significantly higher dry weight (3.22 g) compared with only nematode-treated plants without the addition of fungal suspension.

Furthermore, the effect of the selected fungi was determined on nematode disease burden in tomato plants inoculated with *M. incognita.* Results showed that the application of fungal

suspension caused a significant reduction in nematode infection compared with control. It was found that average galling was in the range of 29 ± 16.2 in *A. thaumasia*- and 43.4 ± 12.8 in *T. cylindrosporum*-treated plants compared with control, which documented approximately 142 ± 5.4 that led to a decrease in galling intensity by 80 and 69%, respectively. Corroborating this, the number of egg masses was approximately 18.6 ± 6.6 and 7.2 ± 3.8 in *A. thaumasia*- and *T. cylindrosporum*-treated plants, respectively, compared with 50.6 ± 4.5 in control plants. Likewise, the number of eggs per egg mass was 325.2 ± 31.1 and 221.2 ± 24.4 in *A. thaumasia*- and *T. cylindrosporum*-treated plants, respectively, compared with 583.4 ± 40.7 in control plants. As a consequence, approximately 63 and 86% reduction in egg masses and 44 and 62% reduction in eggs per egg mass could be obtained in *A. thaumasia*- and *T. cylindrosporum*-treated plants, respectively. Ultimately, approximately 80 and 95% decline in nematode MF was observed in *A. thaumasia*- and *T. cylindrosporum*-treated plants, respectively (**Table 1**).

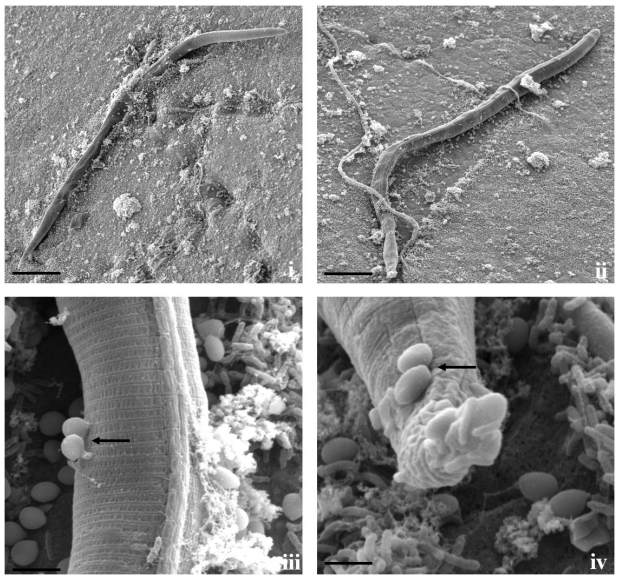


FIGURE 5 | Scanning electron micrographs of interaction between *T. cylindrosporum* Tc_RK and *M. incognita*. **(i,ii)** Fungal colonization and parasitization on nematode cuticle (scale bar = 20 μm), **(iii,iv)** attachment of fungal spores onto nematode cuticle (black arrow) followed by spore germination (scale bar = 2 μm).

Characterization of Volatile and Non-volatile Molecules of the Selected Isolates

Hexane soluble fraction of *A. thaumasia* and *T. cylindrosporum* was subjected to GC–MS analysis to identify VOCs. GC–MS analysis of hexane extract of *A. thaumasia* showed several peaks corresponding to 16 volatile compounds, representing 79.9% of the extract. The compounds are listed based on their retention time in **Table 2**. Interestingly, volatile sulfides such as *n*-propyl-butyl-disulfide (3.04%), bis (1-methylpropyl)-disulfide (2.80%), and dibutyl-disulfide (1.99%) were detected in the total ion chromatogram. 2-Methyl-1-pentanethiol (32.93%) was the major constituent followed by trimethyl-heptadien-4-one (23.34%), hexene-2,5-diol (5.08%), and chloro-3-butyltetrahydropyran (5.08%). Besides, dimethyl-pentanal (1.10%) and nerolic acid (1.10%) were also detected.

Likewise, GC–MS analysis of hexane extract of *T. cylindrosporum* also displayed various major and minor peaks corresponding to 11 VOCs. Compounds are listed as per their elution from the HP-5MS column (**Table 2**). Most of the peaks of the total ion chromatogram of the sample were identified as hydrocarbons and alcohols. Among these, decane (17.70%) was the most abundant. Other major compounds identified were ethyl-3-methyl-benzene (8.10%), octadecenal (7.52%), bis-(dimethyl-ethyl)-phenol (7.04%), dodecane (5.73%), hexadecanol (4.86%), and undecane (4.34%).

Furthermore, UPLC-QToF-ESI-MS analysis of a methanolic extract of *A. thaumasia* revealed various peaks in the total ion chromatogram; however, six metabolites were identified tentatively based on their molecular ion peaks, considering error mass (δ) value within 10 ppm (**Table 3**). These six metabolites were cyclo(L-Pro-L-Val) or piperazinone (1), cyclo(L-Pro-L-Leu), a pyrazine-1,4-dione derivative (2), paganin A (3), talathermophilin E (4), dactylarin (5), and trichodepsipeptide A (6). Among these, cyclo(L-Pro-L-Leu) is a homodetic cyclic peptide consisting of leucyl and prolyl amino acid residues. Furthermore, these metabolites were also confirmed from their possible fragmentation pattern in high-resolution MS. Cyclo(L-Pro-L-Val) or piperazinone was identified from its molecular ion peak at m/z 196.1201. Besides, fragmentation of the molecular ion peak resulted in daughter ion peaks at m/z 154 and m/z 126, originated due to sequential loss of isopropyl (43 amu) and carbonyl (28 amu) moieties. Another metabolite, cyclo(L-Pro-L-Leu), showed a characteristic molecular ion peak at m/z 210.1373, which on fragmentation produced possible daughter ions at m/z 195, 166, and 138, originated due to subsequent breaking of methyl (15 amu), ethyl (29 amu), and carbonyl groups (**Supplementary Figure 4A**). Similarly, the other four metabolites, paganin A, talathermophilin E, dactylarin, and trichodepsipeptide A, were characterized from their respective molecular ion, sodiated or adduct ion peaks at m/z 152.0940, 312.1711, 327.0921, and 711.4034.

UPLC-QToF-ESI-MS analysis of a methanolic extract of *T. cylindrosporum* tentatively revealed the identification of a total of nine metabolites. These metabolites, tolypocladenols A1/A2 (i), tolypyridone A (ii), tolypyridone B (iii), penicillenol B1/B2 (iv), pyridoxatin (v), terpendole E (vi), destruxin A (vii), 4-chloro-2-phenylphenol (viii), and acetamido-6-[(O-methyl-glucopyranosyl)oxy]anthraquinone (ix), were characterized based on their exact monoisotopic mass value (error mass < 10 ppm) and mass fragmentation pattern (**Table 3**). The first metabolite, tolypocladenols A1 or A2, was identified from its adduct ion peak at m/z 356.1852. Fragmentation of the adduct ion produced daughter ion peaks at m/z 327 and 191 originated due to the removal of two methyl (−28 amu) and side hydrocarbon chains (136 amu) (**Supplementary Figure 4B**). Similarly, the remaining eight

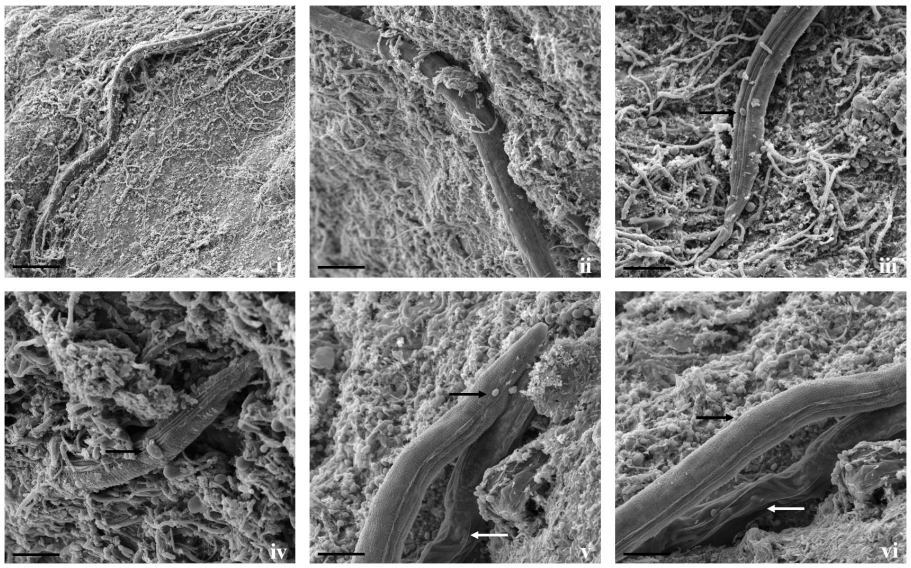


FIGURE 6 | Scanning electron micrographs of interaction between *T. cylindrosporum* Tc_RK and *C. elegans*. **(i,ii)** Fungal colonization and parasitization on nematodes body (scale bar = 50, 20 μm), **(iii,iv)** attachment of fungal spores onto nematode cuticle (black arrow) (scale bar = 20 μm), **(v,vi)** comparison between fungus colonized nematodes having hypha inside its body (white arrows) and not colonized worms (black arrow) (scale bar = 20 μm).

TABLE 1 | Evaluation of tested isolates on *M. incognita* infection and growth parameters of tomato at 45 dpi.

Treatments	Growth parameters			Infection parameters		
	Plant length (cm)	Plant wet weight (g)	Plant dry weight (g)	Number of galls	Number of egg masses	Number of eggs per egg mass
Arthrobotrys thaumasia	66.8 ± 8.01^{b}	13.4 ± 2.3^{b}	3.22 ± 0.36^{b}	29 ± 16.2^{b}	18.6 ± 6.6^{c}	325.2 ± 31.1^{c}
Tolypocladium cylindrosporum	68.2 ± 8.87^{b}	14.92 ± 1.7^{b}	3.04 ± 0.15^{b}	43.4 ± 12.8^{c}	7.2 ± 3.8^{b}	221.2 ± 24.4^{b}
Control Nematode	37.8 ± 3.57^{a}	7.2 ± 0.8^{a}	2.254 ± 0.27^{a}	142 ± 5.4^{d}	50.6 ± 4.5^{d}	583.4 ± 40.7^{d}
Healthy control	79.1 ± 6.34^{c}	19.36 ± 0.82^{c}	3.934 ± 0.56^{c}	0^{a}	0^{a}	0^{a}

Each treatment had five replications. Values in same column followed by different letter(s) are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

metabolites were also characterized from their corresponding fragmentation pattern of the molecular ion peaks.

DISCUSSION

RKNs, *Meloidogyne* spp., are the most damaging endoparasites with a wide range of hosts resulting in huge losses in various crops worldwide. Climatic fluctuations have resulted in the evolution of new races of nematodes that overcome the previous source of resistance and cause diseases. To date, it has been very difficult to recommend a promising nematode management tool that is effective, environmentally safe, economical, and harmless to the non-targets. In this regard, biocontrol agents have been preferred due to their potential against target parasitic nematodes. NTFs hold great potential as biocontrol agents, as they capture nematodes by producing trapping devices from their vegetative mycelia and also produce various metabolites as nematicidal weapons that have antagonistic activity against infective juveniles (Wang X. et al., 2018; Kuo et al., 2020). To understand its importance in the integrated pest management programs and to enrich the options of the fungi to be used as biocontrol agents, the present study was undertaken to characterize nematophagous fungal isolates of *Arthrobotrys* and *Tolypocladium*, which were isolated using *C. elegans* and *M. incognita* as bait (Kassam et al., 2021). These two isolates were used for species identification and evaluation against *C. elegans* and *M. incognita*.

For morphological characterization, structure, nature, and color of the fungal colonies, along with the measurement of taxonomic features such as size and shape of conidia, conidiophores, and phialides, as well as the presence of chlamydospores, were considered. In the case of isolate *Arthrobotrys*, the conidia were inverted pear-shaped, 1–3 septate, and 24.58–60 × 10.15–22.88 μm in size. Furthermore, the trapping device is not a constricting ring but an adhesive network that matches the description of *A. thaumasia* (Wang X. et al., 2017; Zhang et al., 2021). Thus, both the morphological characteristics and nematophagous behavior of our isolate were in conformity with the previous description of *A. thaumasia* that was designated as *A. thaumasia* At_RK. It is important to reiterate that this was isolated from dead *M. incognita*, unlike the other geographical strains reported (Kuo et al., 2020). This is the first

TABLE 2 | Identification of volatile organic compounds (VOCs) of *A. thaumasia* and *T. cylindrosporum* using GC–MS analysis.

Compound Name	RT (min.)	Content (%)	Description
A. thaumasia			
Dimethyl-pentanal	2.17	1.1	–
2-Methyl-1-pentanethiol	2.18	32.93	It was used for preventing, destroying, or mitigating pests. It has a repellent insecticides odor (Matheny, 1980)
Ethylbutyl-hydroperoxide	2.28	0.1	It was found in *Moringa peregrine* leaves extracts as antimicrobial, anticancer, and antioxidant (Al-Owaisi et al., 2014)
Methylpentyl-hydroperoxide	2.34	0.42	
Trimethyl-heptadien-4-one	2.51	23.34	It was also found in *Artemisia lavandulaefolia* extracts works against *Plutella xylostella* and *Rhizoctonia solani* (Huang et al., 2018) It was found in *Malva sylvestris* extract works against *Haemonchus contortus* nematode (Al-Rubaye et al., 2017; Mravčáková et al., 2021)
Hexene-2,5-diol	2.74	5.08	It was isolated from *T. longibrachiatum*, having antibacterial activity against *Bacillus subtilis* (Sarsaiya et al., 2020)
Chloro-3-butyltetrahydropyran	2.85	5.08	–
Dodecadienal	3.1	0.8	It was found in *Ailanthus altissima* extracts, works against *Meloidogyne javanica* (Caboni et al., 2012)
Undecane	4.28	0.47	It was found in neem, *Azadirachta indica* leaf extract (Rady, 2018) It was found in *T. longibrachiatum* having antibacterial activity against *Bacillus subtilis* (Sarsaiya et al., 2020)
Tetramethyl-benzene	4.89	0.7	It was isolated from *T. harzianum* and *Alternaria alternate* PDB (Siddiquee et al., 2012; Encinas-Basurto et al., 2017)
Dibutyl-disulfide	5.71	1.99	It was found in *Ferula assa-foetida* oil, having antibacterial activity against *Streptococcus mutans*, *S. sanguis*, *S. salivarius*, *S. sobrinus*, and *Lactobacillus rhamnosus* (Daneshkazemi et al., 2019)
n-Propyl-butyl-disulfide	5.93	3.04	
Bis (1-methylpropyl)-disulfide	7.4	2.8	
Bicyclo-undeca-pentene	10.1	0.49	–
Ethyl-5-(ethylbutyl)-octadecane	10.22	0.49	It was found in *Salmali Niryasa* having antimicrobial activity against *Klebsiella pneumoniae*, *Staphylococcus aureus*, *E. coli*, and *Candida albicans* (Divya and Prabhu, 2015)
Nerolic acid	12.26	1.1	It was found in *Myrcia ovata* oils having antifungal activity against *Colletotrichum acutatum*, *Plenodomus destruens*, and *Thielaviopsis paradoxa* (White et al., 2019)
Total		79.93	
T. cylindrosporum			
Methyl-hexadecanol	2	2.08	It was found in *Satureja montana* oil having nematotoxic and phytotoxic effects (Faria et al., 2016)
Octadecenal	2.22	7.52	It was found as semiochemicals (attractants) such as pheromones, kairomones, and allomones that act to modify behavior of pests or their natural enemies It was identified as minor components in pheromone gland of female moths as part of the sex pheromone (Chen et al., 2018)
Ethenyloxy-octadecane	2.28	4.34	It was found in *T. longibrachiatum* having antibacterial activity against *Bacillus subtilis* (Sarsaiya et al., 2020)
Ethyl-3-methyl-benzene	2.54	8.1	It was identified from *T. harzianum* and *Alternaria alternate* PDB (Siddiquee et al., 2012; Encinas-Basurto et al., 2017)
Decane	2.81	17.7	It was found in *Capsicum annum* root extract, work against *Meloidogyne incognita* (Kihika et al., 2017) It was found in *Azadirachta indica* leaf extract (Rady, 2018)
Undecane	4.28	4.34	It is found as an active chemical compound of neem *Azadirachta indica* leaf extract (Rady, 2018)
Methylene-1-indene	6.65	0.94	It was found in many natural products and drug candidates with remarkable biological activities (Ye et al., 2010) It is used as a treatment of Alzheimer's disease (Koca et al., 2016)
Dodecane	6.86	5.73	It was found in *Capsicum annum* root extract against *Meloidogyne incognita* (Kihika et al., 2017) It was isolated from *T. longibrachiatum*, having antibacterial activity against *Bacillus subtilis* (Sarsaiya et al., 2020) It was isolated from *Cymbopogon nardus* and *Dysphania ambrosioides,* did not exhibit nematicidal effects (de Freitas Silva et al., 2020)
Bis-(dimethyl-ethyl)-phenol	18.34	7.04	It was found in at least 169 species of bacteria and fungi having antibacterial, insecticidal, and nematicidal activities against *Caenorhabditis elegans* (Zhao et al., 2020)
Ethyl-5-(2-ethylbutyl)-octadecane	21.62	0.18	It was found in *Salmali niryasa* having antimicrobial activity against *Klebsiella pneumoniae*, *Staphylococcus aureus*, *E. coli*, and *Candida albicans* (Divya and Prabhu, 2015)
Hexadecanol	35.14	4.86	It was isolated from *Satureja montana* oil, which has nematotoxic and phytotoxic effects (Faria et al., 2016)
Total		62.83	

TABLE 3 | Identification of metabolites in *A. thaumasia* and *T. cylindrosporum* using UPLC-QToF-ESI-MS analysis.

Metabolites (tentative)	Molecular formula	Neutral mass (Da)	Observed m/z	Error mass (ppm)	Adduct	Description
A. thaumasia						
Cyclo(l-Pro-l-Val)	$C_{10}H_{16}N_2O_2$	196.1212	196.1201	1.53	M^+	It was isolated from different fungi and bacteria as a secondary metabolite (Capon et al., 2007) It was isolated from entomopathogenic fungus *Cordyceps sinensis* (Yang et al., 2011)
Cyclo(l-Pro-l-Leu)	$C_{11}H_{18}N_2O_2$	210.1368	210.1373	2.38	M^+	It was produced by *Achromobacter xylosoxidans*, a bacterium that inhibits Aflatoxin production by *Aspergillus parasiticus* (Yan et al., 2004) It has been isolated from various bacterial and fungal species, including *Streptomyces* (Li et al., 2006)
Paganin A	$C_9H_{11}O_2$	151.0858	152.0940	−3.51	H^+	It was found in *Arthrobotrys entomopa,* enhance adhesive knobs formation (Degenkolb and Vilcinskas, 2016)
Talathermophilin E	$C_{18}H_{21}N_3O_2$	311.1634	312.1711	−3.86	H^+	It was found in *Talaromyces thermophilus,* having a nematicidal activity (Guo et al., 2011)
Dactylarin	$C_{16}H_{16}O_6$	304.0947	327.0921	−8.54	Na^+	It was isolated from *Dactylaria lutea,* having antiprotozoal activity against *Leishmania braziliensis* and *Entamoeba invadens* (Kettner et al., 1973) It was found in *Alternaria* PDB culture (Zheng et al., 2012)
Trichodepsipeptide A	$C_{36}H_{56}N_4O_9$	688.4047	711.4034	−1.19	Na^+	It was isolated from *Trichothecium* sp., showing weak antibacterial activity on *Staphylococcus epidermids* and *Enterococcus duran* (Amagata et al., 2006; Sy-Cordero et al., 2011) It was isolated from many fungi *viz., Acremonium*, *Alternaria*, *Aspergillus*, *Beauveria*, *Fusarium*, *Isaria*, *Metarhizium*, *Penicillium*, and *Rosellina*. Having various biological activities such as antimicrobial and insecticidal (Wang B.L. et al., 2018, Wang X. et al., 2018)
T. cylindrosporum						
Tolypocladenols A1/A2	$C_{21}H_{25}NO_4$	355.1784	356.1852	−5.35	H^+	They were isolated from *Tolypocladium cylindrosporum*, inhabits lichen *Lethariella zahlbruckneri* (Li et al., 2015) They were cytotoxic to human cancer cells (Kebede et al., 2017) They were isolated from *Acremonium* spp. and *Trichoderma hamatum* (Romano et al., 2018)
Tolypyridone A	$C_{21}H_{25}NO_3$	339.4350	363.4416	−6.19	$H^+ + Na^+$	
Tolypyridone B	$C_{15}H_{21}NO_2$	247.3380	248.3480	5.24	H^+	
Pyridoxatin	$C_{15}H_{21}NO_3$	263.1521	526.3010	−6.08	dimer	
Penicillenol B1/B2	$C_{16}H_{25}NO_3$	279.1834	280.1916	−1.79	H^+	They were identified from *Penicillium* spp. associated with *Aegiceras corniculatum* (Lin et al., 2008) They were isolated from *Aspergillus restrictus*, inhibited biofilm formation of pathogenic *Candida albicans* fungus (Wang X. et al., 2017)
Terpendole E	$C_{28}H_{39}NO_3$	437.2930	460.2962	7.31	Na^+	It was isolated from *Neotyphodium coenophialum* endophytic fungus, which decreased population of plant-parasitic nematodes (Takach and Young, 2014; Rogers et al., 2016)
Destruxin A	$C_{29}H_{47}N_5O_7$	577.3475	578.3556	−1.03	H^+	It was isolated from entomopathogenic fungus, *Metarhizium anisopliae* which has broad-spectrum insecticidal effects (Liu and Tzeng, 2012; Shakeel et al., 2018; Wang et al., 2020)
4-Chloro-2-phenylphenol	$C_{12}H_9ClO$	204.0342	205.0446	8.33	H^+	It was isolated as a plant secondary metabolite having nematicidal activity against *M. incognita* (Ohri and Pannu, 2010)
Acetamido-6-[(O-methyl-glucopyranosyl) oxy]anthraquinone	$C_{23}H_{24}NO_9$	458.1442	458.1451 481.1372	1.96	M^+ $H^+ + Na^+$	It was isolated from *Photorhabdus* spp. (symbiotic bacteria of entomopathogenic nematode) having an antimicrobial and mosquitocidal activity (Joyce et al., 2011; Ahn et al., 2013)

report of the presence of *A. thaumasia* in India. In the case of *Tolypocladium* isolate, conidia were hyaline, smooth-walled, short cylindrical, straight, or slightly curved, both ends obtusely rounded, and one-celled 2–4.3 × 1.3–1.7 μm, adhering to the phialide tips in slimy heads. Phialides were 4.5–8.5 × 2–3.2 μm in size and consisted of an inflated ellipsoidal to cylindrical base tapering abruptly to a thin neck, which often gives a bent appearance. All these features corresponded with the description of *T. cylindrosporum* (Bissett, 1983; Samson and Soares, 1984), and hence, we designated our isolate as *T. cylindrosporum* Tc_RK, which is also the first report from India. It is also important to emphasize that this isolate was from the dead nematodes, unlike the isolate used for comparison, which was from insects (Samson and Soares, 1984).

Morphological identification of the selected fungi was further supported by molecular characterization using two molecular

markers. In the case of ITS, the sequence *A. thaumasia* At_RK showed a high similarity to the already reported strain of *A. thaumasia* available in GenBank. Likewise, the sequence of *T. cylindrosporum* Tc_RK also showed maximum identity to the already reported strain of *T. cylindrosporum*. These findings confirmed the identity and presence of *A. thaumasia* and *T. cylindrosporum* in the Indian rhizospheric soils. Our results showed that analysis of molecular variation and maximum composite likelihood analysis using ITS and β-tubulin markers revealed a considerable degree of differentiation between geographical isolates. The results also demonstrate that the ITS and β-tubulin markers are useful for phylogenetic analysis and classification of *Arthrobotrys* and *Tolypocladium* species. Li et al. (2005) also studied phylogenies of NTFs deduced from sequence analyses of 5.8S rDNA, 28S rDNA, and β-tubulin genes and redefined the systematic classification of nematophagous fungi and amended the generic analysis of NTF based on types of trapping devices.

Furthermore, these isolates were studied under different media, incubation temperatures, and pH levels to analyze the growth rates and sporulation characteristics. The SNA media having pH 9 at 30°C showed the best growth rate, whereas RBA media supported high sporulation in the case of *A. thaumasia* At_RK. The results reported herein are in line with those of Wang F.H. et al. (2017), who obtained the highest growth at the optimal temperature at 30°C for the same fungus. In contrast, Fernandez et al. (1999) showed that *A. oligospora* exhibited the best growth rate at 20°C, and another fungus, *Duddingtonia flagrans*, grew at 10°C and formed trapping nets more slowly at this temperature when induced by nematodes. The Indian isolate of *A. thaumasia* At_RK that grew at optimum temperature at 30°C could be advantageous as a biological control agent in subtropical environments that remain at 30°C for a longer period. Additionally, the results of the current study match with the findings of Wang F.H. et al. (2017), who reported optimum growth of *A. thaumasia* on a media having pH 9 and 10. Contrastingly, there are no previous reports about the impact of different media, incubation temperatures, and pH levels on *T. cylindrosporum* growth and sporulation; however, the present study showed the best growth rate of *T. cylindrosporum* Tc_RK in the PDA media, having pH 6 at 20°C, whereas SNA medium supported high sporulation level. These optimized conditions for getting maximum fungal sporulation and growth could be highly useful for the large-scale production of these fungi in the future.

Most importantly, the utility of both fungi was primarily demonstrated by their ability to parasitize *M. incognita* and *C. elegans* under *in vitro* conditions. The selected fungal isolates were found to be effective against both the assessed nematodes after 3 days compared with control. Our results revealed that *A. thaumasia* showed significantly higher parasitism compared with *T. cylindrosporum* and water agar control plates ($p < 0.01$). Direct parasitism of the Indian isolate of *A. thaumasia* At_RK against *M. incognita* was comparatively higher (82%) than the efficacy obtained with the Korean isolate of *A. thaumasia* Nema-1 (55%) (Park et al., 2011). The finer details such as different types of traps and penetration in the region of lateral lines, etc., during *A. thaumasia* parasitizing *M. incognita* J2s was lacking, and the same has been documented for the first time in the present study.

The culture filtrate of *T. cylindrosporum* Tc_RK provided 87.3% mortality of *M. incognita* compared with control. So far, to the best of our knowledge, this is the first report about the ability of *T. cylindrosporum* to parasitize *M. incognita.* Interestingly, the results revealed that mortality of *C. elegans* caused by these two local fungal isolates was comparatively less, although *A. thaumasia* At_RK was isolated from dead *C. elegans*. The possible reason underlying lower mortality of *C. elegans* may be due to the fast movement that could have prevented them from immobilization and paralyzation, and/or body secretion of *M. incognita* could have attracted the trapping fungi relatively more.

During the *in vivo* evaluation of the isolated fungi against *M. incognita*-infected tomato, there was a significant increase in plant growth parameters compared with control plants infected with only nematodes. The tested isolates did not show any promotion in growth parameters compared with healthy control, but they enhanced plant growth in nematode-infected plants as a sign of protection provided against them. Further application of fungal filtrate of *A. thaumasia* At_RK caused a significant reduction in nematode disease burden per plant compared with control. Similar observations have been recorded by Park et al. (2012) using the culture filtrate of *A. thaumasia* on *M. incognita.* Likewise, the application of fungal suspensions of *T. cylindrosporum* Tc_RK in the present study caused a significant reduction in the number of egg masses and eggs per egg mass. This ultimately led to a decline in the nematode MF up to 94.5%. This is the first report on the effect of *T. cylindrosporum* Tc_RK on nematode fecundity.

The successful nematode mortality brought about by both the tested nematophagous fungi in the present study led us to analyze their metabolite profiles, particularly the volatile and non-volatile chemical compounds (VOCs) using GC–MS and UPLC-QToF-ESI-MS to identify the metabolites responsible for the nematicidal activity. The results showed that *A. thaumasia* At_RK secreted both volatile and non-volatile compounds that could be responsible for nematicides. Two volatile compounds, 2-methyl-1-pentanethiol and trimethyl-heptadien-4-one, were identified at higher concentrations in this isolate. The activity of these compounds as a repellent, odor character, insecticides, and nematicides have already been reported earlier by Huang et al. (2018) and Mravčáková et al. (2021). Similarly, the compounds, dodecadienal, undecane, and nerolic acid observed throughout metabolite profiles in the present study have already been reported as nematicidal, antibacterial, and antifungal compounds (Caboni et al., 2012; White et al., 2019; Sarsaiya et al., 2020). Correspondingly, *A. thaumasia* At_RK secreted non-volatile compounds such as aganing, talathermophilin, and dactylarin found in other nematophagous fungi, *A. entomopa*, *Talaromyces thermophilus*, and *Dactylaria lutea* (Kettner et al., 1973; Degenkolb and Vilcinskas, 2016; Wang X. et al., 2017). Interestingly, none of these compounds were detected during the analysis of 100 isolates belonging to three species *A. oligospora, A. thaumasia*, and *A. musiformis*, during their interaction with *C. elegans* (Kuo et al., 2020). Thus, this is the first

general metabolite profiling for *A. thaumasia* isolated from dead *M. incognita*, and hence, it could be promising for commercial exploitation in the future.

Likewise, our study with the Indian isolate of *T. cylindrosporum* Tc_RK showed secretion of volatile compounds, namely, methyl-hexadecanol, hexadecanol, decane, dodecane, and bis-(dimethyl-ethyl)-phenol, and the secreted compounds were reported to have antagonistic activity against nematodes (Faria et al., 2016; Kihika et al., 2017; Zhao et al., 2020). Other chemical compounds having nematicidal activities such as ethyl-3-methyl-benzene and undecane were also observed in the Indian isolate of *T. cylindrosporum* Tc_RK. These compounds were described to have nematicidal activities in the culture filtrate of *Trichoderma harzianum* and leaf extracts of *Azadirachta indica* (Encinas-Basurto et al., 2017; Rady, 2018). On the other hand, *T. cylindrosporum* Tc_RK also secreted non-volatile compounds with activity against nematodes, for instance, tolypocladenols, tolypyridone (A&B), and pyridoxatin, which were reported as metabolites from endolichenic isolate of *T. cylindrosporum*, *Acremonium* spp., and *Trichoderma hamatum* (Li et al., 2015; Romano et al., 2018). In addition to that, the tested isolate in the present study also secreted compounds such as terpendole E, 4-chloro-2-phenylphenol, destruxin A, and acetamido-6-anthraquinone, which were reported to have nematicidal activity (Ohri and Pannu, 2010; Ahn et al., 2013; Rogers et al., 2016; Shakeel et al., 2018; Wang et al., 2020) and not known to be present in *T. cylindrosporum*. It is also important to mention here that *T. cylindrosporum* Tc_RK isolated from dead *C. elegans* could be promising for nematode management due to its secretion of various novel metabolites with nematicidal properties.

CONCLUSION

Despite several reports being available for the efficacy of nematophagous fungi as biocontrol agents, the present investigation established an in-depth study on the Indian isolates of *A. thaumasia* and *T. cylindrosporum* for RKN management. Both these fungi are reported for the first time from India. Furthermore, this is the first report showing the potential of *T. cylindrosporum* for RKN management. Besides, this is also an established report revealing the presence of nematicidal compounds in both fungi using metabolite profiling. In view of the potential demonstrated for both the selected fungi against *M. incognita*, they can be further explored for commercial product development.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

UR conceived, designed, and supervised the experiments. AH and RK analyzed all the experimental data and wrote the original draft of the manuscript. AK analyzed GC–MS and UPLC-QToF-ESI-MS data. UR and AH revised and edited the manuscript. All authors conducted the experiments, read, and approved the final manuscript.

FUNDING

The authors also acknowledge the financial support received from the Indian Council for Cultural Relations for financial assistance to RK.

ACKNOWLEDGMENTS

We thank the Director and the Joint Director (Research), ICAR-Indian Agricultural Research Institute, New Delhi, India, for extending support and facilities to complete the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.790223/full#supplementary-material

REFERENCES

Ahn, J. Y., Lee, J. Y., Yang, E. J., Lee, Y. J., Koo, K. B., Song, K. S., et al. (2013). Mosquitocidal activity of anthraquinones isolated from symbiotic bacteria *Photorhabdus* of entomopathogenic nematode. *J. Asia Pac. Entomol.* 16, 317–320.

Al-Owaisi, M., Al-Hadiwi, N., and Khan, S. A. (2014). GC-MS analysis, determination of total phenolics, flavonoid content and free radical scavenging activities of various crude extracts of *Moringa peregrina* (Forssk.) Fiori leaves. *Asian Pac. J. Trop. Med.* 4, 964–970.

Al-Rubaye, A. F., Kaizal, A. F., and Hameed, I. H. (2017). Phytochemical screening of methanolic leaves extract of *Malva sylvestris*. *Int. J. Pharmacogn. Phytochem. Res.* 9, 537–552.

Amagata, T., Morinaka, B. I., Amagata, A., Tenney, K., Valeriote, F. A., Lobkovsky, E., et al. (2006). A chemical study of cyclic depsipeptides produced by a sponge-derived fungus. *J. Nat. Prod.* 69, 1560–1565. doi: 10.1021/np060178k

Banakar, P., Hada, A., Papolu, P. K., and Rao, U. (2020). Simultaneous RNAi knockdown of three FMRFamide-like peptide genes, *Mi-flp1, Mi-flp12*, and *Mi-flp18* provides resistance to root-knot nematode, *Meloidogyne incognita*. *Front. Microbiol.* 11:2690. doi: 10.3389/fmicb.2020.573916

Barron, G. L. (2003). Predatory fungi, wood decay, and the carbon cycle. *Biodiversity* 4, 3–9. doi: 10.1080/14888386.2003.9712621

Bissett, J. (1983). Notes on Tolypocladium and related genera. *Can. J. Bot.* 61, 1311–1329. doi: 10.1139/b83-139

Caboni, P., Ntalli, N. G., Aissani, N., Cavoski, I., and Angioni, A. (2012). Nematicidal activity of (E, E)-2, 4-decadienal and (E)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*. *J. Agric. Food Chem.* 60, 1146–1151. doi: 10.1021/jf2044586

Capon, R. J., Stewart, M., Ratnayake, R., Lacey, E., and Gill, J. H. (2007). Citromycetins and bilains A–C: new aromatic polyketides and diketopiperazines from Australian marine-derived and terrestrial *Penicillium* spp. *J. Nat. Prod.* 70, 1746–1752. doi: 10.1021/np0702483

Chen, Q. H., Zhu, F., Tian, Z., Zhang, W. M., Guo, R., Liu, W., et al. (2018). Minor components play an important role in interspecific recognition of insects: a basis to pheromone based electronic monitoring tools for rice pests. *Insects* 9:192. doi: 10.3390/insects9040192

Contina, J. B., Dandurand, L. M., and Knudsen, G. R. (2017). Use of GFP-tagged Trichoderma harzianum as a tool to study the biological control of the potato cyst nematode Globodera pallida. *Appl. Soil Ecol.* 115, 31–37. doi: 10.1016/j.apsoil.2017.03.010

Daneshkazemi, A., Zandi, H., Davari, A., Vakili, M., Emtiazi, M., Lotfi, R., et al. (2019). Antimicrobial activity of the essential oil obtained from the seed and oleo-gum-resin of Ferula assa-foetida against oral pathogens. *Front. Dent.* 16, 113–120. doi: 10.18502/fid.v16i2.1362

de Freitas Silva, M., Campos, V. P., Barros, A. F., da Silva, J. C. P., Pedroso, M. P., de Jesus Silva, F., et al. (2020). Medicinal plant volatiles applied against the root-knot nematode *Meloidogyne incognita*. *Crop Prot.* 130:105057.

Degenkolb, T., and Vilcinskas, A. (2016). Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes. *Appl. Microbiol. Biotechnol.* 100, 3799–3812. doi: 10.1007/s00253-015-7233-6

Den Belder, E., Boekestein, A., Van Esch, J. W. J., and Thiel, F. (1993). Low-temperature scanning electron microscopy in fungus-nematode interaction. *Scanning* 15, 37–42. doi: 10.1002/sca.4950150106

Divya, D., and Prabhu, K. (2015). GC-MS analysis, antimicrobial, antioxidant activity of an Ayurvedic medicine, Salmali Niryasa. *J. Chem. Pharm. Res.* 7, 131–139.

Elling, A. A. (2013). Major emerging problems with minor *Meloidogyne* species. *Phytopathology* 103, 1092–1102. doi: 10.1094/PHYTO-01-13-0019-RVW

Encinas-Basurto, D., Valenzuela-Quintanar, M. I., Sánchez-Estrada, A., Tiznado-Hernández, M. E., Rodríguez-Félix, A., and Troncoso-Rojas, R. (2017). Alterations in volatile metabolites profile of fresh tomatoes in response to *Alternaria alternata* (Fr.) Keissl. 1912 infection. *Chil. J. Agric. Res.* 77, 194–201. doi: 10.4067/s0718-58392017000300194

Escudero, N., Lopez-Moya, F., Ghahremani, Z., Zavala-Gonzalez, E. A., Alaguero-Cordovilla, A., Ros-Ibañez, C., et al. (2017). Chitosan increases tomato root colonization by *Pochonia chlamydosporia* and their combination reduces root-knot nematode damage. *Front. Plant. Sci.* 8:1415. doi: 10.3389/fpls.2017.01415

Faria, J. M., Rodrigues, A. M., Sena, I., Moiteiro, C., Bennett, R. N., Mota, M., et al. (2016). Bioactivity of *Ruta graveolens* and *Satureja montana* essential oils on *Solanum tuberosum* hairy roots and *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures. *J. Agric. Food Chem.* 64, 7452–7458. doi: 10.1021/acs.jafc.6b03279

Fernandez, A. S., Larsen, M., Nansen, P., Henningsen, E., Grønvold, J., Wolstrup, J., et al. (1999). The ability of the nematode-trapping fungus *Duddingtonia flagrans* to reduce the transmission of infective *Ostertagia ostertagi* larvae from faeces to herbage. *J. Helminthol.* 73, 115–122. doi: 10.1017/s0022149x99000177

Glass, N. L., and Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61, 1323–1330. doi: 10.1128/aem.61.4.1323-1330.1995

Guo, J. P., Tan, J. L., Wang, Y. L., Wu, H. Y., Zhang, C. P., Niu, X. M., et al. (2011). Isolation of talathermophilins from the thermophilic fungus Talaromyces thermophilus YM3-4. *J. Nat. Prod.* 74, 2278–2281. doi: 10.1021/np200365z

Hada, A., Kumari, C., Phani, V., Singh, D., Chinnusamy, V., and Rao, U. (2020). Host-induced silencing of FMRFamide-like peptide genes, flp-1 and flp-12, in rice impairs reproductive fitness of the root-knot nematode *Meloidogyne graminicola*. *Front. Plant. Sci.* 11:894. doi: 10.3389/fpls.2020.00894

Hada, A., Singh, D., Papolu, P. K., Banakar, P., Raj, A., and Rao, U. (2021b). Host-mediated RNAi for simultaneous silencing of different functional groups of genes in *Meloidogyne incognita* using fusion cassettes in *Nicotiana tabacum*. *Plant Cell Rep.* 40, 2287–2302.

Hada, A., Singh, D., Satyanarayana, K. K. V. V., Chatterjee, M., Phani, V., and Rao, U. (2021c). Effect of fluensulfone on different functional genes of root-knot nematode *Meloidogyne incognita*. *J. Nematol.* 53:e2021-73. doi: 10.21307/jofnem-2021-073

Hada, A., Patil, B. L., Bajpai, A., Kesiraju, K., Dinesh-Kumar, S., Paraselli, B., et al. (2021a). Micro RNA-induced gene silencing strategy for the delivery of siRNAs targeting *Meloidogyne incognita* in a model plant *Nicotiana benthamiana*. *Pest Manag. Sci.* 77, 3396–3405. doi: 10.1002/ps.6384

Huang, X., Ge, S. Y., Liu, J. H., Wang, Y., Liang, X. Y., and Yuan, H. B. (2018). Chemical composition and bioactivity of the essential oil from *Artemisia lavandulaefolia* (Asteraceae) on *Plutella xylostella* (Lepidoptera: Plutellidae). *Fla. Entomol.* 101, 44–48. doi: 10.1653/024.101.0109

Joyce, S. A., Lango, L., and Clarke, D. J. (2011). The regulation of secondary metabolism and mutualism in the insect pathogenic bacterium *Photorhabdus luminescens*. *Adv. Appl. Microbiol.* 76, 1–25. doi: 10.1016/B978-0-12-387048-3.00001-5

Kassam, R., Kamil, D., and Rao, U. (2021). *Caenorhabditis elegans* as bait for isolating promising biocontrol fungi against *Meloidogyne incognita* from soils across India. *Indian Phytopathol.* 74, 739–752.

Kebede, B., Wrigley, S. K., Prashar, A., Rahlff, J., Wolf, M., Reinshagen, J., et al. (2017). Establishing the secondary metabolite profile of the marine fungus: *Tolypocladium geodes* sp. MF458 and subsequent optimisation of bioactive secondary metabolite production. *Mar. Drugs* 15:84. doi: 10.3390/md15040084

Kettner, M., Nemec, P., Kovac, S., and Balanova, J. (1973). Dactylarin, a new antiprotozoal antibiotic from *Dactylaria lutea*. *J. Antibiot.* 26, 692–696.

Kihika, R., Murungi, L. K., Coyne, D., Hassanali, A., Teal, P. E., and Torto, B. (2017). Parasitic nematode *Meloidogyne incognita* interactions with different *Capsicum annum* cultivars reveal the chemical constituents modulating root herbivory. *Sci. Rep.* 7:2903. doi: 10.1038/s41598-017-02379-8

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111–120. doi: 10.1007/bf01731581

Koca, M., Yerdelen, K. O., Anil, B., Kasap, Z., Sevindik, H., Ozyurek, I., et al. (2016). Design, synthesis and biological activity of 1H-indene-2-carboxamides as multi-targeted anti-Alzheimer agents. *J. Enzyme Inhib. Med. Chem.* 31, 13–23. doi: 10.1080/14756366.2016.1186019

Kuo, T. H., Yang, C. T., Chang, H. Y., Hsueh, Y. P., and Hsu, C. C. (2020). Nematode-trapping fungi produce diverse metabolites during predator–prey interaction. *Metabolites* 10:117. doi: 10.3390/metabo10030117

Li, X., Dobretsov, S., Xu, Y., Xiao, X., Hung, O. S., and Qian, P. Y. (2006). Antifouling diketopiperazines produced by a deep-sea bacterium, *Streptomyces fungicidicus*. *Biofouling* 22, 187–194. doi: 10.1080/08927010600780771

Li, X. B., Li, L., Zhu, R. X., Li, W., Chang, W. Q., Zhang, L. L., et al. (2015). Tetramic acids and pyridone alkaloids from the endolichenic fungus *Tolypocladium cylindrosporum*. *J. Nat. Prod.* 78, 2155–2160. doi: 10.1021/np501018w

Li, Y., Hyde, K. D., Jeewon, R., Cai, L., Vijaykrishna, D., and Zhang, K. (2005). Phylogenetics and evolution of nematode-trapping fungi (Orbiliales) estimated from nuclear and protein coding genes. *Mycologia* 97, 1034–1046. doi: 10.3852/mycologia.97.5.1034

Lin, Z. J., Lu, Z. Y., Zhu, T. J., Fang, Y. C., Gu, Q. Q., and Zhu, W. M. (2008). Penicillenols from *Penicillium* sp. GQ-7, an endophytic fungus associated with *Aegiceras corniculatum*. *Chem. Pharm. Bull.* 56, 217–221. doi: 10.1248/cpb.56.217

Liu, B. L., and Tzeng, Y. M. (2012). Development and applications of destruxins: a review. *Biotechnol. Adv.* 30, 1242–1254. doi: 10.1016/j.biotechadv.2011.10.006

Matheny, R. W. (1980). "Federally registered pesticides for vertebrate pest control," in *Proceedings of the 1980 Vertebrate Pest Conference*, Davis, CA.

Mravčáková, D., Sobczak-Filipiak, M., Váradyová, Z., Kucková, K., Čobanová, K., Maršík, P., et al. (2021). Effect of *Artemisia absinthium* and *Malva sylvestris* on Antioxidant Parameters and Abomasal Histopathology in Lambs Experimentally Infected with *Haemonchus contortus*. *Animals* 11:462. doi: 10.3390/ani11020462

Niu, X. M., and Zhang, K. Q. (2011). *Arthrobotrys oligospora*: a model organism for understanding the interaction between fungi and nematodes. *Mycology* 2, 59–78. doi: 10.1080/21501203.2011.562559

Ohri, P., and Pannu, S. K. (2010). Effect of phenolic compounds on nematodes-A review. *J. Appl. Nat. Sci.* 2, 344–350. doi: 10.31018/jans.v2i2.144

Oka, Y., Shapira, N., and Fine, P. (2007). Control of root-knot nematodes in organic farming systems by organic amendments and soil solarization. *Crop Prot.* 26, 1556–1565. doi: 10.1016/j.cropro.2007.01.003

Park, M. H., Kim, J. K., Choi, W. H., and Yoon, M. H. (2011). Nematicidal effect of root-knot nematode (*Meloidogyne incognita*) by biological nematicide. *Korean J. Soil Sci. Fertil.* 44, 228–235. doi: 10.7745/kjssf.2011.44.2.228

Park, M. H., Walpola, B. C., Kim, S. J., and Yoon, M. H. (2012). Control effect of root-knot nematode (*Meloidogyne incognita*) by biological nematicide. *Korean J. Soil Sci. Fertil.* 45, 162–168. doi: 10.7745/kjssf.2012.45.2.162

Ploeg, A., Stoddard, S., and Becker, J. O. (2019). Control of *Meloidogyne incognita* in sweetpotato with fluensulfone. *J. Nematol.* 51, 1–8. doi: 10.21307/jofnem-2019-018

Poveda, J., Abril-Urias, P., and Escobar, C. (2020). Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, mycorrhizal and endophytic fungi. *Front. Microbiol.* 11:992. doi: 10.3389/fmicb.2020.00992

Rady, M. M. (2018). The efficiency of some natural alternatives in root-knot nematode control. *Adv. Plants Agric. Res.* 8, 355–362.

Regmi, H., and Desaeger, J. (2020). Integrated management of root-knot nematode (*Meloidogyne* spp.) in Florida tomatoes combining host resistance and nematicides. *Crop Prot.* 134:105170. doi: 10.1016/j.cropro.2020.105170

Rogers, J. K., Walker, N. R., and Young, C. A. (2016). The effect of endophytic fungi on nematode populations in summer-dormant and summer-active tall fescue. *J. Nematol.* 48, 87–94. doi: 10.21307/jofnem-2017-013

Romano, S., Jackson, S. A., Patry, S., and Dobson, A. D. (2018). Extending the "one strain many compounds" (OSMAC) principle to marine microorganisms. *Mar. Drugs* 16:244. doi: 10.3390/md16070244

Ruiz-Suárez, N., Boada, L. D., Henríquez-Hernández, L. A., González-Moreo, F., Suárez-Pérez, A., Camacho, M., et al. (2015). Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain). *Sci. Total Environ.* 505, 1093–1099. doi: 10.1016/j.scitotenv.2014.10.093

Saha, S., Walia, S., Kundu, A., Kaur, C., Singh, J., and Sisodia, R. (2015a). Capsaicinoids, tocopherol, and sterols content in chili (*Capsicum* sp.) by gas chromatographic-mass spectrometric determination. *Int. J. Food Prop.* 18, 1535–1545.

Saha, S., Walia, S., Kundu, A., Sharma, K., and Paul, R. K. (2015b). Optimal extraction and fingerprinting of carotenoids by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food Chem.* 177, 369–375. doi: 10.1016/j.foodchem.2015.01.039

Samson, R. A., and Soares, G. G. Jr. (1984). Entomopathogenic species of the hyphomycete genus *Tolypocladiu*m. *J. Invertebr. Pathol.* 43, 133–139. doi: 10.1016/0022-2011(84)90130-7

Sarsaiya, S., Jain, A., Fan, X., Jia, Q., Xu, Q., Shu, F., et al. (2020). New insights into detection of a dendrobine compound from a novel endophytic *Trichoderma longibrachiatum* strain and its toxicity against phytopathogenic bacteria. *Front. Microbiol.* 11:337. doi: 10.3389/fmicb.2020.00337

Schouten, A. (2016). Mechanisms involved in nematode control by endophytic fungi. *Annu. Rev. Phytopathol.* 54, 121–142.

Shakeel, M., Xu, X., Xu, J., Li, S., Yu, J., Zhou, X., et al. (2018). Genome-wide identification of Destruxin A-responsive immunity-related microRNAs in diamondback moth, *Plutella xylostella*. *Front. Immunol.* 9:185. doi: 10.3389/fimmu.2018.00185

Siddiquee, S., Cheong, B. E., Taslima, K., Kausar, H., and Hasan, M. M. (2012). Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. *J. Chromatogr. Sci.* 50, 358–367. doi: 10.1093/chromsci/bms012

Siddiqui, I. A., and Shaukat, S. S. (2004). *Trichoderma harzianum* enhances the production of nematicidal compounds *in vitro* and improves biocontrol of *Meloidogyne javanica* by *Pseudomonas fluorescens* in tomato. *Lett. Appl. Microbiol.* 38, 169–175. doi: 10.1111/j.1472-765x.2003.01481.x

Sung, G. H., Hywel-Jones, N. L., Sung, J. M., Luangsa-Ard, J. J., Shrestha, B., and Spatafora, J. W. (2007). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud. Mycol.* 57, 5–59. doi: 10.3114/sim.2007.57.01

Swe, A., Jeewon, R., Pointing, S. B., and Hyde, K. D. (2008). Taxonomy and molecular phylogeny of Arthrobotrys mangrovispora, a new marine nematode-trapping fungal species. *Bot. Mar.* 51, 331–338.

Sy-Cordero, A. A., Graf, T. N., Adcock, A. F., Kroll, D. J., Shen, Q., Swanson, S. M., et al. (2011). Cyclodepsipeptides, sesquiterpenoids, and other cytotoxic metabolites from the filamentous fungus *Trichothecium* sp. (MSX 51320). *J. Nat. Prod.* 74, 2137–2142. doi: 10.1021/np2004243

Takach, J. E., and Young, C. A. (2014). Alkaloid genotype diversity of tall fescue endophytes. *Crop Sci.* 54, 667–678. doi: 10.2135/cropsci2013.06.0423

Tian, B., Yang, J., and Zhang, K. Q. (2007). Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. *FEMS Microbiol. Ecol.* 61, 197–213.

Wang, B. B., Liu, W., Chen, M. Y., Li, X., Han, Y., Xu, Q., et al. (2015). Isolation and characterization of China isolates of *Duddingtonia flagrans*, a candidate of the nematophagous fungi for biocontrol of animal parasitic nematodes. *J. Parasitol.* 101, 476–484. doi: 10.1645/14-715.1

Wang, B. L., Chen, Y. H., He, J. N., Xue, H. X., Yan, N., Zeng, Z. J., et al. (2018). Integrated metabolomics and morphogenesis reveal volatile signaling of the nematode-trapping fungus *Arthrobotrys oligospora*. *Appl. Environ. Microbiol.* 84:e2749-17. doi: 10.1128/AEM.02749-17

Wang, X., Gong, X., Li, P., Lai, D., and Zhou, L. (2018). Structural diversity and biological activities of cyclic depsipeptides from fungi. *Molecules* 23:169.

Wang, F. H., Xu, Q., Wang, B. B., Wang, K. Y., Xue, Y. J., Cai, B., et al. (2017). Isolation, identification and characterisation of the nematophagous fungus *Arthrobotrys thaumasia* (*Monacrosporium thaumasium*) from China. *Biocontrol Sci. Technol.* 27, 378–392.

Wang, X., Li, Y., Zhang, X., Lai, D., and Zhou, L. (2017). Structural diversity and biological activities of the cyclodipeptides from fungi. *Molecules* 22:2026. doi: 10.3390/molecules22122026

Wang, J., Weng, Q., Yin, F., and Hu, Q. (2020). Interactions of Destruxin A with Silkworms' Arginine tRNA Synthetase and Lamin-C Proteins. *Toxins* 12:137. doi: 10.3390/toxins12020137

White, L. A. S., Blank, A. F., Gagliardi, P. R., de Fátima Arrigoni-Blank, M., de Castro Nizio, D. A., Sampaio, T. S., et al. (2019). *In vitro* antifungal activity of Myrcia ovata essential oils and their major compounds against pathogens of citrus, sweet potato, and coconut. *Biosci. J.* 35, 1695–1707.

Whitehead, A. G., and Hemming, J. R. (1965). A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Ann. Appl. Biol.* 55, 25–38.

Wright, D. A., Cummings, N. J., Haack, N. A., and Jackson, T. A. (2009). *Tolypocladium cylindrosporum*, a novel pathogen for sheep blowflies. *N. Z. J. Agric. Res.* 52, 315–321. doi: 10.1080/00288230909510516

Yan, P. S., Song, Y., Sakuno, E., Nakajima, H., Nakagawa, H., and Yabe, K. (2004). Cyclo (L-leucyl-L-prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* 70, 7466–7473. doi: 10.1128/AEM.70.12.7466-7473.2004

Yang, M. L., Kuo, P. C., Hwang, T. L., and Wu, T. S. (2011). Anti-inflammatory principles from *Cordyceps sinensis*. *J. Nat. Prod.* 74, 1996–2000. doi: 10.1021/np100902f

Ye, S., Ren, H., and Wu, J. (2010). Efficient Assembly of 1-Methylene-1 H-indenes via Palladium-Catalyzed Tandem Reaction of 1-(2, 2-Dibromovinyl)-2-alkenylbenzene with Arylboronic Acid. *J. Comb. Chem.* 12, 670–675. doi: 10.1021/cc1000693

Zhang, H., Wei, Z., Zhang, J., and Liu, X. (2021). Classification of dendrocola nematode-trapping fungi. *J. For. Res.* 32, 1295–1304.

Zhang, Y., Qiao, M., Weber, E., Baral, H. O., Hagedorn, G., and Yu, Z. (2010). *Arthrobotrys scaphoides* from China and Europe with a phylogenetic analysis including the type strain. *Mycotaxon* 111, 291–300.

Zhao, F., Wang, P., Lucardi, R. D., Su, Z., and Li, S. (2020). Natural sources and bioactivities of 2, 4-di-tert-butylphenol and its analogs. *Toxins* 12:35.

Zheng, C. J., Shao, C. L., Guo, Z. Y., Chen, J. F., Deng, D. S., Yang, K. L., et al. (2012). Bioactive hydroanthraquinones and anthraquinone dimers from a soft coral-derived *Alternaria* sp. fungus. *J. Nat. Prod.* 75, 189–197. doi: 10.1021/np200766d

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 December 2021
doi: 10.3389/fmicb.2021.774707

Root-Derived Endophytic Diazotrophic Bacteria *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1 Promote Nitrogen Assimilation and Growth in Sugarcane

*Rajesh Kumar Singh[1,2], Pratiksha Singh[1,2,3], Dao-Jun Guo[1,2,4], Anjney Sharma[1,2], Dong-Ping Li[5], Xiang Li[2], Krishan K. Verma[1,2], Mukesh Kumar Malviya[1,2], Xiu-Peng Song[2], Prakash Lakshmanan[1,2,6,7], Li-Tao Yang[2,4] and Yang-Rui Li[1,2,4]**

[1] Key Laboratory of Sugarcane Biotechnology and Genetic Improvement (Guangxi), Ministry of Agriculture, Sugarcane Research Center, Chinese Academy of Agricultural Sciences, Guangxi Key Laboratory of Sugarcane Genetic Improvement, Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China, [2] Guangxi Key Laboratory of Crop Genetic Improvement and Biotechnology, Nanning, China, [3] School of Marine Sciences and Biotechnology, Guangxi University for Nationalities, Nanning, China, [4] State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bio Resources, College of Agriculture, Guangxi University, Nanning, China, [5] Microbiology Institute, Guangxi Academy of Agricultural Sciences, Nanning, China, [6] Interdisciplinary Center for Agriculture Green Development in Yangtze River Basin, College of Resources and Environment, Southwest University, Chongqing, China, [7] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD, Australia

OPEN ACCESS

Edited by:
Prem Lal Kashyap, Indian Institute of Wheat and Barley Research (ICAR), India

Reviewed by:
Sajid Ali, Yeungnam University, South Korea
Shyam Lal Kandel, University of Washington, United States

***Correspondence:**
Yang-Rui Li
liyr@gxaas.net

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *12 September 2021*
Accepted: *12 November 2021*
Published: *15 December 2021*

Citation:
Singh RK, Singh P, Guo D-J, Sharma A, Li D-P, Li X, Verma KK, Malviya MK, Song X-P, Lakshmanan P, Yang L-T and Li Y-R (2021) Root-Derived Endophytic Diazotrophic Bacteria Pantoea cypripedii AF1 and Kosakonia arachidis EF1 Promote Nitrogen Assimilation and Growth in Sugarcane. Front. Microbiol. 12:774707. doi: 10.3389/fmicb.2021.774707

Excessive, long-term application of chemical fertilizers in sugarcane crops disrupts soil microbial flora and causes environmental pollution and yield decline. The role of endophytic bacteria in improving crop production is now well-documented. In this study, we have isolated and identified several endophytic bacterial strains from the root tissues of five sugarcane species. Among them, eleven Gram-negative isolates were selected and screened for plant growth-promoting characteristics, i.e., production of siderophores, indole-3-acetic acid (IAA), ammonia, hydrogen cyanide (HCN), and hydrolytic enzymes, phosphorus solubilization, antifungal activity against plant pathogens, nitrogen-fixation, 1-aminocyclopropane-1-carboxylic acid deaminase activity, and improving tolerance to different abiotic stresses. These isolates had *nifH* (11 isolates), *acdS* (8 isolates), and *HCN* (11 isolates) genes involved in N-fixation, stress tolerance, and pathogen biocontrol, respectively. Two isolates *Pantoea cypripedii* AF1and *Kosakonia arachidis* EF1 were the most potent strains and they colonized and grew in sugarcane plants. Both strains readily colonized the leading Chinese sugarcane variety GT42 and significantly increased the activity of nitrogen assimilation enzymes (glutamine synthetase, NADH glutamate dehydrogenase, and nitrate reductase), chitinase, and endo-glucanase and the content of phytohormones gibberellic acid, indole-3-acetic acid, and abscisic acid. The gene expression analysis of GT42 inoculated with isolates of *P. cypripedii* AF1 or *K. arachidis* EF1 showed increased activity of *nifH* and nitrogen assimilation genes. Also, the inoculated diazotrophs significantly increased plant nitrogen content, which was corroborated by the ^{15}N

isotope dilution analysis. Collectively, these findings suggest that *P. cypripedii* and *K. arachidis* are beneficial endophytes that could be used as a biofertilizer to improve plant nitrogen nutrition and growth of sugarcane. To the best of our knowledge, this is the first report of sugarcane growth enhancement and nitrogen fixation by Gram-negative sugarcane root-associated endophytic bacteria *P. cypripedii* and *K. arachidis*. These strains have the potential to be utilized as sugarcane biofertilizers, thus reducing nitrogen fertilizer use and improving disease management.

Keywords: antifungal activity, endophytes, nitrogen fixation, *Pantoea cypripedii*, *Kosakonia arachidis*, PGP, colonization, sugarcane

INTRODUCTION

Global food security is a major sustainable development goal of United Nations. This is also a major challenge for developing countries with limited resources and scientific capacity. Agriculture is a major consumer of energy and cause of environmental damages, mainly due to the large input of fertilizers, land use practices, and use of fossil fuel (Fraser and Campbell, 2019; Yang et al., 2021). Sustainable intensification of agriculture is a well-recognized concept and is being practiced in the developed world (Tilman et al., 2002; Vanlauwe et al., 2019). It improves resource use efficiency, reduces agricultural inputs, especially fertilizers and other agri-chemicals, and expansion of mechanized farm operations. In this context, considerable research to understand and exploit soil and rhizosphere microbiomes and plant endophytes to reduce fertilizer input and suppress pathogens are now underway globally. This is particularly relevant when 60–90% of the applied chemical fertilizers are wasted depending on the crop and region, and the manufacturing of agri-chemicals like nitrogen (N) fertilizers is a highly energy-intensive process (Bhardwaj et al., 2014). Biofertilizers, which are live cells of microbes, are a potential alternative to chemical fertilizers since they provide nutrients to plants, reduce soil-borne diseases, and improve the health and quality of soil (Bhardwaj et al., 2014; Kour et al., 2020).

Sugarcane (*Saccharum* spp. interspecific hybrids), a member of Poaceae, is a major commercial crop grown in the tropical and subtropical areas of the world. Due to its large contribution to sugar production, manufacture of ethanol, and a source of environmentally sustainable green energy, it is an economically important crop worldwide (Vital et al., 2017). It accounts for more than 80% of global sugar production, with Brazil, India, China, and Thailand accounting for 60% of total output (FAO, 2020a). Global sugar output was around 166.18 million metric tons during the 2019–2020 (United States Department of Agriculture [USDA], 2020). China is the world's third largest producer of sugar, with an annual output of over 13 million tons. Sugarcane accounts for more than 90% of sugar production in China, with Guangxi province accounting for over 65% of total production (Li et al., 2016). Chinese sugarcane crop productivity, however, is lower than the world average. This is largely caused by sub-optimal crop production management and widespread occurrence of diseases and pests (Li et al., 2016). Endophytic bacteria, which survive and grow inside plant tissue, have been extensively studied for control of diseases and amelioration of stresses in a variety of plants (Kumar et al., 2017). The use of plant growth-promoting endophytic bacteria (PGPEB), can therefore be an effective tool for improving crop growth and productivity under non-stresses as well as challenging environmental conditions, including poor soil fertility.

Nitrogen fixation is an important source of N for many crops and both plant growth-promoting bacteria (PGPB) growing on the root surface and PGPEB facilitate plant N availability (Sur et al., 2010; Leghari et al., 2016; Oleńska et al., 2020). The crop productivity depends on N, and use of inorganic N for crop production is still increasing at a global scale (FAO, 2020b) and it undermines efforts to mitigate climate change. The utilization of biological N fixation (BNF) to minimize the input of N fertilizers in sugarcane has been reported, though the BNF microbes are not well characterized. Different bacterial genera such as *Azotobacter*, *Azospirillum, Bacillus*, *Pseudomonas*, and *Enterobacter*, have been shown to be associated with BNF (Martins et al., 2020). The use of PGPEB to improve crop production may have advantages over epiphytic and rhizosphere bacteria as they are more vulnerable to soil and other external conditions (James, 2000). These bacteria produce several phytohormones (auxin, cytokinin, ethylene, and gibberellins) and growth-enhancing compounds (siderophore, hydrogen cyanide, fixed N, and hydrolyzing enzymes) and can be used as biofertilizers (Stefan et al., 2013; Mihalache et al., 2015; Li et al., 2017; Raklami et al., 2019). Our previous research showed BNF in certain varieties of commercially grown sugarcane, and we have identified some rhizobacteria involved in BNF from those varieties (Li et al., 2017; Singh et al., 2020b,c). This research is now extended to study the endophytic bacteria from sugarcane roots, to assess their plant growth-promoting (PGP) potential, including their ability for BNF.

Here, we report the isolation and characterization of non-pathogenic endophytic *Pantoea* and *Kosakonia* diazotrophic bacteria colonized in sugarcane roots. The genera *Pantoea* and *Kosakonia,* belong to Enterobacteriaceae family, are non-spore-forming, rod-shaped, and Gram-negative bacteria (Tambong, 2019). Members of *Pantoea* and *Kosakonia* genera are known to interact and elicit beneficial effects on plant growth (Brock et al., 2018; Chen and Liu, 2019; Romano et al., 2020; Singh et al., 2020b, 2021b; Suman et al., 2020), but little is known about their potential for disease control and N-fixation in sugarcane. Considering the widespread occurrence of diseases and the large reduction in N input required in sugarcane crops in China, we

studied the disease control and BNF properties of members of *Pantoea* and *Kosakonia* genera, and the results are presented here.

MATERIALS AND METHODS

Sampling and Endophytic Bacteria Isolation

Five sugarcane species, *Saccharum officinarum* L. cv Badila, *Saccharum barberi* Jesw. cv Pansahi, *Saccharum robustum*, *Saccharum spontaneum*, and *Saccharum sinense* Roxb. cv Uba were used for this study. All plants were obtained from the experimental farm of Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi (latitude 22° 50′ N, longitude 108° 14′ E, and elevation 70 m), China. The climate in the study region was humid subtropical, with an annual mean temperature of 21.83°C and 1,290 mm of rainfall. At the plant elongation stage, root samples were taken for the isolation of endophytic bacteria according to Dobereiner et al. (1993) method. The soil adhering to the roots was cleaned by thoroughly washing them with tap water, and then rinsed with sterile distilled water, 75% ethanol (5 min), and sodium hypochlorite solution (3% for 5 min). Five root samples were chosen from each sugarcane species. The root samples with white tips, which indicated continued growth, were used to isolate bacteria. Root pieces (1 gm per clone) were transferred to cold, sterilized mortal and pestles, and crushed with 1 mL of sterile 5% sucrose solution. An aliquot of 100 μL from each sample was spread on the different medium (**Supplementary Table 1**) and incubated for 3–5 days at 30 ± 2°C. After incubation, morphologically different bacterial colonies were chosen. The isolated strains were kept at −20°C in a 25% glycerol solution.

Identification of Endophytic Isolates and Analysis of *nifH, acdS*, and Hydrogen Cyanide Biosynthetic Genes

The strains were identified by analyzing their 16S *rRNA* gene sequences amplified from genomic DNA using universal primers pA and pH (**Supplementary Table 2**) (Edwards et al., 1989), and conditions as described by Singh et al. (2021b). Briefly, pure bacterial cultures were grown in Luria-Bertani (LB) broth for 48 h on a shaker incubator and maintained at 32 ± 2°C for 160 rpm. Genomic DNA was isolated from 1.5 mL broth culture using a DNA extraction kit (CWBIO, Beijing-China) following the manufacturer's instructions. The PCR amplification of the 16S *rRNA* gene was completed and the amplified PCR products were purified by using a BioFlux kit (Hangzhou, China) and then sequenced by Sangon Biotech (Shanghai, China). Phylogenetic analysis was performed to verify identities and determine the evolutionary relationship of the isolates with reference strains from the GenBank public database and aligned by ClustalW. Phylogenetic trees were created by MEGAX for the 16S *rRNA* gene (Kumar et al., 2018) via Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were calculated by using the neighbor-joining method (Nei and Kumar, 2000). The bootstrap study (1,000 replicates) was carried out as described earlier (Felsenstein, 1985).

The primer sequences shown in **Supplementary Table 2** were used for the amplification of *nifH* (Poly et al., 2001), *acdS* (Li et al., 2011), and HCN (Ramette et al., 2003) for all selected strains.

Acetylene Reduction Assay and 1-Aminocyclopropane-1-Carboxylate Deaminase Activity

In vivo nitrogenase activity of all bacterial isolates was determined by acetylene reduction assay (ARA) by inoculating pure culture in 10 mL of McCartney vial comprising semi-solid N-free medium and incubated at 30 ± 2°C for 48 h (Hardy et al., 1968).

1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity of bacterial isolates was measured based on their capability to use ACC (3 mM) as a sole N source in the Dworkin and Foster (DF) salt minimal medium (Penrose and Glick, 2003). All strains were spot inoculated on (i) Petri plates comprising DF salts minimal medium containing ACC, (ii) DF minimal medium without of ACC (negative control), and (iii) DF minimal medium with $(NH_4)_2SO_4$ (2 g L^{-1}) (positive control). And, growth was compared with controls after incubation at 32 ± 2°C for 5–7 days. Strains that showed good growth on ACC plates were chosen for quantitative analysis, following Honma and Shimomura (1978) protocol.

Qualitative and Quantitative Evaluation of Plant Growth-Promoting Characteristics of Bacterial Isolates

Phosphate Solubilization

An aliquot of ∼10 μL of freshly produced bacteria was spotted on Pikovskayas agar medium to study the phosphate (P) solubilization capability (Hi-Media). The spotted plates were placed at 30 ± 2°C for 3–5 days and detected for the development of a clear zone around the bacterial colony.

For quantitative P-solubilization, each bacterial isolate was inoculated in 100 mL Erlenmeyer flasks containing 25 mL of Pikovskaya's medium (≈10^8 CFU mL^{-1}) and incubated in a shaker (180 rpm) at 30 ± 2°C for 72 h. Autoclaved medium (uninoculated) served as control. 20 mL of each culture was collected after 72 h of growth and centrifuged for 10 min at 13,000 *g* to obtain cell-free supernatants. The amount of phosphorus in the culture supernatant was measured using Fiske and Subbarow (1925). The pH of the bacterial broth was also measured with a digital pH meter.

Hydrogen Cyanide Production

The Hydrogen cyanide (HCN) produced by each endophytic bacterial isolate was determined by Lorck (1948) process. Briefly, pure bacterial isolate in LB medium with 4.4 g glycine L^{-1} was inoculated in 15 mL broth in a test tube. A sterile Whatman filter paper no. 1 soaked in picric acid (1%) solution was hung in the test tubes once dried. The test tubes were then closed with parafilm and kept for 5–10 days at 30 ± 2°C. After incubation, the change in the color of the filter paper, i.e., yellow to orange-brown, or reddish-brown shows the production of cyanide from bacterial strains.

Siderophore Production

To measure the siderophore production by bacterial isolates, both qualitative and quantitative methods were used. The capacity of bacterial isolates to produce siderophores was evaluated using the universal Chrome azurol S (CAS) agar medium (Schwyn and Neilands, 1987). The pure freshly produced all bacterial isolates were dotted on Petri plates with CAS medium and cultured for 4–5 days at 30 ± 2°C. The formation of an orange zone (hydroxamate-type siderophore) or a purple zone (catechol-type siderophore) surrounding the bacterial colonies on the plates was used to determine siderophore production by endophytic bacteria.

Quantitative estimation (hydroxamate-type siderophore) was completed by taking the supernatant of bacterial cultures grown in a broth of LB medium (Hu and Xu, 2011). Screwcap tubes (20 mL) containing 5 mL of LB broth were autoclaved. Afterward, 10 μL of freshly grown bacterial suspension (≈10^8 CFU mL^{-1}) was inoculated, and the uninoculated broth was maintained as the control. After incubation at 30 ± 2°C for 3 days, bacterial cultures were pelleted by centrifuging at 12,000 rpm for 10 min. at 5°C, and the clear supernatant was used to determine the production of siderophore. 0.5 mL supernatant of each bacterial isolate was mixed with 0.5 mL CAS solution, kept in dark condition for 20 min, and measured its optical density at 630 nm by SPARK® multimode microplate reader (Model-SW Sparkctl. Magellan V2.2 STD 2PC, Austria). Payne (1993) method was used to determine siderophore production in percent siderophore unit (PSU).

Test for Colorimetric Indole-3-Acetic Acid (IAA) Detection

Indole-3-acetic acid synthesis capacity of all isolates were measured the colorimetric method of IAA quantitation as described by Gordon and Weber (1951). Overnight bacterial strains were cultured in LB broth medium at 32 ± 2°C with shaking at 180 rpm after inoculum was prepared (≈10^8 CFU mL^{-1}). The inoculum was added in LB broth medium supplemented with L-tryptophan (0.5 and 1.0 g L^{-1}) as the IAA precursor and incubated for 7 days at 30 ± 2°C. Subsequently, bacterial cells were separated by centrifugation, and the supernatant was used for quantitative measurement of IAA-producing bacteria by using Salkowski's reagent.

Ammonia Production

Freshly cultured bacterial strains were grown in peptone water broth for 5 days at 30 ± 2°C and supernatant was used to measure ammonia production using Nessler's reagent following the method described previously (Goswami et al., 2014). The concentration of ammonia was calculated using the ammonium sulfate standard curve, which ranged from 0.1 to 1 μ moL mL^{-1}.

Biocontrol Assay of Endophytic Bacteria

Using the dual-culture method established by Singh et al. (2014), the chosen bacterial isolates were evaluated for *in vitro* antifungal activity against *Fusarium verticillioides* (*FV*), and *Fusarium oxysporum* f. sp. *cubense* (*FOC*). A fungal disk (5 mm) was placed in the center of a potato dextrose agar (PDA) and nutritional agar (NA) (1:1) plates and bacterial isolates suspended in LB broth (∼6 × 10^8 cell mL^{-1}) were spotted 3 cm away from the fungal disk. Plates with fungal disks (without bacterial isolates) were applied as a control. The plates were maintained at 28 ± 2°C for 5–7 days. By comparing the development of fungal mycelia with and without the tested bacterial isolates, the antifungal activity was determined.

To examine the cell-free crude extract of chosen (dual-culture method) bacteria against the pathogens, strains were cultured in *LB broth* medium for 5–7 days at 120 rpm and 32 ± 2°C on an orbital shaker incubator. The bacterial cells were separated from the broth medium by centrifugation (14,000 rpm for 15 min at 4°C) and filtered using a sterilized membrane (0.22 m pore size; Merck Millipore Ltd.), and the filtrate was stored at −20°C until further use. The pathogen spores were scraped and suspended in sterile distilled water (10 mL) and diluted the spore suspension (10^6 CFU mL^{-1}; colony-forming units), then distributed on Petri dishes comprising PDA. A 5-mm diameter hole was made into the medium with a cork borer, and the well was sealed with sterilized agarose (0.2%). Once the agarose was set and dried, 100 μL of cell-free culture filtrate and LB *broth* medium (control) was applied. The plates were incubated at 26 ± 2°C for 3–5 days.

Bacterial Production of Cell Wall Degrading Enzymes

Use of hydrolytic enzymes is a biocontrol strategy utilized by many microorganisms to restrict the development of fungal pathogens. In this study, the production of hydrolytic enzymes such as chitinase (MM1062O1), protease (MM1206O1), β- 1,3 glucanase (MM91504O1), and cellulase (MM91502O1) by selected strains was studied (Guo et al., 2020). A single strain of freshly grown bacteria was inoculated into 20 mL of LB medium and maintained at 32 ± 2°C for 36–48 h in an incubator shaker, after which the supernatant was centrifuged at 12,000 rpm for 10 min at 4°C and utilized for different enzyme activity measurement using enzyme-linked immunosorbent assays (ELISA) (Wuhan Colorful Gene Biological Technology Co. Ltd., China).

Scanning Electron Microscopy

Biocontrol interaction studied of the fungal plant pathogens and selected diazotrophs were done by scanning electron microscopy (SEM). A small piece of hyphae (∼3 mm) was cut at an interaction point into the plate and control plate only fungal pathogen, fixed in 2% glutaraldehyde for 4 h at room temperature, then rinsed three times with phosphate buffer (0.1 M, pH 7.4) for 15 min and after transferred into blocks with 1% OsO_4 in 0.1 M PB (pH 7.4) for 1–2 h at 20°C. Then, blocks were washed with 0.1 M phosphate buffer (0.1 M, pH 7.4) for 15 min. The samples were desiccated with different concentrations of ethanol, i.e., 30, 50, 70, 80, 90, 95, and 100% for 15 min and at last with isoamyl acetate for 15 min. Samples were dried in a critical point dryer (model K850 Quorum) by a carbon sticker sputter-coated with gold-palladium for the 30 s. Samples were observed with SEM (HITACHI, SU8100, Japan). This technique is also used to study the colony morphology

of selected diazotrophs as well as to verify the colonization in different tissues (root and leaf) of sugarcane plants.

Stress Resistance Test of Endophytic Bacteria

In vitro screening of endophytic isolates for abiotic stress tolerance was analyzed with a broad range of temperatures (20–45°C), pH (5–10), and different NaCl concentrations (7–12%) (Sharma et al., 2019, 2021). 100 μL ($\approx 10^8$ CFU mL^{-1}) of fresh cultures were transferred in 5 mL LB broth medium and incubated in a gyratory shaker set at 120 rpm for 36 h at 32 ± 2°C, and growth was measured at 600 nm using SPARK® multimode microplate reader (Model- SW Sparkctl. Magellan V2.2 STD 2PC, Austria).

Colonization Pattern of Selected Diazotrophs in the Sugarcane Plant

Plasmid Transformation

For this experiment, we selected strains AF1 and EF1, the most potential isolates based on the above studies. Both isolates were resistant to ampicillin and taken as recipients with GFP-pPROBE-pTetr-OT tagging sensitive to kanamycin. Plasmid pPROBE-pTetr-OT comprising the green fluorescent protein (GFP) gene expressed under the Tet^r promoter was inserted by biparental mating with donor strain *Escherichia coli* TG1 (Lin et al., 2012). The plasmid has a wide host range and could be quantified in both Gram-positive and Gram-negative bacteria. In an incubator shaker, the recipient and donor isolates were combined in a 1:2 ratio and maintained at 30 ± 2°C for 48–72 h. A 100 μL aliquot of the above mixture was spread onto LB agar plate and kept overnight at 30 ± 2°C and strains displaying green fluorescence under UV illumination were used for further study.

Inoculation of Micro-Propagated Sugarcane Plantlets

Micro-propagated sugarcane plantlets (variety GT42) were procured from Sugarcane Research Institute, Guangxi Academy of Agriculture Sciences (GXAAS), Nanning, China. Five separate plantlets were moved into a glass bottle containing 50–75 mL of MS liquid medium with sucrose and basal salt mixture. After, 3 days sugarcane plantlets were shifted in other autoclave bottles comprising GFP-tagged bacterial suspension (2.0×10^6 mL^{-1}), and without suspension have been prepared for utilizing as a control. All plantlets were grown in a growth chamber at 30°C with a 14 h photoperiod at 60 μmoL m^{-2} s^{-1} photon flux density.

Laser Scanning Confocal Microscopy

Afterward, 4–5 days sugarcane plantlets both inoculated and un-inoculated were carefully removed from the bottles and washed with autoclaved distilled water, then dried at room temperature. Sugarcane plantlets tissues (root, leaf, and stem) were cut into small parts and mounted on a clean glass slide under a coverslip. Different tissues of sugarcane plantlets were viewed with a Leica DMI 6000 microscope (Mannheim, Germany) using a confocal scanning laser microscope (Olympus SXZ16) at different emission lengths.

Plant Inoculation Studies of Potential Diazotrophic Isolates, i.e., a Pot Experiment

Experimental Design and Treatments

The pot experiment was performed in January, 2020, at the Sugarcane Research Institute, GXAAS, Nanning. Sugarcane variety GT42 was used to establish the interactions between diazotrophic isolates and the host plant. The trial was completed in a greenhouse and the seed canes of test variety were obtained from Sugarcane Research Institute. A disease-free seed cane was used for this experiment. Hot-water treatments of sugarcane stalks (45–50°C for 2 h) were used to disinfect seed cane. Soil was collected from the top 2–20 cm soil depth from the experimental field sites of Sugarcane Research Institute and air-dried, crushed, and its physicochemical properties were analyzed. A plastic pot (30 cm diameter and 40 cm deep) holding 20 kg of soil and sand mixture (3:1 w/w) were used. Approximately similar size and shape of sugarcane plantlets at a three-leaf stage were carefully removed from nursery plants. And finally, the roots were washed slowly with flowing tap water then put in a tub and rinsed carefully until the root was cleaned. The experiment was accomplished in a complete randomized block design with five biological repeats and each pot contained two treated plantlets. The three treatments comprised of: (1) without inoculation-control (WI), (2) inoculation with *P. cypripedii* (AF1), and (3) Inoculation with *K. arachidis* (EF1). The diazotrophic bacteria were cultured in LB medium for 24–36 h (200 rpm, 32 ± 2°C) and centrifuged. Pellet was dissolved with autoclaved distilled water and prepared a bacterial cell suspension were adjusted at 10^8 CFU mL^{-1}, and mixed with 1% autoclaved carboxymethyl cellulose (CMC) solution. All plantlets of sugarcane roots were immersed in CMC solution for 1 h. Following these treatments, plantlets were plotted into plastic pots and kept under controlled environmental conditions at 14 h day/10 h night; 60–70% relative humidity, and 26°C/20°C day/night temperature.

Plant's Harvest, RNA Extraction, Purification, and cDNA Synthesis

Isolation of total RNA of root (100 mg) was done with Trizol reagent (Tiangen, China), and purified by RNeasy Plant Mini Kit (Qiagen), following the manufacturer's commands. Extracted RNA samples were processed with DNase I (Promega, United States) to eliminate contaminating DNA and quantified using a Nano photometer (Pearl, Implen-3780, United States). The Prime-Script™ RT Reagent Kit (TaKaRa, Dalian, China) was used to synthesize single-stranded cDNA from 1 g of total RNA, consistent with the manufacturer's guidelines.

cDNA Amplification

Amplification of the cDNA sequences employed the expression of aminomethyl transferase- *AMT*, nitrate transporter- *NRT*, Nitrate reductases- *NR*, Glutamate synthase- *GS*, Glutamine synthetase- *GOGAT*, N fixation-*nifH*, endo-glucanase- β-*1, 4-GA*, and chitinase- *CHI,* genes in the root tissues of sugarcane during plant-microbes interaction at tillering phase were analyzed in greenhouse condition after treatment with strains (AF1 and EF1)

in GT42 sugarcane varieties with control plants. The primer sequences used in this study are presented in **Supplementary Table 2**. The qRT-PCR reaction mixture consisted of SYBR Premix Ex Tap^{TM} II (TaKaRa, Japan), 10 μL of SYBR Premix, 1 μL of each primer (10 μM), 2 μL of RNA template (10 × diluted cDNA), and 6 μL of ddH_2O in a total volume of 20 μL with five repeats in Real-Time PCR Detection System (Bio-Rad, United States). Amplification was started with a denaturation step of 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 20 s, and 72°C for 30 s, and the last extension at 72°C for 2 min. To standardize qRT-PCR data, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as the reference gene, and the relative expression of all genes was quantified using the $2^{-\Delta\Delta Ct}$ technique (Livak and Schmittgen, 2001).

^{15}N Abundance Plant Analysis

The ^{15}N isotope dilution method quantifies the biological N fixation in the GT42 sugarcane variety (Singh et al., 2020a). This method involves the testing of N-fixing crops with the inoculation of AF1 and EF1 isolates. Soil and sand were mixed with a ratio of 1:3 (w/w) and sterilized two times for 45 min at 121°C. After cooled at room temperature 10 mg ammonium sulfate-^{15}N (10.12 percent atom ^{15}N excess) per kg of soil was added and homogenized for proper distribution of ^{15}N. Twenty kg of ^{15}N soil-sand mixture was filled in the pots and each pot contains two treated sugarcane plantlets for GT42. This experiment was performed in a completely randomized block design with five biological repetitions, and comprised three treatments: (1) without bacterial inoculation (control) (2) inoculation with *P. cypripedii* (AF1), and (3) inoculation with *K. arachidis* (EF1) of each variety. At the tillering phase, plants were harvested and washed with distilled water to eliminate the soil attached to the roots and plants. Roots, leaves, and stems were separated and ground to a fine powder. Five milligrams of powdered root, leaf, and stem materials for all samples were analyzed for ^{15}N isotope content using K05 automatic Kjeldahl N determination equipment (Shanghai Sonnen automated research instrument co. Ltd.), and elementary analysis was done by isotope ratio mass spectrometers (Thermo Fisher Delta V Advantage IRMS). The contribution of N derived from the air (Ndfa) in different tissues of all sugarcane varieties was calculated by Urquiaga et al. (1992).

Determination of Physiological Parameters and Enzymes Associated With Nitrogen Metabolism, and Biocontrol, and Phytohormone Analysis

The experimental plants were collected at the tillering stage. The growth parameters of sugarcane plants including plant height, fresh weight (root and shoot), leaf area (Cl 203 Handheld laser leaf area meter, Bio-Science), chlorophyll content (Chlorophyll meter; SPAD-502 Plus; Konica Minolta Inc.; Japan), net photosynthetic rate, transpiration rate, and stomatal conductance (LI-6,800 compact portable photosynthesis system) were recorded.

Also, the activity of different N-metabolism enzymes (glutamine synthetase-GS, NADH glutamate dehydrogenase, and nitrate reductase-NR), biocontrol-related enzymes (β-1,4 and β-1,3 glucanase-GLU, and chitinase-CHI) (Singh et al., 2021c), and hormones (Gibberellins- GA_3, Indole-3-acetic acid- IAA, and Abscisic acid- ABA) were extracted (Singh et al., 2018) and analyzed by plant ELISA kit (Colorful Gene Biological Technology Co. Ltd., Wuhan, China) according to the manufacturer's guidelines.

Statistical Analysis

All tests were carried out in three repetitions. The standard error was computed using mean values, and the statistical significance threshold was set at $p \leq 0.05$. MS Excel 2016 was utilized for basic statistical analysis of data, i.e., means standard deviation, and bar graphs. Analysis of variance was used to evaluate the statistical significance of the experimental data, followed by multiple comparisons using Tukey's HSD test. A heatmap was also prepared following the method described by to Babicki et al. (2016), a heat map was created.

RESULTS

Isolation of Endophytic Bacteria

A total of 175 endophytic bacterial strains were isolated from the roots of five sugarcane species Among these, only 11 Gram-negative strains of two genera *Pantoea* and *Kosakonia* were selected based on partial sequencing of the 16S *rRNA* gene. Further, the increase in interest in both genera for their potential PGP and nitrogenase activities was also prompted us to select these strains for the study.

Molecular Identification of Endophytic Diazotrophic Bacteria

Sequencing of 16S rRNA Gene

In this present study, molecular identification of all diazotrophic isolates was accomplished through the amplification of 16S *rRNA* gene sequencing (∼1.4–1.5 kb) with the primer's pA and pH. Using BLAST-N searches with type strain database, we found that all the strains belong to the genus *Pantoea* and *Kosakonia* with *rRNA* sequences similarity values at ≈97.77–99.58%. All sequences of the isolates were deposited to NCBI GenBank under MZ497007 - MZ497017 accessions numbers (**Table 1**).

Phylogenetic Analysis

The sequences of the 16S *rRNA* gene were aligned and used to build a phylogenetic tree using the neighbor-joining method. We found that all isolates were distributed into four major clusters using a total of 1,000 bootstrap samples of representative isolates compared to type strains of related taxa. According to this, all endophytic diazotroph bacterial isolates were grouped into different clusters with cluster I formed with two isolates (BA4- *Pantoea ananatis* and BB2- *Pantoea allii*), cluster II also formed with two isolates (AE2- *Pantoea dispersa* and AF1- *P. cypripedii*), cluster III formed with one isolate (CF1- *Pantoea agglomerans*), and cluster IV formed with six isolates (AE3- *Kosakonia oryzae*, EA1- *Kosakonia radicincitans*, EF1-

TABLE 1 | Identification of selected isolates through 16S *rRNA* gene and their nitrogenase activity via acetylene reduction assay, hydrogen cyanide production, and the presence of gene amplification with an accession number.

Isolates	Strain name	16S accession numbers	ARA (nmoL C_2H_4 mg protein h^{-1})	*nifH* gene	*nifH* accession numbers	HCN production	HCN gene	HCN accession numbers
AE2	*Pantoea dispersa*	MZ497007	15.30 ± 0.23^{e}	+	MZ502257	+	+	MZ502268
AE3	*Kosakonia oryzae*	MZ497008	10.29 ± 0.15^{g}	+	MZ502258	–	+	MZ502269
AF1	*Pantoea cypripedii*	MZ497009	23.24 ± 0.35^{b}	+	MZ502259	+	+	MZ502270
BA4	*Pantoea ananatis*	MZ497010	18.64 ± 0.28^{c}	+	MZ502260	+	+	MZ502271
BB2	*Pantoea allii*	MZ497011	12.59 ± 0.19^{f}	+	MZ502261	–	+	MZ502272
CF1	*Pantoea agglomerans*	MZ497012	10.08 ± 0.15^{g}	+	MZ502262	–	+	MZ502273
EA1	*Kosakonia radicincitans*	MZ497013	10.50 ± 0.16^{g}	+	MZ502263	+	+	MZ502274
EF1	*Kosakonia arachidis*	MZ497014	35.14 ± 0.52^{a}	+	MZ502264	+	+	MZ502275
ACCR4	*Kosakonia oryziphila*	MZ497015	9.46 ± 0.14^{h}	+	MZ502265	+	+	MZ502276
ACCR21	*Kosakonia quasisacchari*	MZ497016	17.39 ± 0.26^{d}	+	MZ502266	+	+	MZ502277
ACCE1	*Kosakonia pseudosacchari*	MZ497017	6.95 ± 0.10^{i}	+	MZ502267	–	+	MZ502278

Means followed by the similar letter are not significantly different ($p \leq 0.05$).

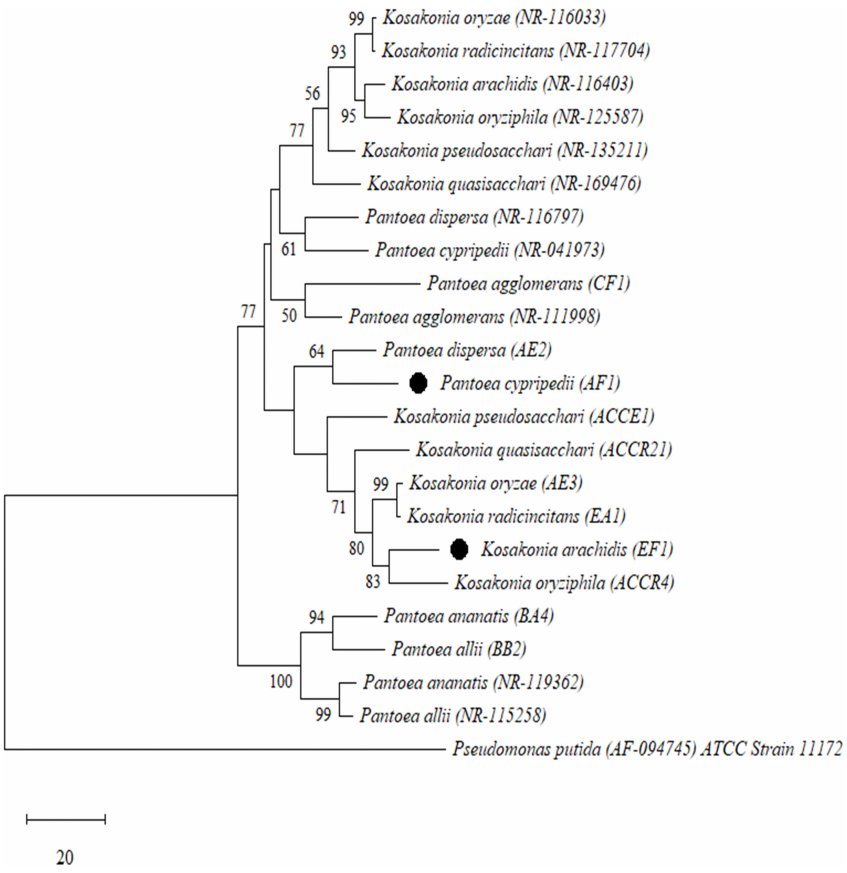


FIGURE 1 | Phylogenetic tree analysis based on the partial 16S rRNA gene sequences of all selected Gram-negative endophytic nitrogen-fixing bacterial isolates isolated from sugarcane root. Scale bar means the number of changes per base location and *Pseudomonas putida* was used as an outgroup.

K. arachidis, ACCR4- *K. oryziphila*, ACCE1- *K. pseudosacchari,* and ACCR21- *Kosakonia quasisacchari*) as shown in **Figure 1**.

Detection of *nifH*, *acdS*, and *HCN* Genes

The PCR-based molecular detection of *nifH*, *acdS*, and *HCN* genes that promote plant development directly or indirectly was studied. Results showed that all strains had positive *nifH* gene amplification (**Supplementary Figure 1A**), and the evolutionary relationship of the *nifH* phylogenetic tree is presented (**Supplementary Figure 1D**). After sequencing, all sequences were matched with *nifH* gene sequences obtained from NCBI by BLASTN search. The *nifH* sequences of test isolates

TABLE 2 | *In vitro* screening for assessing the endophytic plant growth-promoting, 1-Aminocyclopropane-1-carboxylate deaminase activity and antifungal activities for selected bacterial isolates obtained from sugarcane root.

Isolates	ACC deaminase activity		Phosphate		Siderophore		Ammonia		Antifungal activities			
	A	B	A	B (PSI)	A	B (PSU)	A	B (μ moL mL^{-1})	C (FV)	D (*FV*)	C (*FOC*)	(D) (*FOC*)
AE2	+	676.70 ± 10.04^{c}	–	–	+++	59.35 ± 1.19^{b}	+++	6.18 ± 0.14^{a}	56.67 ± 0.65^{b}	5.8	64.71 ± 0.53^{c}	6.1
AE3	+	221.35 ± 3.28^{f}	++	2.81	++	39.16 ± 0.75^{cd}	++	3.65 ± 0.10^{f}	30.00 ± 1.04^{e}	–	40.00 ± 0.89^{h}	–
AF1	+	1325.62 ± 19.67^{a}	++	2.68	+++	71.23 ± 1.66^{a}	+++	6.33 ± 0.14^{a}	64.44 ± 0.53^{a}	5.1	74.12 ± 0.39^{a}	5.5
BA4	+	359.11 ± 5.33^{d}	–	–	++	32.60 ± 1.59^{de}	+++	5.72 ± 0.13^{b}	24.44 ± 1.12^{f}	–	44.71 ± 0.82^{g}	–
BB2	+	323.77 ± 4.80^{e}	+++	3.34	+	06.57 ± 3.74^{f}	+++	5.16 ± 0.12^{cd}	20.00 ± 1.19^{g}	–	29.41 ± 1.05^{i}	–
CF1	+	209.68 ± 3.11^{f}	++	2.54	++	40.37 ± 4.80^{cd}	++	4.33 ± 0.11^{e}	35.56 ± 0.96^{d}	–	60.00 ± 0.60^{d}	3.4
EA1	+	204.91 ± 3.04^{f}	+	2.22	++	29.74 ± 1.22^{e}	+++	4.74 ± 0.08^{de}	44.44 ± 0.83^{c}	–	55.29 ± 0.67^{e}	–
EF1	+	824.33 ± 12.23^{b}	++	2.77	+++	63.46 ± 1.16^{ab}	+++	6.21 ± 0.13^{a}	66.11 ± 0.50^{a}	6.5	70.59 ± 0.44^{b}	6.3
ACCR4	+	103.37 ± 1.53^{g}	+	2.34	+	8.47 ± 3.33^{f}	+	3.27 ± 0.09^{f}	34.44 ± 0.98^{d}	–	54.12 ± 0.68^{e}	–
ACCR21	+	344.99 ± 5.12^{de}	+++	3.56	++	23.90 ± 2.45^{e}	+++	5.55 ± 0.12^{bc}	45.56 ± 0.81^{c}	–	52.94 ± 0.70^{e}	–
ACCE1	+	93.35 ± 1.38^{g}	–	–	++	43.89 ± 1.72^{c}	+	2.37 ± 0.17^{g}	23.33 ± 1.14^{f}	–	49.41 ± 0.75^{f}	–

A, qualitative analysis; B, quantitative analysis (μmoL mg^{-1} h^{-1}); (+++), high production; (++), medium production; (+), low production; PSI, phosphate solubilization index; PSU, percent siderophore unit; C, dual culture (% inhibition); D, well diffusion (mm), FV; Fusarium verticillioides, FOC; Fusarium oxysporum f. sp. cubense. Different letters indicate significant differences among treatments at $p \leq 0.05$.

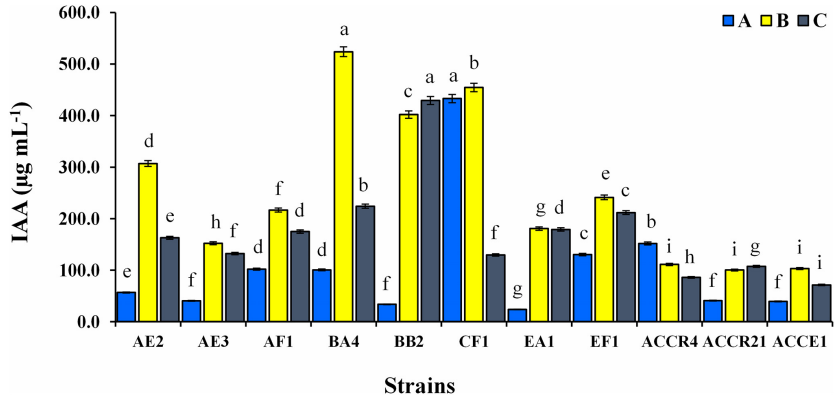


FIGURE 2 | Indole-3-acetic acid production of endophytic bacterial isolates at different concentrations of L-tryptophan (A) absence of L-tryptophan and (B,C) presence of L-tryptophan (0.5 and 1.0 g L^{-1}). Dissimilar lowercase letters show a significant difference at $p \leq 0.05$.

were submitted to NCBI GenBank with accession numbers MZ502257 – MZ502267 (**Table 1**).

Amplification of the *acdS* gene revealed that nine strains had ACC deaminase gene (**Supplementary Figure 1B**). The HCN gene was present in all 11 strains at ∼587 bp of amplification (**Supplementary Figure 1C**). All sequences showed 95–100% similarity with other HCN genes present in the NCBI database. Accession numbers of HCN genes of seven isolates are MZ502268 – MZ502278 (**Table 1**).

Nitrogenase Activity

The nitrogenase activity obtained from isolates varied from 6.95 ± 0.10 to 35.14 ± 0.52 C_2H_4 mg protein h^{-1} nmoL (**Table 1**). The maximum nitrogenase activity was obtained in EF1 followed by AF1, BA4, ACCR21, and AE2 (which is ≥15 nmoL C_2H_4 mg protein h^{-1}, respectively). Isolate ACCE1 exhibited ≤10 nmol C_2H_4 mg protein h^{-1} level of nitrogenase activity as compared with other isolates (**Table 1**).

1-Aminocyclopropane-1-Carboxylate Deaminase Activity

1-aminocyclopropane-1-carboxylate deaminase activity was measured to determine isolates' ability to utilize ACC as a nitrogen source to grow. After 4–5 days of incubation at 32 ± 2°C, all of the endophytic isolates were able to grow on medium supplemented with 3 mMoL L^{-1} of ACC (**Table 2**). Based on this result, the ACCD enzyme activity was determined quantitatively and the amount of α-ketobutyrate breakdown during ACC by the ACCD enzyme. It was observed that AF1, EF1, and AE2 isolates used maximum ACC and produced 1325.62 ± 19.67, 824.33 ± 12.23, and 676.70 ± 10.04 α-ketobutyrate μmoL mg^{-1} h^{-1}, respectively (**Table 2**).

Plant Growth Promoting Traits of Endophytic Isolates

A total of 11 selected endophytic bacterial isolates (from AE2 to ACCE1) were tested for their potential PGP activities, such as

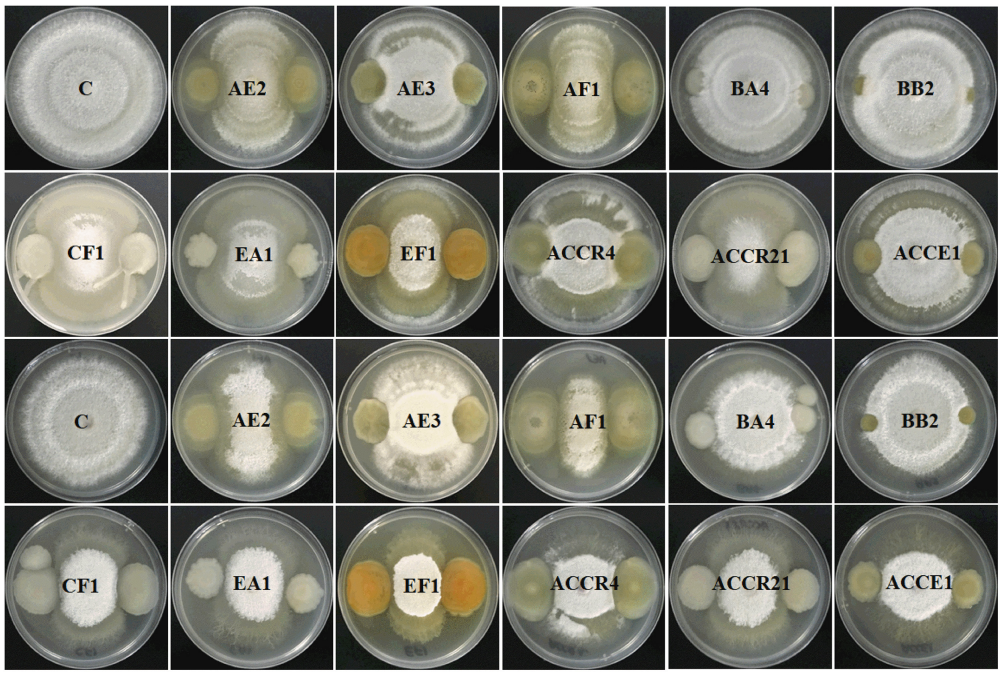


FIGURE 3 | The activity of all selected Gram-negative endophytic antagonistic bacteria against *Fusarium verticillioides* and *Fusarium oxysporum* f. sp. *cubense* pathogens in dual culture and inhibition percentage by culture filtrate method.

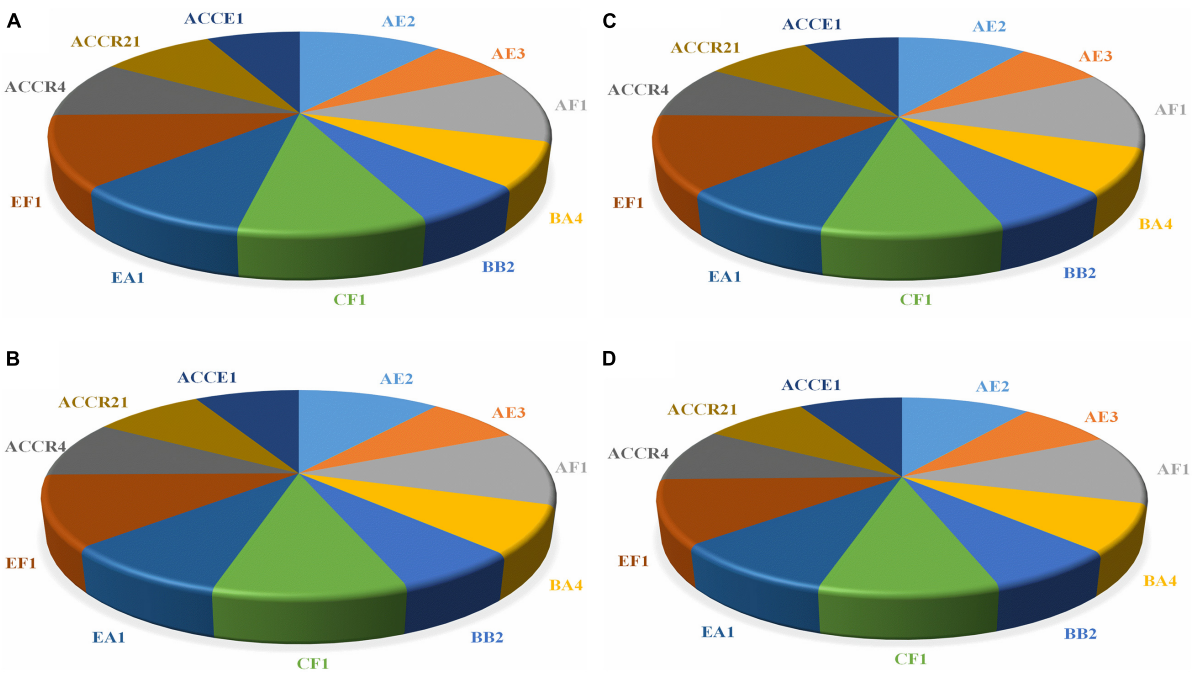


FIGURE 4 | The endophytic isolates displaying different concentrations of hydrolytic enzymes activity and proficient to degrade the cell wall of fungal pathogens. **(A)** Chitinase, **(B)** Protease, **(C)** Cellulase, and **(D)** Endoglucanase.

the production of siderophores, phosphate, HCN, and ammonia (**Tables 1, 2** and **Supplementary Figure 2**).

After 5–7 days of incubation, eight (73%) endophytic isolates displayed positive phosphate solubilization on the Pikovskaya agar medium plate containing $Ca_3(PO_4)_2$, with a clear zone around the colony (**Table 2**). Isolates BB2 and ACCR21 showed phosphate solubilization index ≥3 PSI; while the other six (AE3, AF1, CF1, EA1,

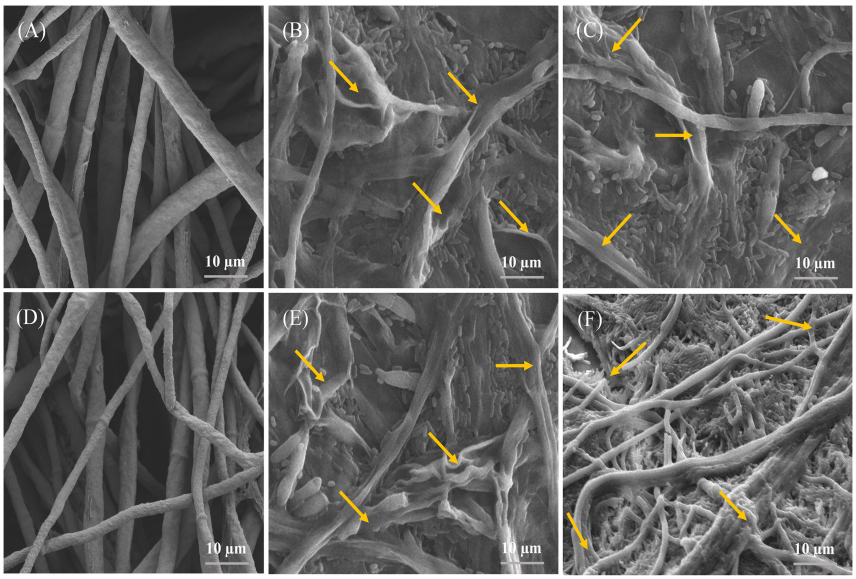


FIGURE 5 | Images obtained by scanning electron microscopy of the antagonistic bacteria interacting with hyphae of selected fungal pathogens on NA: PDA medium after 5 days incubation. **(A,D)** indicating a normal hyphae of *F. verticillioides* and *F. oxysporum* f. sp. *cubense*, whereas **(B,C,E,F)**, indicating abnormal hyphae of selected fungal pathogens.

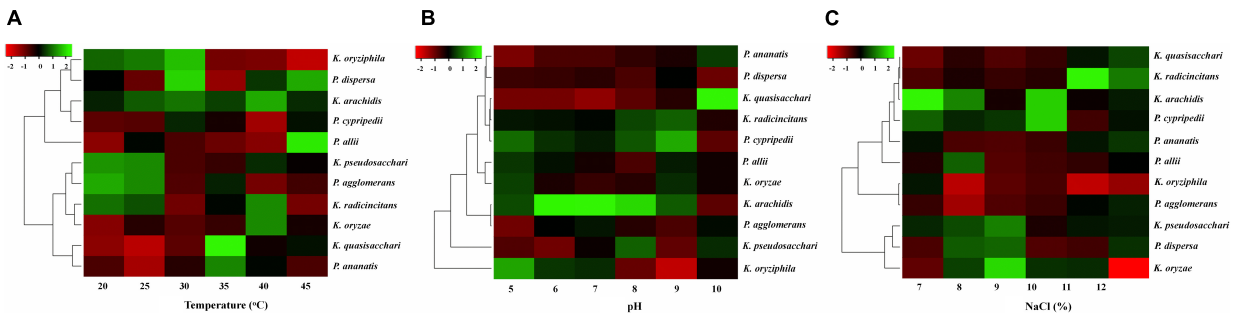


FIGURE 6 | Effects of different abiotic stresses on selected isolates. **(A)** Temperature, **(B)** pH level, and **(C)** Different concentrations of salt.

EF1, and ACCR4) isolates showed from 2.81 to 2.34 PSI (**Table 2**).

The HCN-producing ability of all endophytic isolates was tested. Seven (64%) isolates exhibited an orange-brown color of Whatman filter paper no. 1 (soaked in 2% sodium carbonate in 0.1% picric acid solution) that confirmed a positive result and four isolates (AE3, BB2, CF1, and ACCE1) could not produce HCN (**Table 1**).

In CAS agar plate media, an orange-colored halo zone formed around the colonies, indicating that bacterial strains were producing siderophores. All the bacterial isolates selected in the study produced siderophores, and strains AE2, AF1, and EF1 had the greatest capacity for siderophore production (**Table 2**). The concentration of siderophore generated by bacterial strains ranged from 06.57 ± 1.59 to 71.23 ± 1.66 PSU. The AF1, EF1, and AE2 isolates displayed the greatest production of siderophores (71.23 ± 1.66, 63.46 ± 1.16, and 59.35 ± 1.19 PSU) among the tested isolates (**Table 2**).

The quantitative estimation of IAA production of all endophytic isolates is shown in **Figure 2**. In the presence of L-tryptophan (0.5 and 1%), IAA production was higher but it showed considerable variation depending on the amount of tryptophan (100.35 ± 0.75–523.65 ± 9.50 and 71.42 ± 1.30–429.39 ± 7.79 µg mL^{-1}). AF1, BA4, CF1, EF1, EA1, and ACCR4 strains displayed significantly higher amounts of IAA production with an increase in tryptophan concentration up to ≥100–432 µg mL^{-1} without addition of L-tryptophan. The maximum IAA production without supplementation of L-tryptophan in the medium (432.94 ± 7.85 µg mL^{-1}) was found in CF1 isolate (**Figure 2**).

Ammonia production is another important PGP feature of PGPEB. This feature was seen in all chosen endophytic isolates that used peptone as a substrate to produce ammonia (**Table 2**). AE2, AF1, and EF1 isolates produced maximum ammonia, which was 6.18 ± 0.14, 6.33 ± 0.14, and 6.24 ± 0.13 µmoL mL^{-1}. Isolates BA4, ACCR21 and BB2, showed in descending

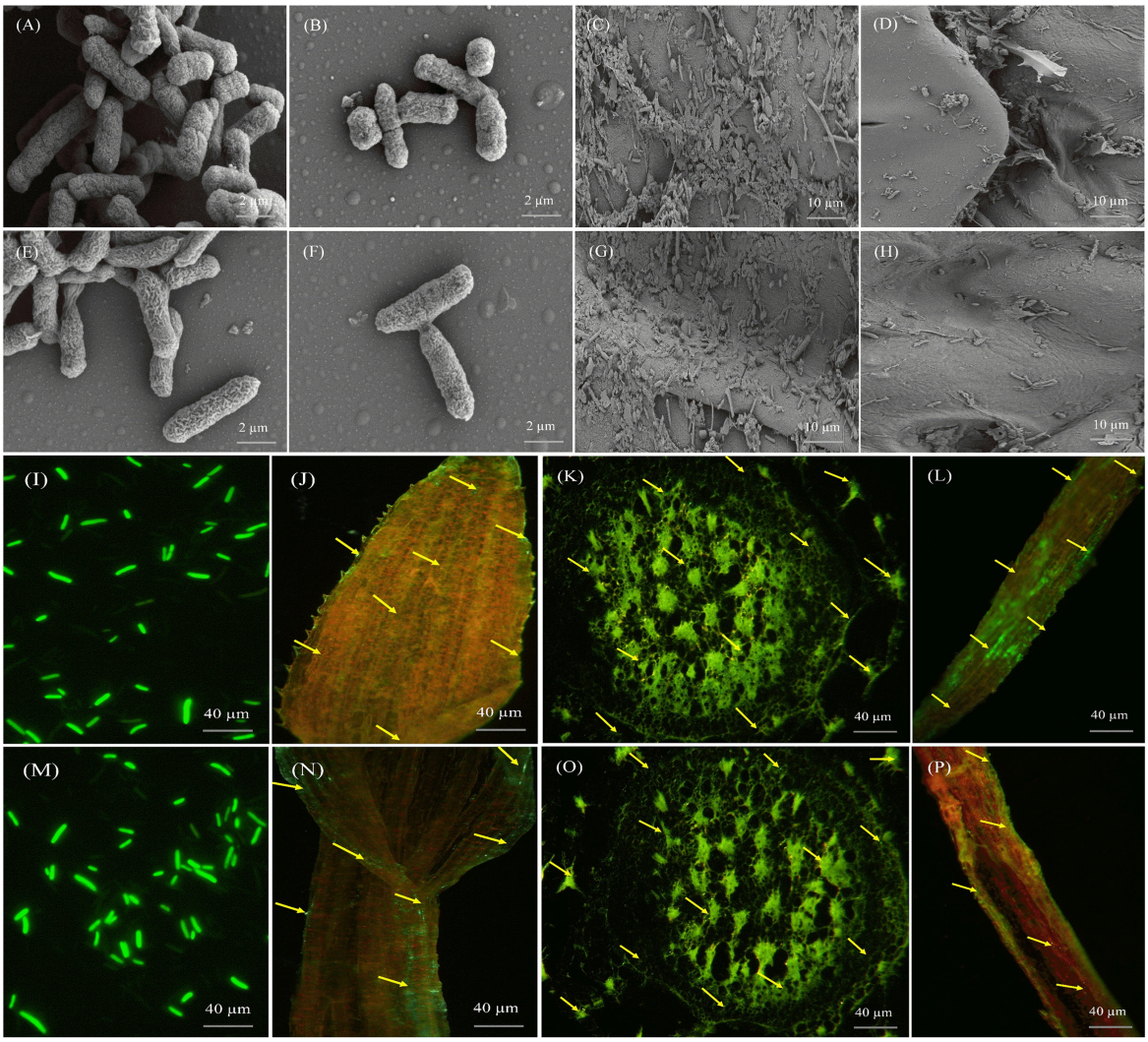


FIGURE 7 | Microscopic pictures presenting the morphology and colonization of *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1 in sugarcane (GT42). **(A,B,E,F)** Scanning electron micrographs (SEM) displaying morphology (rod-shaped) of AF1 and EF1 bacteria. **(C,D,G,H)** Colonization of AF1 and EF1 in root and stem tissues of sugarcane plant. **(I,M)** Confocal laser scanning micrographs (CLSM) displaying morphology of GFP tagged AF1 and EF1 strains. **(J–L,N–P)** Colonization of AF1 and EF1 strains as green dots in leaf, stem, and root tissues of sugarcane.

rank depending on ammonia production capacity ≥5 moL mL^{-1} (**Table 2**).

Biocontrol Activity and Hydrolytic Enzymes Production of Endophytic Bacterial Isolates

The antagonistic potential of 11 identified endophytic strains (*Pantoea dispersa*, *Kosakonia oryzae*- 2, *P. cypripedii*, *P. ananatis, P. agglomerans*- 2, *K. pseudosacchari*, *K. oryziphila, K. arachidis*, and *K. radicincitans*) was examined against two fungal pathogens (*FV* and *FOC*). Results showed that reduction of *FV* mycelia by strains AE2, AF1, and EF1 was more than 55%, whereas AE2, AF1, CF1, EA1, and EF1 strains were able to reduce the growth of *FOC* pathogen. Among all, AF1 and EF1 strains showed (≥ 60%) maximum inhibition of both pathogens (**Figure 3**).

Figure 4, showing the quantitative estimation of enzymes, i.e., chitinase, protease, cellulase, and endoglucanase of all selected endophytic strains. Endophytic isolates of sugarcane roots were able to generate a large quantity of hydrolytic enzyme activities, which ranged from 386.87–711.37, 137.39–239.85, 741.56–1363.02, and 1530.26–2388.28 IU mL^{-1}, respectively. The strain AF1 showed the maximum enzymatic activities of all enzymes as compared to EF1 and other potential strains (**Figure 4**).

Scanning Electron Microscopy of Bacteria-Fungal Pathogens Interaction

Inhibition of both pathogens' growth by AF1 and EF1 isolates was further validated *in vitro* by SEM investigations (**Figure 5**). Control plates- without strains AF1 and EF1 had healthy mycelia of *FV* and *FOC* pathogens, i.e., with regular in shape and

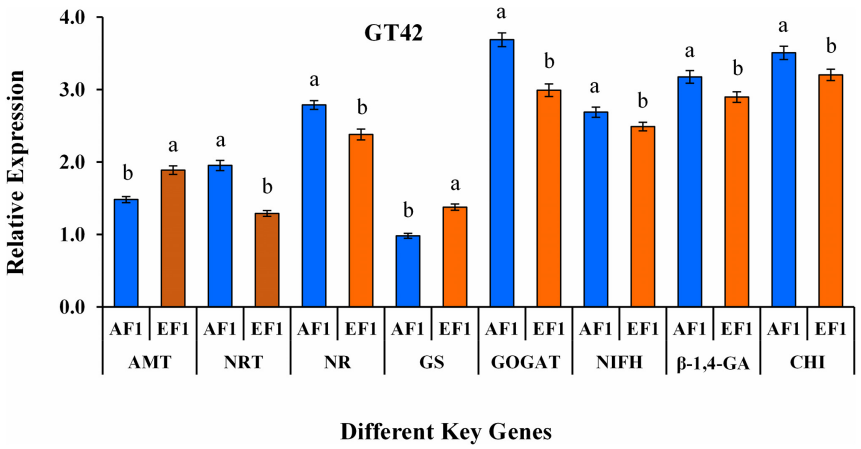


FIGURE 8 | qRT-PCR study of aminomethyl transferase- *AMT*, nitrate transporter- *NRT*, nitrate reductases- *NR*, glutamate synthase- *GS*, glutamine synthetase- *GOGAT*, nitrogen fixation- *nifH*, endo-glucanase- β-*1,4-GA,* and chitinase- *CHI* genes in root tissue of sugarcane variety (GT42) inoculated with *P. cypripedii* AF1 and *K. arachidis* EF1 strains. The GAPDH expression level was used to standardize the data. All data points are viewed as the mean ± SE (n = 3). Different letters specify significant differences among the treatments at $p \leq 0.05$.

cylindrical (**Figures 5A,D**), whereas, morphological changes were observed in the mycelia of *FV* and *FOC* inoculated with pathogens AF1 and EF1 isolates had broken mycelial surface and fragmentation of mycelia (**Figures 5B,C,E,F**).

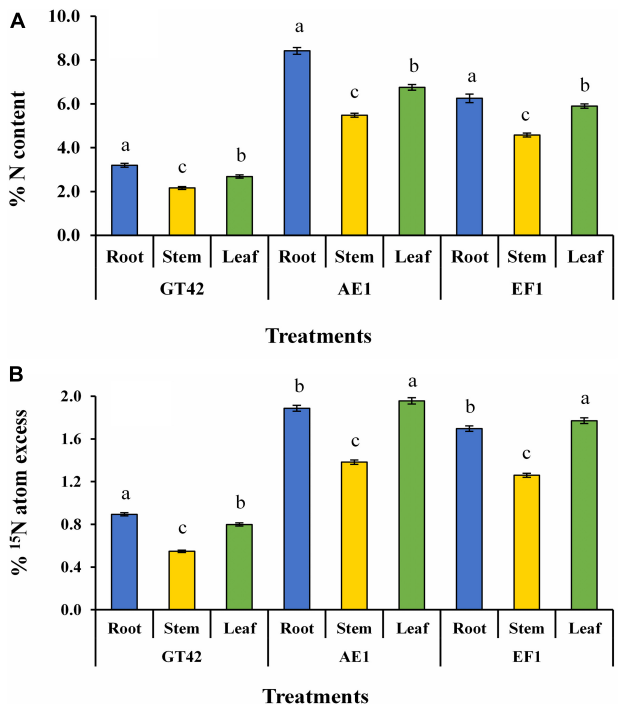


FIGURE 9 | Estimation of biological nitrogen fixation in sugarcane tissues (dry matter yield of root, stem, and leaf) by *P. cypripedii* AF1 and *K. arachidis* EF1 cultivated in ^{15}N-labeled soil (values are the average of three repeats) **(A)** % N content and **(B)** % ^{15}N atom excess. Significant changes among treatments at $p \leq 0.05$ are showed by dissimilar letters.

Effect of Abiotic Stress Factors

In this study, growth was measured for all isolates in various abiotic stress environments, i.e., temperature (20–45°C), pH (5–10), and NaCl (7–12%) and the results are presented using a heatmap (**Figure 6**).

All isolates showed maximum favorable growth at 25–35°C. However, at 40°C the growth of the isolates was decreased, and at 45°C growth was observed but decreased remarkably with only a few isolates showed little growth, as shown in **Figure 6A**. The result of diverse pH values (5–10) on the growth of all diazotrophs is shown in **Figure 6B**. All diazotrophs showed little growth at pH 5 but grew well from pH 6 to 9. Also, it was observed that all isolates showed optimum growth up to 7–8% NaCl concentration, but increasing NaCl concentration beyond that level (9–12% NaCl) decreased growth, and only a few strains showed very limited growth at 12% NaCl (**Figure 6C**).

Localization of Diazotrophs in the Sugarcane Plant

Scanning electron microscopy was also used to confirm the colony morphology and colonization of AF1 and EF1 in the root and stem of sugarcane plants (**Figures 7A–H**). In general, inoculated plants showed the colonization of bacterial isolates on the root and stem with high density and it was dispersed throughout the plant body. This result suggests that both diazotrophic isolates are aggressive endophytic colonizers of the sugarcane cultivated regions.

We also examined the colonization of AF1 and EF1isolates by CLSM in sugarcane plant tissues (root, stem, and leaf). **Figure 7** clearly shows the colonies of bacterial isolates inside the root, stem, and leaf tissues. Both isolates colonized root hairs and showed their presence in root epidermal cells when inoculated separately. Green fluorescence of GFP-tagged bacterial isolates was detected in numerous cells in different tissues throughout the plant (**Figure 7**). At 40× resolution,

GFP-tagged cells were localized in inter-cellular regions, within the vascular tissues, and in fissures at the points of lateral root emergence (**Figures 7J–L,N–P**).

Studies on N Metabolism and Pathogen-Control Related Genes

RT-qPCR was used to quantify the expression level of N metabolism (*AMT*, *NRT*, *NR*, *GS*, *GOGAT*, and *NifH*) and biocontrol (β-*1,4-GA*, and *CHI*) related genes in root tissues of sugarcane plant (Variety- GT42) after AE1 and EF1 inoculation (**Figure 8**). The results showed significant changes in the expression of all the analyzed genes in sugarcane after AE1 and EF1 inoculation as compared to control. Whereas AF1-inoculated plants showed higher expression levels of *NRT*, *NR*, *GOGAT*, *NifH*, β-*1,4-GA*, and *CHI* genes, and expression of *AMT* and *GS* was maximum in EF1inoculated plants (**Figure 8**).

TABLE 3 | The assessing of diazotrophic endophytes *Pantoea cypripedii* AF1 and *Kosakonia arachidis* EF1 inoculum on the sugarcane (variety GT42) in the greenhouse experiment.

Parameters	Treatments	Tillering stage	% Change over control
Plant height (cm)	GT42	36.55 ± 0.35^{c}	–
	AF1	53.39 ± 0.50^{a}	46.08
	EF1	48.31 ± 0.46^{b}	32.18
Root weight (g)	GT42	3.26 ± 0.03^{c}	–
	AF1	5.63 ± 0.05^{a}	72.70
	EF1	4.28 ± 0.04^{b}	31.29
Shoot weight (g)	GT42	32.38 ± 0.31^{c}	–
	AF1	87.25 ± 0.82^{a}	169.46
	EF1	72.46 ± 0.68^{b}	123.78
Leaf area (cm^2)	GT42	204.46 ± 1.93^{c}	–
	AF1	448.39 ± 4.24^{b}	119.30
	EF1	465.11 ± 4.39^{a}	127.48
Leaf length (cm)	GT42	114.24 ± 5.73^{b}	–
	AF1	221.85 ± 3.34^{a}	94.20
	EF1	208.80 ± 9.30^{a}	82.77
Leaf width (cm)	GT42	2.29 ± 0.14^{b}	–
	AF1	2.83 ± 0.11^{a}	23.44
	EF1	2.66 ± 0.10^{a}	16.16
Plant diameter (mm)	GT42	10.28 ± 0.44^{c}	–
	AF1	13.69 ± 0.52^{a}	33.20
	EF1	12.32 ± 0.48^{b}	19.84
Total protein (μg g^{-1} Fresh weight)	GT42	1337.50 ± 12.64^{b}	–
	AF1	1755.77 ± 16.59^{a}	31.27
	EF1	1783.23 ± 16.85^{a}	33.33
Chlorophyll content (SPAD units)	GT42	33.80 ± 0.32^{c}	–
	AF1	43.42 ± 0.41^{a}	28.47
	EF1	41.44 ± 0.39^{b}	22.62
Photosynthesis (μ mol CO_2 m^{-2} s^{-1})	GT42	10.38 ± 0.10^{c}	–
	AF1	19.82 ± 0.19^{a}	90.97
	EF1	18.55 ± 0.18^{b}	78.72
Transpiration rate (mmol H_2O m^{-2} s^{-1})	GT42	1.14 ± 0.01^{c}	–
	AF1	2.64 ± 0.02^{a}	131.80
	EF1	2.52 ± 0.02^{b}	120.84
Stomatal conductance (mmol H_2O m^{-2} s^{-1})	GT42	39.63 ± 0.37^{b}	–
	AF1	82.42 ± 0.78^{a}	107.97
	EF1	81.31 ± 0.77^{a}	105.18

Different alphabets indicate significant differences between treatments at $p \leq 0.05$.

Biological Nitrogen Fixation Contribution by the ^{15}N Isotopic Dilution Method

^{15}N isotope dilution method showed that N content was higher in sugarcane plants (GT42) after inoculation of AE1 and EF1 strains as compared to control plants (**Figure 9**). The maximum tissue concentration of N was found in roots than leaf and stem tissues, respectively (**Figure 9A**). Whereas, % ^{15}N atom excess of sugarcane leaf was higher than the root and stem tissues after bacterial inoculation (**Figure 9B**). Therefore, biological N fixation in sugarcane by selected isolates was confirmed.

Greenhouse Assay for Diazotrophic Isolates

We measured the different physiological growth parameters such as plant height, root and shoot weight, leaf area, chlorophyll content, photosynthesis, transpiration rate, stomatal conductance, and total protein content of sugarcane (GT42) inoculated with (AF1 and EF1) strains under greenhouse conditions. As compared to control, isolates AF1 and EF1 showed increased plant height, root weight, and shoot weight, i.e., 46, 32, and 72% and 31, 169, and 123%, respectively (**Table 3**). Whereas, no more significant difference was observed in leaf area and total protein content of sugarcane (**Table 3**). **Table 3** shows significantly higher chlorophyll content (SPAD values) in inoculated sugarcane leaves than in control plants. In general, the SPAD values were lower in EF1 than AF1. The photosynthesis rate was marginally higher in plants-inoculated with AF1 (19.82 ± 0.19 μ mol CO_2 m^{-2} s^{-1}) than those with EF1 (18.55 ± 0.18 μ mol CO_2 m^{-2} s^{-1}). And, it was also found that the transpiration rate in the sugarcane plant was significantly lower in EF1 than AF1 inoculated bacteria but as compared to the control plant both isolates increased up to 131 and 120% (**Table 3**). When comparing both strain's stomatal conductance of sugarcane plants, there were no more significant differences observed, but over control, the observed percent increase values were 107 and 105 for AF1 and EF1 strains (**Table 3**). Overall, these results suggest that both selected diazotrophic isolates are effective in sugarcane growth enhancement, and the application of strain AF1 showed more significant improvement as compared to EF1 in GT42.

Measurement of N Metabolism and Biocontrol Enzymes Activities

An increased level of selected N metabolism and biocontrol-related enzymes was observed in sugarcane plants (GT42) treated with diazotrophs (AF1 and EF1) (**Figure 10**). The plants inoculated with AF1 and EF1 showed 61 and 68% greater GS activity than control plants (**Figure 10A**), whereas, increase in NADH-GDH content was higher in AF1 (43%) inoculated plants than EF1 (36%) as compared to their controls (**Figure 10B**). Following inoculation with isolates, NR activity was higher in

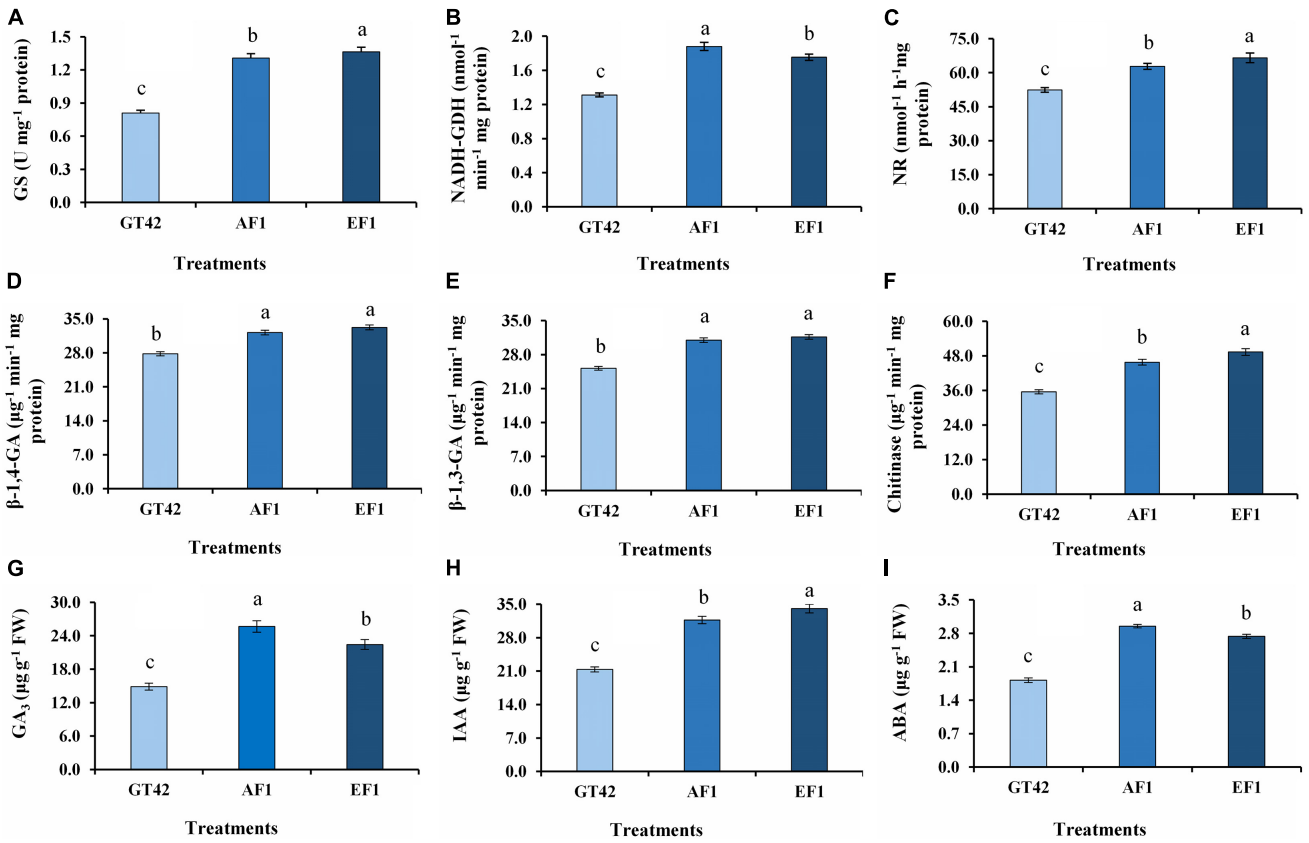


FIGURE 10 | Analysis of N-metabolism enzymes, hydrolytic enzymes, and hormone activities in root tissues of sugarcane (GT42) inoculated with *P. cypripedii* AF1 and *K. arachidis* EF1. **(A)** Glutamine synthetase, **(B)** glutamate dehydrogenase, **(C)** nitrate reductase, **(D,E)** β-1,4 and β-1,3 glucanase, **(F)** chitinase, **(G)** gibberellins, **(H)** indole-3-acetic acid, and **(I)** abscisic acid. Different lowercase letters display a significant difference at $p \leq 0.05$.

EF1 and AF1 inoculated plants (66.56 ± 2.11 and 62.78 ± 1.33 n mol^{-1} h^{-1} mg proteins) than control (52.39 ± 0.98 n mol^{-1} h^{-1} mg proteins) (**Figure 10C**). Inoculation with endophytic diazotroph EF1 and AF1 significantly enhanced 19 and 16% β-1,4-glucanase activity in the root as compared to non-inoculated sugarcane plants (**Figure 10D**). A similar pattern of β-1,3-glucanase activity was observed in sugarcane inoculated with both isolates (AF1 and EF1) (**Figure 10E**). A significant increase in chitinase activity (28 and 38%) was observed in roots of GT42 inoculated with AF1and EF1 as compared to non-inoculated sugarcane plants (**Figure 10F**).

In this study, the significant difference in GA_3, IAA, and ABA level was observed between the roots of bacteria-inoculated and uninoculated sugarcane plants (**Figure 10**). The treatment of plants with AF1 and EF1 increased GA production in root by 72 and 51%, respectively, as compared to control (**Figure 10G**). The IAA production in the root was increased more with the EF1 inoculation (**Figure 10H**). EF1 and AF1 inoculation increased IAA content by 59 and 48%, respectively, in inoculated plants, as compared to the non-inoculated plants. ABA production by sugarcane plants inoculated with AF1 and EF1 strains in 62 and 50%, respectively, higher compared with the non-inoculated plants.

DISCUSSION

Diazotrophs, known for their BNF properties, also produce antibiotics, HCN, and siderophores, as well as build systemic resistance in plants, to inhibit a wide spectrum of plant fungal pathogens (Das et al., 2017; Ali et al., 2020). Hence, the development of diazotrophic bacteria as a biofertilizer with multi-functional traits might help boost plant growth and crop production. In a previous survey of sugarcane-producing regions in Guangxi, China, Lin et al. (2012) found that *Klebsiella* is the most abundant plant-associated N-fixing bacteria. Thus, the screening and selection of different diazotrophic bacteria with PGP activities will be valuable for sustainable sugarcane agriculture, especially in low fertile soils. In this research, a culture-independent approach was used to discover the potential Gram-negative endophytic diazotrophic bacteria along with PGP features for their potential application as biofertilizers in sugarcane. A total of 175 endophytic isolates were isolated and after 16S *rRNA* gene sequencing, we selected 11 strains of genus *Kosakonia* (*K. oryzae*, *K. radicincitans*, *K. arachidis*, *K. oryziphila*, *K. quasisacchari*, and *K. pseudosacchari*) and *Pantoea* (*P. dispersa*, *P. cypripedii*, *P. ananatis*, *P. allii*, and *P. agglomerans*). A number of different diazotrophic bacterial species isolated from sugarcane

can also be opportunistic pathogens, but these microbes usually do not cause diseases in a healthy host (Fishman, 2013; Berg et al., 2014). The strains selected in this study also showed abiotic stress tolerance properties as they grew on a wide range of temperatures (20–45°C), pH (5–10), and salinity (7–12% NaCl), suggesting that these isolates may tolerate stressful crop production environments. Two strains *P. cypripedii* AF1 and *K. arachidis* EF1, which exhibited multiple PGP traits, were selected for detailed study.

The use of N-fixing beneficial microbes in agriculture might reduce the usage of chemical N fertilizers in agriculture, and reducing their negative environmental impacts (Noar and Bruno-Bárcena, 2018). Both selected strains showed the presence of *nifH* gene, which encodes the nitrogenase reductase enzyme. We observed the enhanced expression of N metabolism (*AMT, NRT*, *NR, GS*, and *GOGAT*) and BNF-related genes (*nifH*) in sugarcane after inoculation with AF1 or EF1 strains. *AMT* and *NRT* facilitate nitrate-N and ammonium-N uptake in the roots (Singh et al., 2016). The expression of *NRT, NR*, and *GOGAT* was higher in EF1-inoculated GT42 roots compared with AF1-inoculated ones with, indicating the strain variability for plant N metabolism regulation. Nitrate is taken up by roots and converted into glutamine and glutamate, which are used by plants to make other amino acids and nitrogenous compounds (Pratelli and Pilot, 2014). The primary assimilation of nitrate-N and ammonium-N in plants is mediated by two important N-assimilation enzymes, NR and GS (Funayama et al., 2013). The activities of N metabolism-related enzymes (GS, NADH-GDH, and NR) in AF1- and EF1-inoculated plants show that these bacteria had considerably increased N use efficiency in sugarcane (**Figure 10**). Glutamate synthase is a vital enzyme in N assimilation, metabolism, and remobilization, and its activity is influenced by environmental factors (Miflin and Habash, 2002; Stitt et al., 2002). It was observed that the activity of GS was higher in EF1 than that observed for AF1 (**Figure 10**). The high concentration of GS1 transcripts in sugarcane leaves implies that this isoform plays an important role in C4 plants for N metabolism (Nogueira et al., 2005). Ammonia production by PGP diazotrophic isolates contributes N to the host plants and supports biomass production (Marques et al., 2010). Our results showed that all Gram-negative endophytic bacteria produced ammonia, with *P. cypripedii* AF1 and *K. arachidis* EF1 being more efficient than the other isolates.

The technique of ^{15}N isotope dilution has been frequently utilized to measure the contribution of associative N-fixing systems to graminaceous crops like sugarcane (Urquiaga et al., 1992; Asis et al., 2002; Montañez et al., 2009; Thaweenut et al., 2011; Lin et al., 2012; Li et al., 2017; Singh et al., 2020c, 2021b). This study was performed in pots that used total N balance and enriched ^{15}N fertilizer isotope-dilution methods, and the outcome was impressive. The findings of the ^{15}N enrichment indicated that there was a significant amount of non-labeled N present, which could only have originated from BNF (Martins et al., 2020). Previous, investigations have found that Brazilian sugarcane cultivars were able to acquire 40–100 kg N ha^{-1} yr^{-1} from BNF utilizing plants without any treatment. A significant development of plant growth and N accumulation using diazotrophic bacteria was also observed in different sugarcane varieties (Li et al., 2017; Singh et al., 2020b). However, even after three decades of research, our understanding as to how the diazotrophs work, fix N, and transfer the fixed N to the crop remains limited (Martins et al., 2020).

Plant growth and crop productivity can be improved by siderophore-producing diazotrophic bacteria by improving Fe accessibility and boosting plant growth (Ahmed and Holmström, 2014). As expected, all chosen strains were capable of generating iron-chelating siderophores at various levels, and this trait is usually present in *Pantoea* and *Kosakonia* genera (Chen et al., 2017; Lambrese et al., 2018; Romano et al., 2020). Also, in this study, only eight diazotrophic isolates (73%) were able to solubilize phosphate. In the soil, Phosphate (P) is present abundantly in most a crop land but mostly unavailable to the plants, hence this is one of the main growth-limiting nutrients for plants in agricultural systems. It's also critical for root formation, early shoot growth and tillering, early productivity, and plant stem elongation. Previously, a few strains of genus *Kosakonia* with P solubilizing ability were reported (Chakdar et al., 2018; Singh et al., 2020b). And, Chen and Liu (2019), studies found the genus *Pantoea* a highly effective P-solubilizing bacterium.

Hydrogen cyanide is a volatile secondary metabolite generated by several endophytic bacterial isolates involved in disease suppression and protection of the plants, particularly from fungal pathogens (Olanrewaju et al., 2017). The results showed that only 63% of endophytic isolates produced HCN. Strains that tested positive for HCN production were shown to be effective against sugarcane pathogens in this study. In many respects, these PGP results are similar to previous studies (Li et al., 2017; Guo et al., 2020; Singh et al., 2021a).

The sugarcane plant is affected by several fungal pathogens, therefore we screened 11 endophytic isolates for the antifungal property. Among them two selected strains showed good antagonistic activity against fungal pathogens, which was further corroborated by the degradation of fungal mycelia seen during SEM analysis. This finding supports earlier observations that, some diazotrophic bacterial strains isolated from sugarcane can suppress the plant fungal pathogens (Li et al., 2017; Guo et al., 2020). Additionally, all isolates were also tested for an important role in the production of hydrolytic enzymes such as chitinase, protease, cellulase, and endoglucanase. The role of these enzymes in biological control of various fungal pathogens has been previously reported (Huang et al., 2005; Singh et al., 2013; Passari et al., 2017, 2018). Pot experiment also showed the interaction of *P. cypripedii* AF1 and *K. arachidis* EF1 strains enhanced the production of CHI and GLU in root tissues of sugarcane. Similarly, CHI and GLU produced by different strains of genera *Pantoea* and *Kosakonia* improved sugarcane growth (Quecine et al., 2012; Singh et al., 2021b). The colonization of endophytic bacteria on plant tissues is also playing a crucial role in disease management and plant growth enhancement (Li et al., 2017; Guo et al., 2020). We examined the colonization of selected endophytic strains individually with genetically tagged GFP and found that both strains effectively colonized all plant tissues.

Our findings revealed a substantial influence of diazotrophic bacterial inoculation of sugarcane plant (GT42) with GA_3, IAA, and ABA content. Sugarcane inoculated with selected isolates had considerably greater levels of all phytohormones than GT42 control. These hormones promote plant growth, root development, fertilizer absorption and assimilation, water uptake and a variety of metabolic processes in response to abiotic and biotic stresses (Graham, 2003; Stepanova et al., 2007; Singh et al., 2019). 1-aminocyclopropane-1-carboxylate deaminase activity is one of the principal mechanisms of endophytic bacteria that can assist plant growth in the presence of various biotic and abiotic stresses. All endophytic isolates selected in this study synthesized ACC deaminase, and the presence of *acdS* gene was confirmed in eight isolates. The reason for the inability to detect this gene in three isolates remains unclear. Endophytic bacteria protect host plants from a range of stress conditions by generating chemicals and enzymes such as ACC deaminase to decrease ethylene production (Glick, 2014; Mercado-Blanco and Lugtenberg, 2014). Apart from conferring stress tolerance, ACC deaminase also promotes root growth, which will have a positive effect on healthy shoot development (Belimov et al., 2002; Glick et al., 2007). Bacterial isolates with ACC deaminase activity have been reported, but it did not show the presence of *acdS* gene (Nascimento et al., 2014).

CONCLUSION

Despite the variety of bacterial species identified in sugarcane-producing habitats, there is little information regarding the diversity of Gram-negative bacteria, their ecology, and biotechnological potential in commercial sugarcane production. The significance of this study is highlighted by the promotion of plant growth and development by the Gram-negative endophytic diazotrophs *P. cypripedii* AF1 and *K. arachidis* EF1, and this is the first report to show that these two diazotrophic bacteria reside in sugarcane roots and potentially contributing to sugarcane growth, development, and disease suppression. These isolates exhibited a variety of PGP characteristics, including antifungal action against plant pathogens, N fixation, and the synthesis of enzymes and phytohormones. Further research into these diazotrophic isolates is needed to evaluate their commercial value as bio-fertilizers for improved sugarcane agricultural productivity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

RS, PS, and Y-RL designed the experiments. RS, PS, and D-JG conducted the majority of the experiments. RS and PS wrote the article. PL, L-TY, and Y-RL reviewed and editing the article. The other authors assisted in experiments and discussed the results. All authors read and approved this manuscript.

FUNDING

This research was funded by the Guangxi Innovation Teams of Modern Agriculture Technology (nytxgxcxtd-2021-03-01) and Fund of Guangxi Academy of Agricultural Sciences (2021YT011).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.774707/full#supplementary-material

REFERENCES

Ahmed, E., and Holmström, S. J. (2014). Siderophores in environmental research: roles and applications. *Microbial Biotechnol.* 7, 196–208. doi: 10.1111/1751-7915.12117

Ali, J. G., Casteel, C., Mauck, K., and Trase, O. (2020). Chemical ecology of multitrophic microbial interactions: plants, insects, microbes and the metabolites that connect them. *J. Chem. Ecol.* 46, 645–648. doi: 10.1007/s10886-020-01209-y

Asis, C. A. Jr., Kubota, M., Ohta, H., Arima, Y., Ohwaki, Y., Yoneyama, T., et al. (2002). Estimation of the nitrogen fixation by sugarcane cultivars NiF-8 using 15N dilution and natural 15N abundance techniques. *Soil Sci. Plant Nutr.* 48, 283–285.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., et al. (2016). Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* 44, W147–W153. doi: 10.1093/nar/gkw419

Belimov, A. A., Safronova, V. I., and Mimura, T. (2002). Response of spring rape (*Brassica napus* L. var. Oleifera) to inoculation with plant growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. *Can. J. Microbiol.* 48, 189–199. doi: 10.1139/w02-007

Berg, G., Erlacher, A., Smalla, K., and Krause, R. (2014). Vegetable microbiomes: is there a connection among opportunistic infections, human health and our "gut feeling"? *Microb. Biotechnol.* 7, 487–495. doi: 10.1111/1751-7915.12159

Bhardwaj, D., Ansari, M. W., Sahoo, R. K., and Tuteja, N. (2014). Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microbial Cell Factories* 13:66. doi: 10.1186/1475-2859-13-66

Brock, A. K., Berger, B., Schreiner, M., Ruppel, S., and Mewis, I. (2018). Plant growth-promoting bacteria *Kosakonia radicincitans* mediate anti-herbivore defense in *Arabidopsis thaliana*. *Planta* 248, 1383–1392. doi: 10.1007/s00425-018-2964-0

Chakdar, H., Dastager, S. G., Khire, J. M., Rane, D., and Dharne, M. S. (2018). Characterization of mineral phosphate solubilizing and plant growth promoting bacteria from termite soil of arid region. *3 Biotech* 8:463. doi: 10.1007/s13205-018-1488-4

Chen, C., Xin, K., Liu, H., Cheng, J., Shen, X., Wang, Y., et al. (2017). Pantoea alhagi, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat. *Sci. Rep.* 7:41564.

Chen, Q., and Liu, S. (2019). Identification and characterization of the phosphate-solubilizing bacterium *Pantoea sp.* S32 in reclamation Soil

in Shanxi, China. *Front. Microbiol.* 10:2171. doi: 10.3389/fmicb.2019.02171

Das, K., Prasanna, R., and Saxena, A. K. (2017). Rhizobia: a potential biocontrol agent for soilborne fungal pathogens. *Folia Microbiol.* 62, 425–435. doi: 10.1007/s12223-017-0513-z

Dobereiner, J., Reis, V. M., Paula, M. A., and Olivares, F. L. (1993). "Endophytic diazotrophs in sugar cane, cereals and tuber plants," in *New Horizons in Nitrogen Fixation*, eds R. Palacios, J. Mora, and W. E. Newton (Dordrecht: Kluwer Academic), 671–676. doi: 10.1007/978-94-017-2416-6_55

Edwards, U., Rogall, T., Blöcker, H., Emde, M., and Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 17, 7843–7853. doi: 10.1093/nar/17.19.7843

FAO (2020a). *FAO Agricultural Outlook. Agriculture Statistics*. Rome: FAO.

FAO (2020b). *World Fertilizer Trends and Outlook to 2018*. Rome: FAO.

Felsenstein, J. (1985). Confidence limits on phylogenies, an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Fishman, J. A. (2013). Opportunistic infections–coming to the limits of immune suppression? *Cold Spring Harb. Perspect. Med.* 3:a015669. doi: 10.1101/cshperspect.a015669

Fiske, C. H., and Subbarow, Y. (1925). A colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375–400.

Fraser, E. D. G., and Campbell, M. (2019). Agriculture 5.0: reconciling production with planetary health. *One Earth* 1, 278–280.

Funayama, K., Kojima, S., Tabuchi-Kobayashi, M., Sawa, Y., Nakayama, Y., Hayakawa, T., et al. (2013). Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots. *Plant Cell Physiol.* 54, 934–943. doi: 10.1093/pcp/pct046

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Glick, B. R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., and McConkey, B. (2007). Promotion of plant growth by bacterial ACC deaminase. *Crit. Rev. Plant Sci.* 26, 227–242.

Gordon, S. A., and Weber, R. P. (1951). Colorimetric estimation of indole acetic acid. *Plant Physiol.* 26, 192–195.

Goswami, D., Dhandhukia, P., Patel, P., and Thakker, J. N. (2014). Screening of PGPR from saline desert of Kutch: growth promotion in *Arachis hypogea* by Bacillus licheniformis A2. *Microbiol. Res.* 169, 66–75. doi: 10.1016/j.micres.2013.07.004

Graham, N. (2003). Dissecting *Arabidopsis* lateral root development. *Trends Plant Sci.* 8, 165–171.

Guo, D. J., Singh, R. K., Singh, P., Li, D. P., Sharma, A., Xing, Y. X., et al. (2020). Complete genome sequence of *Enterobacter roggenkampii* ED5, a nitrogen fixing plant growth promoting endophytic bacterium with biocontrol and stress tolerance properties, isolated from sugarcane root. *Front. Microbiol.* 11:580081. doi: 10.3389/fmicb.2020.580081

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. (1968). The acetylene ethylene assay for N fixation, laboratory and field evaluation. *Plant Physiol.* 43, 1185–1207. doi: 10.1104/pp.43.8.1185

Honma, M., and Shimomura, T. (1978). Metabolism of 1-aminocyclopropane-1-carboxylic acid. *Agric. Biol. Chem.* 42, 1825–1831.

Hu, Q. P., and Xu, J. G. (2011). A simple double-layered chrome azurol S agar (SDCASA) plate assay to optimize the production of siderophores by a potential biocontrol agent *Bacillus*. *Afr. J. Microbiol. Res.* 5, 4321–4327.

Huang, C. J., Wang, T. K., Chung, S. C., and Chen, C. Y. (2005). Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *J. Biochem. Mol. Biol.* 38, 82–88.

James, E. K. (2000). Nitrogen fixation in endophytic and associative symbiosis. *Food Crop Res.* 65, 197–209.

Kour, D., Rana, K. L., Yadav, A. N., Yadav, N., Kumar, M., Kumar, V., et al. (2020). Microbial biofertilizers: bioresources and eco-friendly technologies for agricultural and environmental sustainability. *Biocatal. Agric. Biotechnol.* 23:101487. doi: 10.1016/j.bcab.2019.101487

Kumar, M., Grader, G., Sessitsch, A., Maki, A., van Elsas, J. D., and Nissien, R. (2017). Plants assemble species specific bacterial communities from common core taxa in three arcto-alpine climate zones. *Front. Microbiol.* 8:12. doi: 10.3389/fmicb.2017.00012

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X, molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Lambrese, Y., Guiñez, M., Calvente, V., Sansone, G., Cerutti, S., Raba, J., et al. (2018). Production of siderophores by the bacterium Kosakonia radicincitans and its application to control of phytopathogenic fungi. *Bioresour. Technol. Rep.* 3, 82–87.

Leghari, S. J., Wahocho, N. A., Laghari, G. M., Laghari, A. H., Bhabhan, G. M., Talpur, K. H., et al. (2016). Role of nitrogen for plant growth and development: a review. *Adv. Environ. Biol.* 10, 209–218.

Li, H. B., Singh, R. K., Singh, P., Song, Q. Q., Xing, Y. X., Yang, L. T., et al. (2017). Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas species* isolated from sugarcane rhizosphere. *Front. Microbiol.* 8:1268. doi: 10.3389/fmicb.2017.01268

Li, Y. R., Song, X. P., Wu, J. M., Li, C. N., Liang, Q., Liu, X. H., et al. (2016). Sugar industry and improved sugarcane farming technologies in China. *Sugar Tech.* 18, 603–611. doi: 10.1007/s12355-016-0480-488

Li, Z., Chang, S., Lin, L., Li, Y., and An, Q. (2011). A colorimetric assay of 1- aminocyclopropane-1-carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase. *Lett. Appl. Microbiol.* 53, 178–185. doi: 10.1111/j.1472-765X.2011.03088.x

Lin, L., Li, Z., Hu, C., Zhang, X., Chang, S., Yang, L., et al. (2012). Plant growth-promoting nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi. China. *Microbes Environ.* 27, 391–398. doi: 10.1264/jsme2.me11275

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-11Ct method. *Methods* 25, 402–408.

Lorck, H. (1948). Production of hydrocyanic acid by bacteria. *Physiol. Plant.* 1, 142–146.

Marques, A. P. G. C., Pires, C., Moreira, H., Rangel, A. O. S. S., and Castro, P. M. L. (2010). Assessment of the plant growth promotion abilities of six bacterial isolates using Zea mays as indicator plant. *Soil Biol. Biochem.* 42, 1229–1235.

Martins, D. S., Reis, V. M., Schultz, N., Alves, B. J. R., Urquiaga, S., Pereira, W., et al. (2020). Both the contribution of soil nitrogen and of biological N2 fixation to sugarcane can increase with the inoculation of diazotrophic bacteria. *Plant Soil.* 454, 155–169.

Mercado-Blanco, J., and Lugtenberg, B. (2014). Biotechnological applications of bacterial endophytes. *Curr. Biotechnol.* 3, 60–75.

Miflin, B. J., and Habash, D. Z. (2002). The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J. Exp. Bot.* 53, 979–987. doi: 10.1093/jexbot/53.370.979

Mihalache, G., Zamfirache, M. M., and Stefan, M. (2015). Root associated bacteria—friends or enemies? a review. *Mem. Sci. Sect. Rom. Acad.* 38, 27–54.

Montañez, A., Abreu, C., Gill, P. R., Hardarson, G., and Sicardi, M. (2009). Biological nitrogen fixation in maize (*Zea mays* L.) by 15N isotope-dilution and identification of associated culturable diazotrophs. *Biol Fert Soils.* 45, 253–263.

Nascimento, F. X., Rossi, M. J., Soares, C. R. F. S., McConkey, B. J., and Glick, B. R. (2014). New insights into 1-Aminocyclopropane1-carboxylate (ACC) deaminase phylogeny. evolution and ecological significance. *PLoS One* 9:e99168. doi: 10.1371/journal.pone.0099168

Nei, M., and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York, NY: Oxford University Press.

Noar, J. D., and Bruno-Bárcena, J. M. (2018). Azotobacter vinelandii: the source of 100 years of discoveries and many more to come. *Microbiology* 164, 421–436. doi: 10.1099/mic.0.000643

Nogueira, E. D. M., Olivares, F. L., Japiassu, J. C., Vilar, C., Vinagre, F., Baldani, J. I., et al. (2005). Characterization of glutamine synthetase genes in sugarcane genotypes with different rates of biological nitrogen fixation. *Plant Sci.* 169, 819–832.

Olanrewaju, O. S., Glick, B. R., and Babalola, O. O. (2017). Mechanisms of action of plant growth promoting bacteria. *World J. Microbiol. Biotechnol.* 33: 197.

Oleńska, E., Małek, W., Wójcik, M., Swiecicka, I., Thijs, S., and Vangronsveld, J. (2020). Beneficial features of plant growth-promoting rhizobacteria for improving plant growth and health in challenging conditions: a methodical

review. *Sci. Total Enviorn.* 743:140682. doi: 10.1016/j.scitotenv.2020.140682

Passari, A. K., Lalsiamthari, P. C., Zothanpuia, Leo, V. V., Mishra, V. K., Yadav, M. K., et al. (2018). Biocontrol of *Fusarium wilt* of *Capsicum annuum* by rhizospheric bacteria isolated from turmeric endowed with plant growth promotion and disease suppression potential. *Eur. J. Plant Pathol.* 150, 831–846.

Passari, A. K., Mishra, V. K., Singh, G., Singh, P., Kumar, B., Gupta, V. K., et al. (2017). Insights into the functionality of endophytic actinobacteria with a focus on their biosynthetic potential and secondary metabolites production. *Sci. Rep.* 7:11809.

Payne, S. M. (1993). Iron acquisition in microbial pathogenesis. *Trends Microbiol.* 1, 66–69. doi: 10.1016/0966-842x(93)90036-q

Penrose, D. M., and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant.* 118, 10–15. doi: 10.1034/j.1399-3054.2003.00086.x

Poly, F., Monrozier, L. J., and Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. *Res. Microbiol.* 152, 95–103. doi: 10.1016/s0923-2508(00)01172-4

Pratelli, R., and Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *J. Exp. Bot.* 65, 5535–5556. doi: 10.1093/jxb/eru320

Quecine, M. C., Araújo, W. L., Rossetto, P. B., Ferreira, A., Tsui, S., Lacava, P. T., et al. (2012). Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1. *Appl. Environ. Microbiol.* 78, 7511–7518. doi: 10.1128/AEM.00836-12

Raklami, A., Bechtaoui, N., Tahiri, A. I., Anli, M., Meddich, A., and Oufdou, K. (2019). Use of rhizobacteria and mycorrhizae consortium in the open field as a strategy for improving crop nutrition, productivity and soil fertility. *Front. Microbiol.* 10:1106. doi: 10.3389/fmicb.2019.01106

Ramette, A., Frapolli, M., Defago, G., and Moenne-Loccoz, Y. (2003). Phylogeny of HCN synthase-encoding hcnBC genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. *Mol. Plant Microb. Interact.* 16, 525–535. doi: 10.1094/MPMI.2003.16.6.525

Romano, I., Ventorino, V., Ambrosino, P., Testa, A., Chouyia, F. E., and Pepe, O. (2020). Development and application of low-cost and eco-sustainable bio-stimulant containing a new plant growth-promoting strain *Kosakonia pseudosacchari* TL13. *Front. Microbiol.* 11:2044. doi: 10.3389/fmicb.2020.02044

Saitou, N., and Nei, M. (1987). The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Sharma, A., Kashyap, P. L., Srivastava, A. K., Bansal, Y. K., and Kaushik, R. (2019). Isolation and characterization of salt-tolerant bacilli from chickpea (*Cicer arietinum* L.) rhizosphere for plant growth promotion and biocontrol traits. *Eur. J. Plant Pathol.* 153, 787–800.

Sharma, A., Singh, R. K., Singh, P., Vaishnav, A., Guo, D.-J., Verma, K. K., et al. (2021). Insights into the bacterial and nitric oxide-induced salt tolerance in sugarcane and their growth promoting abilities. *Microorganisms* 9: 2203.

Singh, A., Kumar, P., Gautam, V., Rengasamy, B., Adhikari, B., Udayakumar, M., et al. (2016). Root transcriptome of two contrasting indica rice cultivars uncovers regulators of root development and physiological responses. *Sci. Rep.* 6:39266. doi: 10.1038/srep39266

Singh, P., Singh, R. K., Li, H. B., Guo, D. J., Sharma, A., Lakshmanan, P., et al. (2021b). Diazotrophic bacteria pantoea dispersa and *Enterobacter asburiae* promote sugarcane growth by inducing nitrogen uptake and defense-related gene expression. *Front. Microbiol.* 11:600417. doi: 10.3389/fmicb.2020.600417

Singh, P., Singh, R. K., Song, Q. Q., Li, H. B., Guo, D. J., Malviya, M. K., et al. (2021c). Comparative analysis of protein and differential responses of defense related gene and enzyme activity reveals the long-term molecular responses of sugarcane inoculated with *Sporisorium scitamineum*. *J. Plant Interact.* 16, 12–29.

Singh, P., Singh, R. K., Guo, D.-J., Sharma, A., Singh, R. N., Li, D.-P., et al. (2021a). Whole genome analysis of sugarcane root-associated endophyte *Pseudomonas aeruginosa* B18—A plant growth-promoting bacterium with antagonistic potential against *Sporisorium scitamineum*. *Front. Microbiol.* 1:628376. doi: 10.3389/fmicb.2021.628376

Singh, R. K., Singh, P., Li, H. B., Guo, D. J., Song, Q. Q., Yang, L. T., et al. (2020b). Plant-PGPR interaction study of plant growth-promoting diazotrophs Kosakonia radicincitans BA1 and *Stenotrophomonas maltophilia* COA2 to enhance growth and stress-related gene expression in *Saccharum spp*. *J. Plant Interact.* 15, 427–445.

Singh, R. K., Singh, P., Li, H. B., Song, Q. Q., Guo, D. J., Solanki, M. K., et al. (2020c). Diversity of nitrogen-fixing rhizobacteria associated with sugarcane, a comprehensive study of plant-microbe interactions for growth enhancement in *Saccharum spp*. *BMC Plant Biol.* 20:220. doi: 10.1186/s12870-020-02400-9

Singh, P., Singh, R. K., Song, Q. Q., Li, H. B., Yang, L. T., and Li, Y. R. (2020a). Methods for estimation of nitrogen components in plants and microorganisms. *Methods Mol. Biol.* 2057, 103–112. doi: 10.1007/978-1-4939-9790-9_10

Singh, P., Song, Q. Q., Singh, R. K., Li, H. B., Solanki, M. K., Malviya, M. K., et al. (2019). Proteomic analysis of the resistance mechanisms in sugarcane during *Sporisorium scitamineum* infection. *Int. J. Mol. Sci.* 20:569. doi: 10.3390/ijms20030569

Singh, P., Song, Q. Q., Singh, R. K., Li, H. B., Solanki, M. K., Yang, L. T., et al. (2018). Physiological and molecular analysis of sugarcane (varieties- F134 and NCo310) during *Sporisorium scitamineum* interaction. *Sugar Tech.* 21, 631–644.

Singh, R. K., Kumar, D. P., Singh, P., Solanki, M. K., Srivastava, S., Srivastva, et al. (2014). Multifarious plant growth promoting characteristics of chickpea rhizosphere associated *Bacilli* help to suppress soil-borne pathogens. *Plant Growth Regul.* 73, 91–101.

Singh, R. K., Kumar, D. P., Solanki, M. K., Singh, P., Srivastva, A. K., Kumar, S., et al. (2013). Optimization of media components for chitinase production by chickpea rhizosphere associated *Lysinibacillus fusiformis* B-CM18. *J. Basic Microbiol.* 53, 451–460. doi: 10.1002/jobm.201100590

Stefan, M., Munteanu, N., Stoleru, V., and Mihasan, M. (2013). Effects of inoculation with plant growth promoting rhizobacteria on photosynthesis, antioxidant status and yield of runner bean. *Rom. Biotechnol. Lett.* 18, 8132–8143.

Stepanova, A. N., Yun, J., Likhacheva, A. V., and Alonso, J. M. (2007). Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. *Plant Cell* 19, 2169–2185. doi: 10.1105/tpc.107.052068

Stitt, M., Müller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., et al. (2002). Steps towards an integrated view of nitrogen metabolism. *J. Exp. Bot.* 53, 959–970. doi: 10.1093/jexbot/53.370.959

Suman, A., Shukla, L., Marag, P. S., Verma, P., Gond, S., and Prasad, J. S. (2020). Potential use of plant colonizing Pantoeaas generic plant growth promoting bacteria for cereal crops. *J. Environ. Biol.* 41, 987–994. doi: 10.1128/AEM.00582-21

Sur, S., Bothra, A. K., and Sen, A. (2010). Symbiotic nitrogen fixation-a bioinformatics perspective. *Biotech* 9, 257–273.

Tambong, J. T. (2019). Taxogenomics and systematics of the genus pantoea. *Front. Microbiol.* 10:2463. doi: 10.3389/fmicb.2019.02463

Thaweenut, N., Hachisuka, Y., Ando, S., Yanagisawa, S., and Tadakatsu, Y. (2011). Two seasons' study on nifH gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum spp*. hybrids): expression of nifH genes similar to those of rhizobia. *Plant Soil.* 338, 435–449.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671–677. doi: 10.1038/nature01014

United States Department of Agriculture [USDA] (2020). *Sugar: World Markets and Trade*. Washington, D.C: USDA.

Urquiaga, S., Cruz, K. H. S., and Boddey, R. M. (1992). Contribution of nitrogen fixation to sugarcane: nitrogen-15 and nitrogen-balance estimates. *Soil Sci. Soc. Am. J.* 56, 105–114.

Vanlauwe, B., Hungria, M., Kanampiu, F., and Giller, K. E. (2019). The role of legumes in the sustainable intensification of African smallholder agriculture: lessons learnt and challenges for the future. *Agric. Ecosyst. Environ.* 284:106583. doi: 10.1016/j.agee.2019.106583

Vital, C. E., Giordano, A., de Almeida Soares, E., Rhys Williams, T. C., Mesquita, R. O., Vidigal, P., et al. (2017). An integrative overview of the molecular and physiological responses of sugarcane under drought conditions. *Plant Mol. Biol.* 94, 577–594. doi: 10.1007/s11103-017-0611-y

Yang, L., Deng, Y., Wang, X., Zhang, W., Shi, X., Chen, X., et al. (2021). Global direct nitrous oxide emissions from the bioenergy crop sugarcane (*Saccharum spp*. inter-specific hybrids). *Sci. Total Enviorn.* 752:141795. doi: 10.1016/j.scitotenv.2020.141795

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 December 2021
doi: 10.3389/fmicb.2021.797889

Genome Characteristics Reveal the Biocontrol Potential of Actinobacteria Isolated From Sugarcane Rhizosphere

Zhen Wang[1,2,3†], Manoj Kumar Solanki[4†], Zhuo-Xin Yu[3], Muhammad Anas[3], Deng-Feng Dong[3], Yong-Xiu Xing[3], Mukesh Kumar Malviya[2], Fei Pang[1] and Yang-Rui Li[2,3]**

[1] Guangxi Key Laboratory of Agricultural Resources Chemistry and Biotechnology, College of Biology and Pharmacy, Yulin Normal University, Yulin, China, [2] Key Laboratory of Sugarcane Biotechnology and Genetic Improvement (Guangxi), Guangxi Key Laboratory of Sugarcane Genetic Improvement, Ministry of Agriculture, Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences, Nanning, China, [3] Agricultural College, Guangxi University, Nanning, China, [4] Plant Cytogenetics and Molecular Biology Group, Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Katowice, Poland

To understand the beneficial interaction of sugarcane rhizosphere actinobacteria in promoting plant growth and managing plant diseases, this study investigated the potential role of sugarcane rhizospheric actinobacteria in promoting plant growth and antagonizing plant pathogens. We isolated 58 actinobacteria from the sugarcane rhizosphere, conducted plant growth-promoting (PGP) characteristics research, and tested the pathogenic fungi *in vitro*. Results showed that BTU6 (*Streptomyces griseorubiginosus*), the most representative strain, regulates plant defense enzyme activity and significantly enhances sugarcane smut resistance by regulating stress resistance-related enzyme (substances (POD, PAL, PPO, TP) in sugarcane) activity in sugarcane. The genomic evaluation indicated that BTU6 has the ability to biosynthesize chitinase, β-1,3-glucanase, and various secondary metabolites and plays an essential role in the growth of sugarcane plants under biotic stress. Potential mechanisms of the strain in improving the disease resistance of sugarcane plants and its potential in biodegrading exogenous chemicals were also revealed. This study showed the importance of sugarcane rhizosphere actinobacteria in microbial ecology and plant growth promotion.

Keywords: actinobacteria, sugarcane, genome, biocontrol, smut

OPEN ACCESS

Edited by:
Prem Lal Kashyap, Indian Institute of Wheat and Barley Research (ICAR), India

Reviewed by:
Karthik Loganathan, Salem Microbes Pvt Ltd., India
Jen-Tsung Chen, National University of Kaohsiung, Taiwan
Rafael Soares Correa De Souza, State University of Campinas, Brazil
Leticia Bianca Pereira, State University of Campinas, Brazil

***Correspondence:**
Fei Pang
pangf123@foxmail.com
Yang-Rui Li
liyr5745@126.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *19 October 2021*
Accepted: *06 December 2021*
Published: *23 December 2021*

Citation:
Wang Z, Solanki MK, Yu Z-X, Anas M, Dong D-F, Xing Y-X, Malviya MK, Pang F and Li Y-R (2021) Genome Characteristics Reveal the Biocontrol Potential of Actinobacteria Isolated From Sugarcane Rhizosphere. Front. Microbiol. 12:797889. doi: 10.3389/fmicb.2021.797889

INTRODUCTION

The plant rhizosphere is a reservoir of microbial interactions that regulate plant activities. Plant roots exudates provide nutrients and energy substances to microbes (Fan et al., 2001; Bertin et al., 2003), and plant defense against pathogens in the rhizosphere by secreting antibiotics, inducing systemic resistance, or by direct antagonism against the pathogen for nutrient and space (Bakker et al., 2003); the roots also produce auxin (IAA), siderophores, dissolved inorganic phosphorus, which promote nutrient absorption and plant growth (Burdman et al., 2000).

Microorganisms are known to regulate plant gene expression, metabolism, growth and provide protection against multiple stress-causing factors (Ortíz Castro et al., 2009). Actinobacteria, which are gram-positive bacteria with high G + C DNA content that constitute one of the largest bacterial phyla, secrete several extracellular metabolites and antibiotics used as plant growth promoters (Strap, 2011). After biocontrol bacteria induce the host plant, it produces plant defense enzymes and disease-related proteins; lignin and phenol induction also directly improves the plant defense mechanisms (Zhu et al., 1995). A few studies have shown that *Streptomyces*, *Actinomadura*, *Micromonospora*, *Nocardia,* and other plant rhizosphere actinomycetes can enhance plant growth-promoting substances that increase plant biomass (Mishra et al., 1987; El-Tarabily et al., 2010). Moreover, several *Streptomyces* spp. have been recognized as potential biocontrol agents that indirectly increase plant disease resistance when pathogens invade the plant rhizosphere (Nimnoi et al., 2010). *Streptomyces* is widely utilized as a biocontrol agent due to its high sporulation capacity and the thick cell wall of its spores that result in long-term survival (Samac et al., 2003); this bacterium has a fast growth rate and is used as a bioinoculum in different forms, including powder, liquid, and cell biomass culture. For example, Mycostop, a biocontrol agent that contains actinomycetes cells, can control several soil-borne diseases such as *Rhizoctonia* spp., *Pythium* spp., *Fusarium* spp., *Phytophthora* spp. (Kortemaa et al., 1997; Hansen et al., 2010; Aggarwal et al., 2016), and Actinovate (containing 1% *Streptomyces lydicus* WYEC 108) and *Streptomyces griseoviridis* K61 have also been registered as biopesticides in the United States and France, respectively, and used in horticultural crops to manage soil-borne diseases, including *Fusarium* wilt and *Rhizoctonia* root rot (Yuan and Crawford, 1995; Minuto et al., 2006). In addition, *Streptomyces* secrete several antimicrobial compounds such as chitinase, glucanase, cellulase, organic acids, steroidal compounds, vitamins, and enzyme inhibitors (Mahadevan and Crawford, 1997; Palaniyandi et al., 2013). These metabolites plays an important role in plant diseases management and plant growth promotion (Olanrewaju and Babalola, 2019).

Plant diseases cause up to 25% of annual crop yield losses (Prashar et al., 2013). Plant growers well accept Microbe-based biological control technology in developed countries due to its low cost, environmental friendliness, and chemical-free residue. In recent years, microbes have been accepted as alternate sources for the biological control of plant diseases and plant growth promotion (Montesinos et al., 2002; Islam et al., 2016; Liu et al., 2018). Microbial biocontrol agents inhibit pathogens by different mechanisms, including antibiotics secretion, competition for food and space, direct parasitism, induced resistance of plant, and growth promotion of plant in stressed conditions (Van Wees et al., 2008; Kannan and Sureendar, 2009; de Jesus Sousa and Olivares, 2016; Viaene et al., 2016). However, the role of sugarcane rhizosphere actinomycetes in plant growth management and plant growth promotion is not well documented. In our previous studies, the actinobacterial genome has provided new insights on microorganisms that can improve drought resistance in plants (Wang et al., 2018). This study aimed to characterize actinomycetes in the sugarcane rhizosphere and their growth-promoting activity in order to provide new references for microorganisms that can antagonize plant pathogens and improve plant health.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Rhizosphere soil samples were collected from experiment fields of Sugarcane Research Institute of Guangxi University, Nanning (Guangxi, China). The rhizosphere soil sampling was performed according to the procedure of Gobran and Clegg (1996). The sugarcane rhizosphere soil samples were collected from the root surface (0.5–5 mm) for the study. We used a modified method of Shahidi Bonjar et al. (2005) to isolate the actinomycetes. Briefly, 1 g of rhizosphere soil sample was added into a centrifuge tube containing 9 mL sterile water and incubated in a shaker at 100 rpm for 30 min. The suspension was subjected to gradient dilution and 200 μL of the diluted soil suspension was added to Gauze medium (Solarbio Biotech Co., Ltd., Beijing, China), containing 20 μg mL^{-1} nalidixic acid, and cultured at 30°C until colony appeared. Pure colonies were separately transferred into the Gauze agar plate. The purified strains were stored in 20% glycerol at –80°C. *Streptomyces chartreusis* WZS021 (Wang et al., 2016, 2018) was used as a reference strain for phylogenetic analysis and PGP characteristics.

Plant Growth-Promoting Characteristics

To assess the production of indole acetic acid (IAA), 5 mL Luria broth was modified 0.5 mg mL^{-1} tryptophan, and the test strain was inoculated and incubated for 3 days on an incubator shaker at 180 rpm on 30°C. After incubation, the broth was centrifuged and to 1 mL of supernatant, 50 μL of 10 mmol L^{-1} orthophosphoric acid was added followed by the addition of 2 mL of Salkowski's reagent for color development at 25°C incubation for 30 min in the dark; the optical density was taken at 530 nm. The different concentrations of IAA standard solution were used for the standard curve to quantify the IAA production (Gordon and Weber, 1951). For the siderophore test, the active strain was streaked onto the chrome azurol S (CAS) plate (Alexander and Zuberer, 1991) and incubated at 30°C. After 5 days, the presence or absence of an orange-yellow halo around the colonies on the medium was observed. The phosphate solubilizing ability of the strain was determined by inoculating the active strain in a phosphate dissolving medium (Hopebio Biological, Qingdao, China) and incubating for 5 days at 30°C; the halo area around the colony indicated that the bacteria had the ability to dissolve phosphorus. ACC deaminase activity was determined by measuring the production of α-ketobutyrate and ammonia produced by ACC cleavage according to (Penrose and Glick, 2003).

DNA Extraction of Actinomycetes and Amplification of *16S* rRNA Sequences

Bacterial genomic DNA extraction kit (CWBIO Biotechnology Co., Ltd., Beijing, China) was used to isolate genomic DNA, and DNA purity and quantity were monitored using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington,

DE, United States). PCR amplification of the *16S* rRNA and secondary metabolites synthase genes were carried out using the isolated genomic DNA of actinomycetes. The PCR system and reaction conditions are shown in **Supplementary Tables 1**, **2**. The amplified PCR product was purified using the SanPrep column DNA gel recovery kit (Sangon Biotech Co., Ltd., Shanghai, China) and Sanger sequencing was performed in Sangon Biotech Co., Ltd. (Shanghai, China). The sequence reads in Fasta format were aligned in the NCBI database and accession numbers were obtained (**Table 1**). NCBI GenBank database using a BLASTn program. Identification to the species level was determined as maximum homology (C97%) to a type strain sequence in the GenBank. Evolutionary trees for the data sets were inferred by the neighbor-joining method of Saitou and Nei (1987) by using the neighbor-joining programby MEGA 6.0 software (Tamura et al., 2013) to construct the phylogenetic tree of actinomycetes.

Detection of Antifungal Activity of Actinomycetes

To test the antagonism of actinomycetes, nine economically important plant pathogens were used in the dual culture assay (**Supplementary Table 3**). A 5 mm disc of full-grown pathogenic fungi was inoculated in the center of the potato dextrose agar plate, and an active actinomycetes strain was streaked 30 mm away from the disc of the pathogenic fungi. In each experiment, three replicates were performed, and pathogenic fungi without active strains were treated as controls. All plates were incubated at 28°C for 7 days, and mycelial growth and zone of inhibition were calculated as compared to control.

Greenhouse-Based Biocontrol Assay Against the Smut Pathogen

The experiment was carried out in the sugarcane greenhouse located in the Guangxi University (Nanning, China). The indoor temperature was 24–32°C. The characteristics of the soil were as follows: pH, 6.76; organic matter, 21.42 $g{\cdot}kg^{-1}$; total nitrogen, 0.94 $g{\cdot}kg^{-1}$; total phosphorus, 2.73 $g{\cdot}kg^{-1}$; total potassium, 8.02 $g{\cdot}kg^{-1}$; alkaline nitrogen, 108 $mg{\cdot}kg^{-1}$; available phosphorus, 96 $mg{\cdot}kg^{-1}$; and available potassium, 79 $mg{\cdot}kg^{-1}$. We used three different treatments in this experiment: T1 (no inoculation, only water), T2 (inoculation of smut pathogen only), T3 (inoculation of smut pathogen and BTU6 as biocontrol agent). Thirty sugarcane plants were used per treatment and the experiment was repeated three times. Sugarcane stems with single shoots were soaked in water for 30 min at 52°C for hot water treatment, then soaked in a bacterial suspension ($\sim 10^6$ $cfu{\cdot}mL^{-1}$) for 1 h at room temperature, followed by planting. A month later, T2 and T3 were treated with 100 mL of smut pathogen suspension ($\sim 10^6$ $cfu{\cdot}mL^{-1}$) and T1 was treated with sterile water. After 2 months, the disease was analyzed and the leaves of three healthy sugarcane plants were collected for biochemical assays. Peroxidase (POD) activity was determined by the guaiacol method (Sakharov and Ardila, 1999). Phenylalanine ammonia-lyase (PAL) (Cat. No. BC0210), total phenol (TP) (Cat. No. BC1340), lignin (Cat. No. BC4200), and polyphenol oxidase (PPO) (Cat. No. BC0190) were determined using kits (Solarbio Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions.

Colonization of BTU6 in the Roots of Sugarcane

Sugarcane seedlings were maintained in plastic pots (20 cm in diameter, 18 cm in depth) filled with sterilized sand, which was moistened with sterile Hoagland nutrient solution, and kept in the greenhouse (28/22°C, 16/8 h light/dark cycle). BTU6 (10 mL, $\sim 10^6$ $cfu{\cdot}mL^{-1}$) was inoculated into a pot, and root samples were collected on day 14. After overnight fixation with 2.5% glutaraldehyde, the sample was dehydrated in a gradient series of acetone (30, 50, 70, 80, 90, and 100%; incubation of 20 min with each concentration of acetone). After drying with hexamethyldisilazane, the sample was coated with gold using an ion sputtering equipment (EM ACE200; Leica Microsystems, Wetzlar, Germany) and observed under a scanning electron microscope (SU8020; Hitachi High-Tech Instruments, Tokyo, Japan).

Genome Sequencing of BTU6, Assembly, and Annotation

Cellular genome extraction is described with reference to the manufacturer's protocol of bacterial genomic DNA extraction kit (CWBIO Biotechnology Co., Ltd., Beijing, China). The purity of extracted DNA was assayed with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). The BTU6 genome was sequenced by Oxford Nanopore PromethION sequencing platform. Sequencing was carried out at the Biomarker Biotechnology Co., Ltd. (Beijing, China). After obtaining raw sequencing data, low-quality readings were filtered by SMRT 2.3.0 (Berlin et al., 2015; Koren and Phillippy, 2015) and the filtered sub-reads were assembled using Canu v1.5 software (Koren et al., 2017). Pilon software (Walker et al., 2014) was used to further correct the assembled genome, and a contig with higher accuracy and no gap was obtained.

We analyzed the Genome function using Gene Ontology (GO) (Ashburner et al., 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2004, 2006), Clusters of Orthologous Groups (COG) (Tatusov et al., 2003), Non-Redundant Protein Database databases (NR) (Li et al., 2002), Transporter Classification Database (TCDB) (Saier et al., 2014), Swiss-Prot (Bairoch and Apweiler, 2000), and TrEMBL (Magrane and Consortium, 2011). We analyzed and predicted the secondary metabolite synthetase genes of strain BTU6 using antiSMASH (Blin et al., 2019). The respected genomic sequence data was submitted to GenBank under the BioProject PRJNA549819 (accession number CP041168). Using the predicted genomic information, the application software Circos (Krzywinski et al., 2009) was used to draw a genomic circle map.

Statistical Analysis

All data were analyzed using SPSS version 26 (IBM Corp., Armonk, NY, United States). One-way analysis of variance and Duncan's multiple range test were used to determine differences

TABLE 1 | Identification and PGP characteristics of actinobacteria.

Isolates	Type strain match (% similarity)	No. of nucleotides	Accession number	PGP traits				
				[a]IAA	[b]ACC Deaminase	Siderophore	P-solibilization	N-free medium growth
TU2	*Nocardioides lutues* KCTC 9575^T (99.22)	1,416	MH497623	–	0.28^g	+	–	+
TU3	*Streptomyces cinereoruber* NBRC 15396^T (98.45)	1,429	MH482864	–	–	+	–	–
TU4	*Streptomyces gramineus* JR043^T (97.99)	1,442	MH482865	21.89^m	0.06^k	–	–	–
TU5	*Streptomyces recifensis* NBRC 12813^T (98.17)	1,426	MH482866	–	0.26^g	–	–	–
TU6	*Streptomyces lannensis* TA408^T (99.51)	1,424	MH482867	27.55^{kl}	–	–	–	–
TU7	*Streptomyces griseoluteus* NBRC 13375^T (98.38)	1,424	MH482868	–	–	–	–	–
TU8	*Streptomyces filipinensis* NBRC 12860^T (98.60)	1,426	MH482869	25.83^l	–	–	–	–
TU10	*Streptomyces bambusae* T110^T (98.95)	1,426	MH482870	–	–	–	+	+
TU11	*Streptomyces cyaneus* NRRL B02296^T (99.16)	1,427	MH482871	49.05^h	–	–	+	–
TU12	*Streptomyces shaanxiensis* CCNWHQ 0031^T (98.73)	1,426	MH482872	43.75^i	–	–	–	–
TU13	*Streptomyces neopeptinius* KNF 2047^T (98.71)	1,422	MH482873	75.55^c	0.51^f	+	–	–
TU14	*Streptomyces filipinensis* NBRC 12860^T (98.45)	1,423	MH482874	–	–	–	–	–
TU15	*Streptomyces pseudovenezuelae* DSM 40212^T (98.88)	1,430	MH482875	48.01^h	0.01^k	–	–	–
TU16	*Streptomyces griseorubiginosus* DSM 40469^T (99.02)	1,429	MH482876	58.67^{de}	0.55^f	–	–	–
TU17	*Streptomyces griseoluteus* NBRC 13375^T (98.10)	1,427	MH482877	–	–	–	–	–
TU19	*Streptomyces lannensis* TA408^T (99.30)	1,431	MH482878	–	–	–	–	+
TU20	*Amycolatopsis xylanica* CPCC 202699^T (99.15)	1,414	MH497624	36.02^j	–	–	+	–
TU21	*Streptomyces griseoluteus* NBRC 13375^T (98.29)	1,408	MH482879	29.05^{kl}	–	–	–	–
TU22	*Streptomyces recifensis* NBRC 12813^T (99.51)	1,425	MH482880	–	–	–	–	+
TU23	*Streptomyces lannensis* TA408^T (99.23)	1,424	MH482881	–	–	–	–	+
TU32	*Streptomyces pratensis* ch24^T (100)	1,427	MH482882	–	–	+	–	–
TU33	*Streptomyces neopeptinius* KNF 2047^T (98.85)	1,424	MH482883	40.71^i	0.52^f	–	–	–
BTU1	*Streptomyces shaanxiensis* CCNWHQ 0031^T (99.01)	1,423	MH482884	56.52^{ef}	0.24^g	+	+	–
BTU2	*Streptomyces neopeptinius* KNF 2047^T (98.93)	1,425	MH482885	60.96^d	0.07^{jk}	–	+	–
BTU3	*Streptomyces gramineus* JR043^T (97.99)	1,423	MH482886	–	–	–	–	+
BTU4	*Streptomyces griseoluteus* NBRC 13375^T (97.82)	1,423	MH482887	43.25^i	–	–	–	–
BTU5	*Streptomyces recifensis* NBRC 12813^T (97.82)	1,426	MH482888	–	–	–	–	+
BTU6	*Streptomyces griseorubiginosus* DSM 40469^T (99.02)	1,428	MH482889	50.87^{gh}	0.08^{ijk}	+	–	+
BTU8	*Streptomyces amritsarensis* MTCC 11845^T (99.16)	1,428	MH482890	84.85^a	–	+	+	–

(Continued)

TABLE 1 | (Continued)

Isolates	Type strain match (% similarity)	No. of nucleotides	Accession number	PGP traits				
				[a]IAA	[b]ACC Deaminase	Siderophore	P-solibilization	N-free medium growth
BTU9	*Streptomyces pseudovenezuelae* DSM 40212^T (99.16)	1,423	MH482891	88.65^{a}	1.47^{d}	–	–	–
BTU10	*Streptomyces canus* DSM 40017^T (98.95)	1,426	MH482892	56.99^{def}	0.15^{hij}	–	–	–
BTU11	*Streptomyces pseudovenezuelae* DSM 40212^T (98.95)	1,426	MH482893	79.45^{b}	0.16^{hi}	–	–	–
BTU12	*Streptomyces neopeptinius* KNF 2047^T (98.93)	1,428	MH482894	54.30^{fg}	0.07^{ijk}	–	–	+
BTU13	*Streptomyces amritsarensis* MTCC 11845^T (99.30)	1,424	MH482895	+	–	–	+	–
BTU14	*Streptomyces neopeptinius* KNF 2047^T (99.00)	1,427	MH482896	–	0.26^{g}	–	+	–
BTU16	*Streptomyces cyaneus* NRRL B02296^T (98.94)	1,421	MH482897	59.89^{de}	1.56^{c}	–	–	–
BTU17	*Streptomyces pseudovenezuelae* DSM 40212^T (98.88)	1,426	MH482898	87.47^{a}	0.08^{h-k}	–	–	–
BTU18	*Streptomyces shaanxiensis* CCNWHQ 0031^T (99.01)	1,425	MH482899	51.16^{gh}	0.08^{ijk}	+	–	+
BTU19	*Streptomyces pratensis* ch24^T (100)	1,424	MH482900	–	0.76^{e}	–	–	+
BTU20	*Streptomyces neopeptinius* KNF 2047^T (98.64)	1,424	MH482901	56.52^{ef}	0.16^{h}	–	+	+
BTU21	*Streptomyces gramineus* JR043^T (98.13)	1,429	MH482902	–	0.30^{g}	–	–	–
BTU22	*Leifsonia soli* TG-S248^T (98.64)	1,425	MH497612	–	–	–	–	–
GEN1	*Streptomyces gramineus* JR043^T (97.99)	1,422	MH482903	–	–	–	–	–
GEN2	*Streptomyces violaceorubidus* LMG 20319^T (98.31)	1,423	MH482904	20.36^{m}	–	–	–	–
GEN5	*Streptomyces glauciniger* CGMCC 4.1858^T (99.15)	1,420	MH482905	28.76^{kl}	–	–	–	+
GEN7	*Streptomyces lannensis* TA408^T (99.79)	1,404	MH482906	21.89^{m}	–	–	–	–
GEN8	*Streptomyces rhizophilus* JR041^T (99.15)	1,417	MH482907	35.20^{j}	–	–	–	–
GEN15	*Streptomyces glauciniger* CGMCC 4.1858^T (99.29)	1,419	MH482908	–	–	–	–	+
WZS021	*Streptomyces chartreusis* NBRC 12753^T (99.63)	1,484	KX775948	30.50^{k}	3.03^{a}	+	–	+
WZS023	*Leucobacter aridicollis* CIP 108388^T (99.37)	1,427	MH497608	26.22^{l}	–	+	+	–
WZS027	*Streptomyces badius* NRRL B02567^T (99.86)	1,403	MH482909	–	–	+	–	–
WZS028	*Streptomyces pratensis* ch24^T (99.78)	1,425	MH482910	–	–	–	+	–
WZS030	*Streptomyces pratensis* ch24^T (99.81)	1,429	MH482911	–	–	–	–	–
WZS031	*Streptomyces violascens* ISP 5183^T (99.93)	1,424	MH482912	–	–	+	–	–
WZS035	*Nocardioides lutues* KCTC 9575^T (100)	1,368	MH497610	21.32^{m}	–	–	–	–
WZS050	*Streptomyces pratensis* ch24^T (100)	1,430	MH482913	–	–	–	+	–
WZS051	*Streptomyces pratensis* ch24^T (99.89)	1,428	MH482914	–	2.05^{b}	+	–	–
WZS221	*Brevibacterium epidermidis* NBRC 14811^T (98.73)	1,424	MH559626	29.87^{kl}	–	–	–	+

+, positive; –, negative. [a]mg·mL^{-1}, SE: 2.07, DMRT (p = 0.05): 1.87, CV (%): 4.4; [b]μmol α-ketobutyrate·mg^{-1} protein·h^{-1}, SE: 0.09, DMRT (p = 0.05): 0.04, CV (%): 16.5. Different small letters as superscript represent significant difference at 95% confidence intervals based on DMRT test.

between samples, with statistically significant differences set at a 5% level.

RESULTS

Identification of Rhizosphere Actinomycetes

We isolated a total of 58 actinobacteria from the sugarcane rhizosphere; of these, 52 strains were identified as *Streptomyces* (89%), and the remaining 6 non-*Streptomyces* actinomycetes strains were identified as *Leucobacter* (2%), *Nocardioides* (3%), *Leifsonia* (2%), *Amycolatopsis* (2%), and *Brevibacterium*(2%). Phylogenetic relationships among the isolates are shown in **Figure 1**.

PGPR Characteristics of Actinomycetes

Among all isolates, 55% of strains had the ability to secrete IAA, with BTU9 showing the maximum IAA production of 88.65 mg mL^{-1}. Moreover, 40% of active strains were able to secret ACC deaminase in the range 0.01–3.03 μmol α-ketobutyrate mg^{-1} protein h^{-1}. The siderophores assay results indicated that 22% of strains had iron chelation ability. Among all strains, 21% of active strains had the ability to dissolve inorganic phosphorus (**Table 1**).

Detection of Actinomycetes Activity Against Pathogenic Fungi in *in vitro*

The antagonism assays revealed that 72% actinomycetes showed antagonism against *Botrytis cinerea*, followed by 59% strains that antagonized against brown spot disease-causing *Alternaria brassicicola,* and 60% against the rice sheath blight-causing *Rhizoctonia solani* and banana wilt causing *Fusarium oxysporum* f. sp. *Cubense*. Among all strains, 37 actinomycetes antagonized more than four pathogenic fungi and only 2 strains showed antifungal activity against all tested pathogenic fungi (**Figure 2A** and **Supplementary Table 3**). However, some strains, including BTU6, BTU8, BTU13, and WZS031, had strong antagonistic effects on the *Sporisorium scitamineum*, and based on potential antagonistic ability, we used strain BTU6 for the greenhouse experiment.

PCR Detection of Secondary Metabolite Synthase Genes

The secondary metabolite synthase gene detection test showed that the highest number (83%) of strains had *PKS II* gene, followed by *PKS I* gene (62%); the least number of strains (45%) had the *CYP* gene. Interestingly, 17 strains had five secondary metabolite synthase genes were commonly detected, and three secondary metabolite synthetase genes were widely detected in 51 strains (**Figure 2B** and **Supplementary Table 4**).

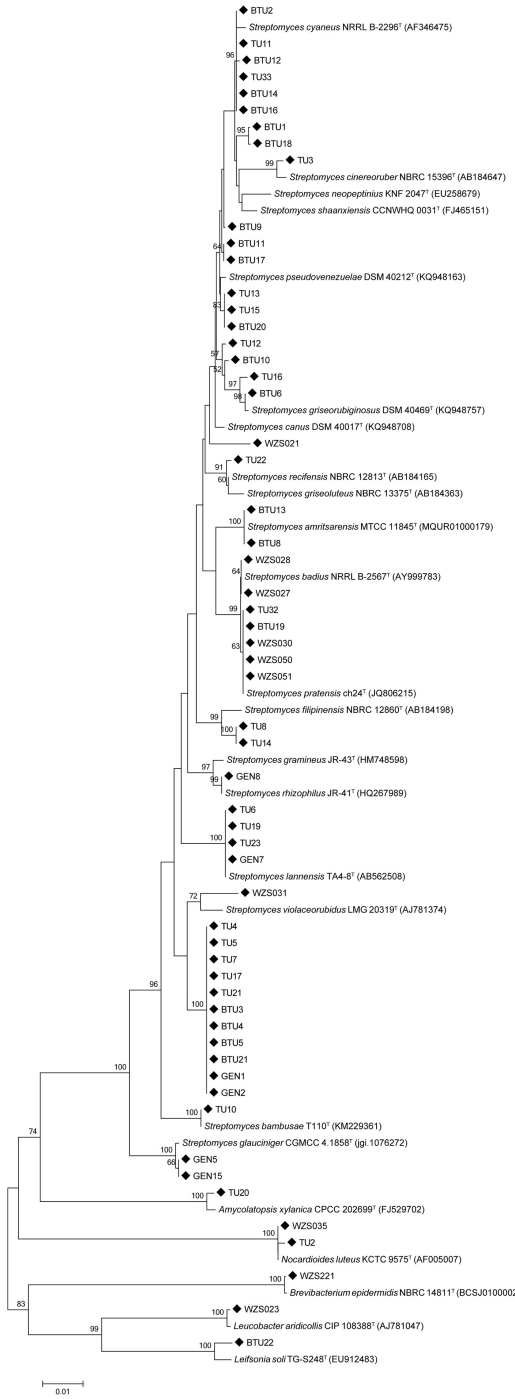


FIGURE 1 | The phylogenetic tree was constructed using the 16S rDNA sequence of sugarcane rhizosphere actinomycetes and similar type strains through the neighbor-joining method.

Greenhouse Based Biocontrol Assay Against the Smut Pathogen

We assessed plant defense enzyme activity and disease-related substances, to examine the vital effect of BTU6 inoculation on plant antagonism against the sugarcane smut disease. The difference in POD, PPO, and PAL activity of the three treatments reached significant levels, with activity patterns in the respected treatments recorded as follows: T3 > T2 > T1. Compared with T1, The TP content in T2 and T3 increased by 54.5 and 121.2%, respectively. There was no significant difference in lignin

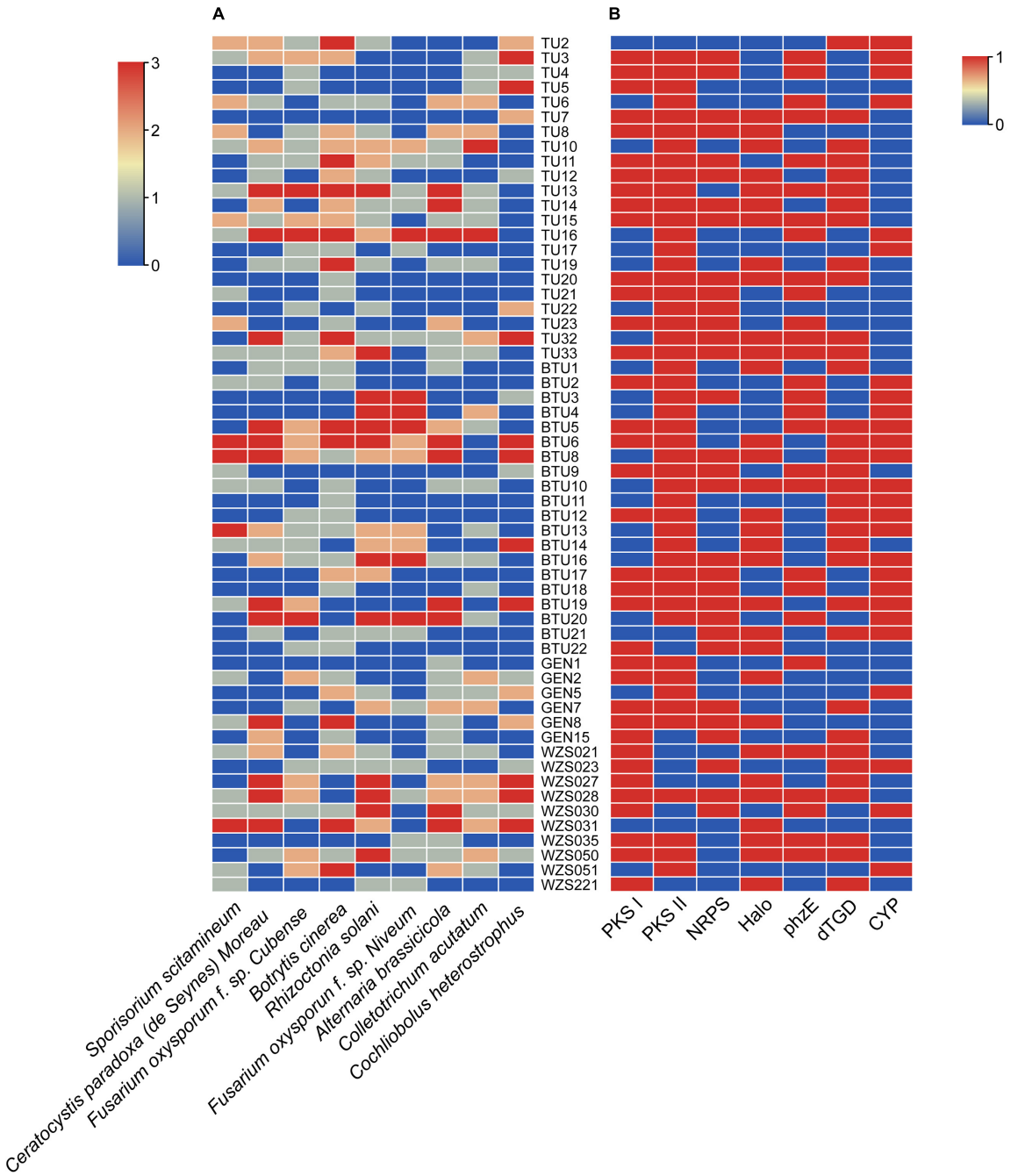


FIGURE 2 | *In vitro* antagonism of actinobacteria and phytopathogenic fungi **(A)**, and screening of synthetic genes for secondary metabolites of actinobacteria **(B)**. **(A)** Growth definitely retarded with zone of inhibition (1) near the colony, (2) of ≥ 10 mm, and (3) of ≥ 15 mm. (0) Represents no inhibition. **(B)** (0) Negative; (1) positive.

in the leaves of inoculated and uninoculated plants (**Table 2**). The fresh weights of T2 and T3 were significantly higher than that of T1 (increased by 18.0 and 20.1%, respectively), but no significant difference in plant height was observed. Moreover, the presence of BTU6 enhanced the resistance of sugarcane to smut (**Supplementary Figure 1**). The smut infection rate of T3 was 12% higher than that of T1, but 10% lower than that of T2. The sterile sand inoculation test in the greenhouse also showed that BTU6 can colonize the root surface and root hair area of sugarcane (**Figure 3**).

Genomic Traits and Genes Associated With Antagonistic Diseases

After sequencing, the complete genome sequence of *Streptomyces griseorubiginosus* BTU6 was obtained. The genome contained

TABLE 2 | Comparison of defensive enzyme activities, active substances and susceptibility rates of plant disease resistance in different treatments in greenhouse experiments.

Treatment		POD ($U \cdot g^{-1}$)	PPO ($U \cdot g^{-1}$)	PAL ($U \cdot g^{-1}$)	TP ($mg\ g^{-1}$)	Lignin ($mg\ g^{-1}$)	Infection rate (%)	Fresh weight (g)		Plant height (m)
								Shoot	Root	
T1		90.1^{c}	78.2^{c}	26.9^{c}	3.3^{c}	153^{a}	5.6^{c}	154.9^{b}	12.2^{b}	0.62^{a}
T2		105.9^{b}	101.4^{b}	57.4^{b}	5.1^{b}	161.5^{a}	45.6^{b}	174.4^{a}	22.7^{a}	0.67^{a}
T3		138.6^{a}	131.6^{a}	67.4^{a}	7.3^{a}	157.9^{a}	78.9^{a}	182.1^{a}	18.6^{a}	0.63^{a}
	SE	7.41	8.14	6.13	0.58	4.32	10.67	4.23	1.69	0.01
	LSD ($p = 0.05$)	5.57	7.19	1.79	0.22	11.70	3.51	3.49	2.0	0.02
	CV (%)	6.6	7.8	12.1	11.0	2.7	24.6	2.9	9.4	1.8

T1 (no inoculation-only water), T2 (inoculation of smut pathogen only), T3 (inoculation of smut pathogen and actinomycetes BTU6 as biocontrol agent). Different small letters as superscript represent significant difference at 95% confidence intervals based on DMRT test.

a 9.22 Mb circular chromosome with a GC content of 71.15% (**Figure 4** and **Supplementary Table 5**). Strain BTU6 contained 8,110 predicted protein-coding genes, 18S rRNA genes, 73 tRNA genes, and 42 ncRNA genes, related to the COG and GO functional categories (**Supplementary Figures 2, 3**). There were 305 genes involved in the biosynthesis, transport, and catabolism of secondary metabolites, 614 for amino acid transporters and metabolic genes, 319 for lipid transport and metabolic genes, 311 for inorganic ion transport and metabolism genes, and 616 for carbohydrate transport and metabolic genes. Combined with antiSMARSH results, strain BTU6 could produce a variety of peptidoglycans and polyketose-resistant compounds such as polyketide, t2pks, terpene, lanthipeptide, γ-butyrolactone (**Supplementary Table 6**). The synthesis of these secondary metabolites might help to inhibit the growth of the pathogen. A total of 34 biosynthetic gene clusters (BGCs) were identified in BTU6, of which 23 had a similarity more significant than 20%. The products encoded by these BGCs are the primary source of antibacterial activity of BTU6; of these products, atratumycin was demonstrated to be unique to BTU6. Upon a comparative analysis of the BTU6 and 16 *Streptomyces* genomes with similar genetic relationships, a total of 735 BGCs were predicted; the number of BGCs for a single strain ranged from 27 to 65. The differences in the number of BGCs among the strains were not significantly correlated with the genome size ($R^2 = 0.0898$). According to the antiSMASH classification standard, these BGCs belonged to 38 categories, of which polyketide synthases (PKSs), terpenes, and non-ribosomal peptide synthetases (NRPSs) were the main types, accounting for a total of 306 (approximately 41.6%) of all BGCs (**Figure 5**).

DISCUSSION

Streptomyces are one of the most important actinobacteria genus associated with the plant rhizosphere and endosphere. The phylogenetic tree analysis showed that all *Streptomyces* were in the same clade, and other actinibacterial strains such as TU20, WZS035, and TU2, separated out in another clade. Strains WZS023 and BTU22 displayed a close relationship with each other.

Berg et al. (2006) reported that 35% of microorganisms isolated from the rhizosphere have the effect of inhibiting the growth of phytopathogenic microorganisms, while for results of microbial isolation by Fürnkranz et al. (2009), about two-thirds of the alternate microorganisms promoted plant growth. Hamdali et al. (2008) isolated 8 strains for dissolving inorganic phosphorus from mineral-containing phosphate soils in Morocco. The PGP properties screening results showed the strains with the highest IAA capacity were found (32, 55%), followed by the strains with ACC deaminase activity (23, 40%), and 89% of the strains had at least one kind of PGP ability. Strains indicating that sugarcane rhizosphere actinobacteria have the ability to promote plant growth.

Moreover, previous studies have discussed the secondary metabolite production of *Streptomyces*. Strobel (2003) reported that *Streptomyces* NRRL30562 strain produced four peptide antibiotics, Munumbicins A, B, C, and D, that could inhibit a variety of phytopathogenic bacteria, molds, and malaria parasites. Clermont et al. (2010) also revealed that geldanamycin produced by *Streptomyces melanosporofaciens* EF-76 had antagonistic effects on a variety of gram-positive bacteria and fungi. However, Yuan et al. (2014) confirmed that the PCR technology was a rapid method for detecting the secondary metabolite genes. In the present study, we also used PCR method to detect *PKS I*, *PKS II*, *NRPS*, *phz*E, *dTGD*, *Halo*, *CYP* genes. Interestingly, 83% strains had *the PKS II* gene (**Supplementary Table 4**) and 59% of strains had the *dTGD* gene. These results aligned with previous studies that reported on the important role of *dTGD* gene in the activity, toxicity, and solubility of natural products (Kirschning et al., 1997; Poulsen et al., 2000). D-glucose undergoes a series of complex actions, resulting in a structurally diverse 6-deoxyhexose, therefore, a very large family of 6-deoxyhexoses (Chen et al., 2011). From the highly conserved nature of the *dTGD* gene, the potential for the synthesis of 6-deoxyhexose by the *Streptomyces* strain was determined (Yu et al., 2004). Results showed that the *CYP* gene encoded a key enzyme in the biosynthesis of polyene antibiotics, cytochrome

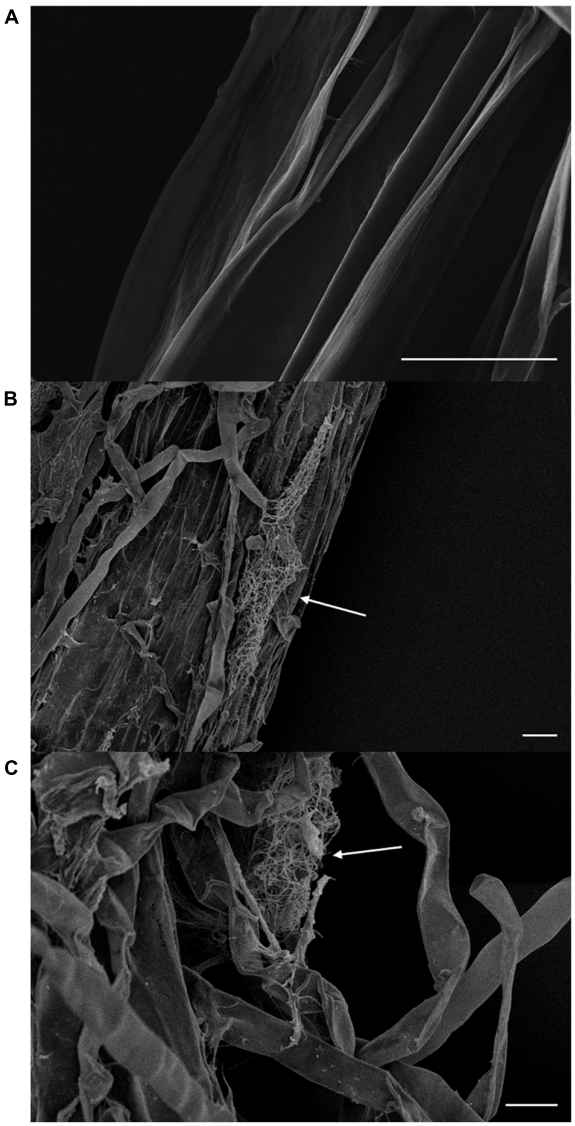


FIGURE 3 | *Streptomyces griseorubiginosus* BTU6 colonizes the root surface **(B)** and hair area **(C)** of sugarcane and forms a mycelial cluster.
(A) Represents a control sugarcane plant without BTU6 inoculation. Scale bar = 20 μm.

P450 hydroxylase (Lee et al., 2006). The halogenase gene in the molecule plays an important role in the biological activity of the entire compound. $FADH_2$-dependent halogenase is the most important halogenase in the secondary metabolic halide biosynthesis pathway (Van Pee and Patallo, 2006), and the biosynthesis of many antibiotics is based mainly on $FADH_2$-dependent halogenase (Van Pee, 2001). However, some strains have antibacterial activity, but no secondary metabolite genes have been detected; also, selected primers for amplifying metabolite genes have not been suitable for all actinobacteria due to differences in genotypes, although some of these genotypes could still be used as a basic understanding of biosynthetic genes. In the present study, the *PKS II* gene was detected in 83% of the strains, indicating that most of the sugarcane rhizosphere actinobacteria have the potential to synthesize type II polyketides. Even the smallest strain with the *CYP* gene was almost half the number of tested strains (26, 45%). Together with the PCR test of bioactive substance synthase, we also conducted an antagonistic test *in vitro*. All actinobacteria antagonized at least one pathogenic fungus, demonstrating the antagonistic properties of the actinobacteria.

Based on the *in vitro* results, we selected strain BTU6 for the sugarcane smut disease management experiment in the greenhouse, and results indicated that strain BTU6 increased plant disease resistance by regulating plant defense enzymes. Results on plant defense enzymes such as POD, PAL, and PPO in the BTU6 inoculated with smut pathogen showed that the activity levels were significantly higher ($p < 0.05$) than without BTU6, indicating that BTU6 inoculation enhanced the plant defense response against the smut pathogen. Ehret et al. (2010) also reported plant defense enzymes associated with disease resistance, of which POD, PAL, and PPO were essential enzymes involved in the plant protection process against the pathogen infection; these enzymes are often used as an important indicator of plant defense response (Lambais and Mehdy, 1995). Intermediates of PAL synthesis, such as phenolic substances and lignin are important antimicrobial substances in plants (Pellegrini et al., 1994). POD functions in strengthening plant cell walls, resisting the invasion of pathogens, and as a protective enzyme for scavenging reactive oxygen species in cells (Ray et al., 1998). PPO oxidizes phenolic substances into highly toxic terpenoids, thereby killing invading pathogens (Volpin et al., 1995). Lignin and its analogs are produced in large quantities when pathogens invade plants or are mechanically damaged (Vance et al., 1980; Hammerschmidt and Kuć, 1982; Nicholson and Hammerschmidt, 1992; Boudet et al., 1995). Lignified cell walls enhance the resistance of herbaceous and woody plants against pathogens (Hammerschmidt and Kuć, 1982; Southerton and Deverall, 1990; Dushnicky et al., 1998). Total phenolic content is also closely related to the disease resistance of plants. The total phenolic content of the cowpea varieties resistant to a mosaic disease was higher than the susceptible genotype (Shilpashree et al., 2013). Similarly, the content of phenolic substances in highly resistant red rot varieties was significantly higher than that in susceptible varieties. The change in total phenolic content could be used as a basis for assessing the degree of disease resistance of sugarcane (Beato et al., 1970). In this study, the total phenolic content of the three treatments was significantly different, indicating that phenolic substances also played a role in improving sugarcane smut resistance. Ippolito et al. (2000) found that *Aureobasidium pullulans* can significantly increase the activity of chitinase and β-1,3 glucanases in apple fruit, and degrade the fungal cell wall to inhibit fungal growth. In addition, Luo et al. (2012) found that the antagonistic yeast (*Pichia membranefaciens*) can induce the increase in POD activity in citrus fruits, and promote the formation of phenols and flavonoids, thereby improving the disease resistance of citrus. Zhao et al. (2008) studied the biocontrol effect of *Pichia guilliermondii* on tomato root rot (*Rhizopus nigricans*) and found that *Pichia guilliermondii* could induce the increase of

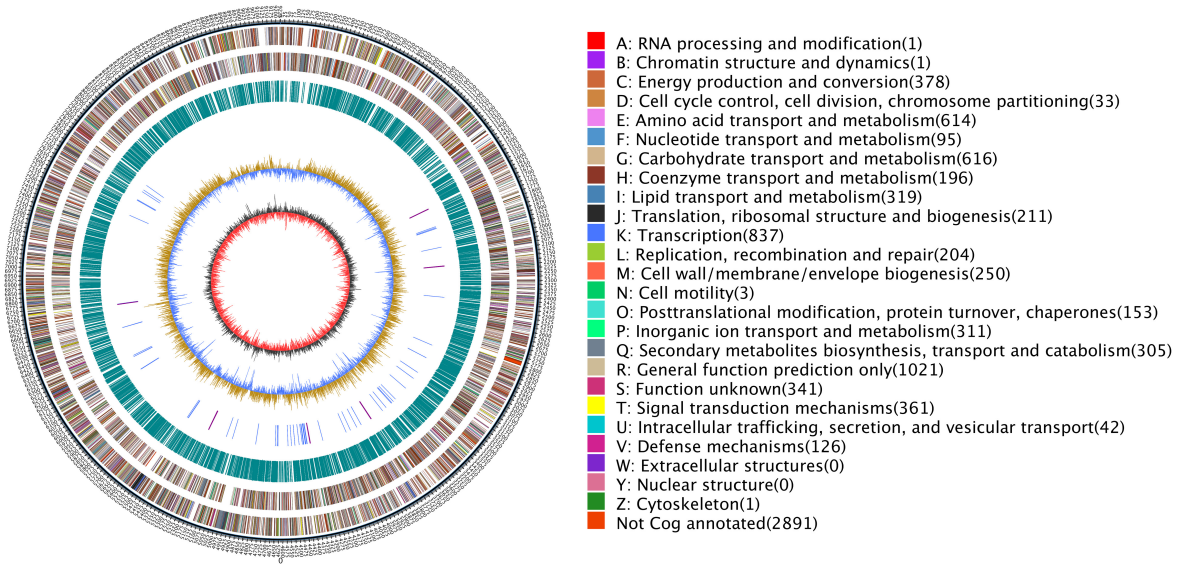


FIGURE 4 | Genome overview map of *Streptomyces griseorubiginosus* BTU6. Circles from the outside to the inside represent the genome size, COG function classification, repetitive sequence, RNA classification, GC content, and GC-s.

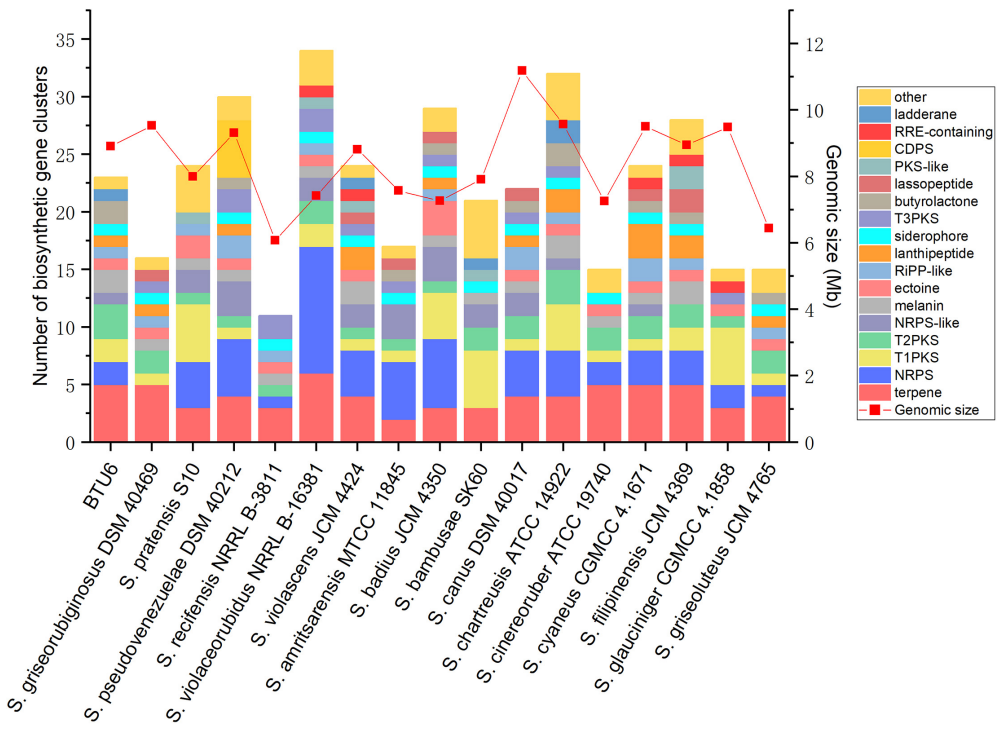


FIGURE 5 | Comparison of 17 *Streptomyces* biosynthetic gene clusters and genome size, with similarity ≥ 20%.

SOD, CAT and POD activities in tomato fruit. This study also demonstrated that antagonistic bacteria could induce defense-related enzymes of sugarcane.

To explore the characteristics and commonalities between BTU6 and other *Streptomyces* strains, the BGCs of 17 *Streptomyces* strains were predicted and compared. Overall, different types of unknown gene clusters were identified in *Streptomyces*. PKS- and NRPS-related BGCs were the most prevalent; these two types of BGCs synthesize active substances of macrolides non-ribosomal peptides, respectively (Theobald et al., 2019). These observed differences between *Streptomyces* may be related to their complex living environment, thus

representing adaptation events that they underwent. Moreover, these results confirmed the abundance of *Streptomyces*-derived naturally active substances in the sugarcane rhizosphere, which may be helpful for the development of new antibiotics. In particular, all *Streptomyces* were found to contain ectoine and siderophore BGCs. In the long-term evolutionary process, most microorganisms have developed an osmotic pressure mechanism that accumulates compatible solutes in the cytoplasm to counteract the changing external environment. Among them, ectoine is an important compatible solute (Fenizia et al., 2020), which enables *Streptomyces* to better adapt to the complex and changeable environment. Siderophores affect plant health by promoting plant nutrient absorption, improving plant resistance, and inhibiting the growth of pathogenic microbes (Verbon et al., 2017). In the present study, many heterozygous gene clusters were also identified in *Streptomyces*, thereby indicating that these bacteria exhibit a high degree of horizontal gene transfer in the long-term evolutionary process, which has essential ecological functions. Atratumycin is a cyclodepsipeptide that actively targets *Mycobacterium tuberculosis*. It is generally isolated from deep-sea *Streptomyces*, but it has also been reported in terrestrial *Streptomyces* (Sun et al., 2019). Considering the unique BGCs observed, BTU6 strains exhibit remarkable potential for application in the development of antimicrobial agents. Taken together, the common BGCs of *Streptomyces*, as well as the unique clusters identified, can explain the broad-spectrum antagonistic effect of BTU6 on common pathogens, mainly smut.

In vivo and *in vitro* experiments showed that BTU6 has the ability to enhance plant growth and also can improve plant defense against pathogens. These results motivated us to identify the genomic information to understand the plant growth promotion and stress regulation mechanisms. Interestingly, 34 secondary metabolic gene clusters in the genome of BTU6 concluded our results regarding the biocontrol potential. Several past studies have described the importance of chitinase to break down the chitinolytic cell wall of pathogens (Alexopoulos et al., 1996; Baharlouei et al., 2010; Chater et al., 2010; Shen et al., 2016). Polyketide compounds are one of the most diverse natural products of function and structure, with biological activities, including antibacterial, antiviral, antitumor, anti-pathogenic, anti-tuberculosis and immunosuppression (Miyanaga, 2017). NRPS can be used as an antibiotic, immunosuppressant, lipid-lowering and antifungal, and is widely used in medicine and agriculture (Misiek and Hoffmeister, 2007; Maansson et al., 2016). The metabolites of *Streptomyces* isolated from the soil also contain large amounts of terpenes (Wang C. et al., 2013; Wang Z. et al., 2013). Terpenoids not only have an antibacterial effect (Gallucci et al., 2009), but also have a strong killing effect on root-knot nematodes (Ntalli et al., 2011). Eisenman and Casadevall (2012) reported that melanin has anti-ultraviolet radiation and scavenging free radicals in organisms and can improve the ability of organisms to survive and compete. Lanthipeptide is a large class of cyclic peptide compounds containing thioether bonds (Cooper et al., 2010; Knerr and van der Donk, 2012) and has strong antibacterial activity against Gram-positive bacteria (Delves-Broughton et al., 1996). In this study, the defense enzyme activity of BTU6-treated sugarcane plants was different from that of untreated control plants and had a direct or indirect relationship with the expression of corresponding genes. *Streptomyces* is an essential class of biocontrol bacteria in agricultural production. Traditional experimental analysis and identification methods have limitations in analyzing *Streptomyces* active substances and cannot fully exploit their antibacterial potential; however, bioinformatics tools make up for these shortcomings. Bioinformatics provides an in-depth understanding of the nature and function of organisms from the perspective of the genome, and provides new approaches for microbial research (Bentley et al., 2002; Udwary et al., 2007; Kersten et al., 2011; Ziemert et al., 2012).

Persistent organic pollutants (POPs) have the characteristics of persistence, semi-volatility, easy bioaccumulation, and high toxicity, which bring great harm to human health and the environment (Lohmann et al., 2007; Lapworth et al., 2012; Tran et al., 2013). The microbial degradation method has received extensive attention due to its mild reaction conditions, thorough degradation, low operating costs, and no secondary pollution (Shokrollahzadeh et al., 2008; Hassan and Sorial, 2009; Rene et al., 2010). The genomic information generated from this study demonstrated the potential of BTU6 to biodegrade persistent organic pollutants. For example, hexachlorocyclohexanes (HCH) is the most common type of persistent organic pollutant. It was also the organic synthetic pesticide discovered and applied. Due to its excellent insecticidal effect, it is widely used in agriculture (Quintero et al., 2008). Benzoates, styrene, and caprolactam pose greater risks to environmental organisms (Marczynski et al., 2000; Dahlhoff et al., 2001; Willis and Ling, 2003). Bisphenol A (BPA) is often used as a compound to manufacture plastics, epoxies, and other materials. It is also an endocrine disruptor that interferes with the synthesis and metabolism of hormones and is associated with multiple pathologies in the human reproductive system (Howdeshell et al., 1999; Maffini et al., 2006; Diamanti-Kandarakis et al., 2009). Dioxins are often associated with waste incineration, which are carcinogenic, teratogenic, mutagenic, and are included in the first batch of Stockholm Convention on Persistent Organic Pollutants list (Everaert and Baeyens, 2002; Wang et al., 2012). The natural and synthetic polycyclic aromatic hydrocarbons are dispersed throughout the world with the flow of the atmosphere. Polycyclic aromatic hydrocarbons enter the vegetation from the atmosphere and eventually lead to enrichment in the food chain, which is the leading organic pollutant affecting human health (Blumer, 1976; Morehead et al., 1986). Atrazine was once considered one of the most difficult to degrade herbicides, posing a threat to aquatic ecosystems and human drinking water sources (Storrs and Kiesecker, 2004; Fan and Song, 2014). In addition, glycine oxidase in microorganisms degrades glyphosate to amine methylphosphonic acid (AM-PA) and glyoxylic acid to reduce glyphosate residues (Nishiya and Imanaka, 1998). BTU6 could degrade a variety of exogenous chemicals, especially those that are difficult to degrade by artificial methods, which provides a new solution to ecological protection.

CONCLUSION

The sugarcane rhizosphere is rich in actinobacteria that promote plant growth, and most have the potential to antagonize plant pathogens. *Streptomyces griseorubiginosus* BTU6 enhances the resistance of plants to smut by regulating the stress resistance related enzyme activity and substances (POD, PAL, PPO, TP) in sugarcane. Bacterial genomic information also provides evidence that BTU6 produces a variety of secondary metabolites that antagonize fungi, further elucidating the molecular mechanisms of bacterial-plant interactions. In addition, the genome showed that BTU6 has the potential for biodegrading of a variety of exogenous chemicals, which can provide new insights into green solutions to various environmental options.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZW, Y-RL, D-FD, and FP designed the study. ZW, MS, and Z-XY conducted the experiments. MA and Y-XX analyzed the data. ZW, MS, Y-RL, and MM wrote the manuscript. Y-RL and D-FD revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The present study was supported by the National Natural Science Foundation of China (32101836, 31171504, and 31471449), the Guangxi Special Fund for Scientific Base and Talent (GKAD17195100), the Guangxi Special Funds for Bagui Scholars and Distinguished Experts (2013-3), and the Guangxi Sugarcane Innovation Team of National Agricultural Industry Technology System (gjnytxgxcxtd-03-01).

ACKNOWLEDGMENTS

We thank Hai-Yan Wu and Qiong He of Guangxi University for providing the related fungal pathogens for plant, and Dr. Cheng-Xi Li of Guangxi University for helping in genome informatics analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.797889/full#supplementary-material

REFERENCES

Aggarwal, N., Thind, S. K., and Sharma, S. (2016). "Role of secondary metabolites of *Actinomycetes* in crop protection," in *Plant Growth Promoting Actinobacteria*, eds G. Subramaniam, S. Arumugam, and V. Rajendran (Singapore: Springer), 99–121. doi: 10.1007/978-981-10-0707-1_7

Alexander, D. B., and Zuberer, D. A. (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol. Fertil. Soils* 12, 39–45. doi: 10.1007/BF00369386

Alexopoulos, C. J., Mims, C. W., and Blackwell, M. (1996). *Introductory Mycology*. New York, NY: John Wiley and Sons.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. *Nat. Genet.* 25:25. doi: 10.1038/75556

Baharlouei, A., Sharifi-Sirchi, G. R., and Bonjar, G. H. S. (2010). Identification of an antifungal Chitinase from a potential biocontrol agent, *Streptomyces plicatus* strain 101, and its new antagonistic spectrum of activity. *Philipp. Agric. Sci.* 93, 439–445.

Bairoch, A., and Apweiler, R. (2000). The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Res.* 28, 45–48. doi: 10.1093/nar/28.1.45

Bakker, P.a.H.M., Ran, L. X., Pieterse, C. M. J., and Loon, L. C. V. (2003). Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. *Can. J. Plant Pathol.* 25, 5–9. doi: 10.1080/07060660309507043

Beato, M., Homoki, J., Doenecke, D., and Sekeris, C. E. (1970). Phenolic content of sugarcane in relation to red rot disease. *Experientia* 26, 1074–1076. doi: 10.1007/BF02112682

Bentley, S. D., Chater, K. F., Cerdeño-Tárraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417:141. doi: 10.1038/417141a

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R., et al. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol. Ecol.* 56, 250–261. doi: 10.1111/j.1574-6941.2005.00025.x

Berlin, K., Koren, S., Chin, C. S., Drake, J. P., Landolin, J. M., and Phillippy, A. M. (2015). Assembling large genomes with single-molecule sequencing and locality-sensitive hashing. *Nat. Biotechnol.* 33, 623–630. doi: 10.1038/nbt.3238

Bertin, C., Yang, X., and Weston, L. A. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256, 67–83. doi: 10.1023/A:1026290508166

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., et al. (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.* 47, W81–W87. doi: 10.1093/nar/gkz310

Blumer, M. (1976). Polycyclic aromatic compounds in nature. *Sci. Am.* 234, 35–45. doi: 10.1038/scientificamerican0376-34

Boudet, A. M., Lapierre, C., and Grima-Pettenati, J. (1995). Biochemistry and molecular biology of lignification. *New Phytol.* 129, 203–236. doi: 10.1111/j.1469-8137.1995.tb04292.x

Burdman, S., Jurkevitch, E., and Okon, Y. (2000). Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. *Microb. Interact. Agric. For.* 2, 229–250.

Chater, K. F., Biró, S., Lee, K. J., Palmer, T., and Schrempf, H. (2010). The complex extracellular biology of *Streptomyces*. *FEMS Microbiol. Rev.* 34, 171–198. doi: 10.1111/j.1574-6976.2009.00206.x

Chen, F., Lin, L., Wang, L., Tan, Y., Zhou, H., Wang, Y., et al. (2011). Distribution of dTDP-glucose-4,6-dehydratase gene and diversity of potential glycosylated natural products in marine sediment-derived bacteria. *Appl. Microbiol. Biotechnol.* 90, 1347–1359. doi: 10.1007/s00253-011-3112-y

Clermont, N., Legault, G., Lerat, S., and Beaulieu, C. (2010). Effect of biopolymers on geldanamycin production and biocontrol ability of *Streptomyces melanosporofaciens* strain EF-76. *Can. J. Plant Pathol.* 32, 481–489. doi: 10.1080/07060661.2010.512121

Cooper, L. E., Li, B., and Van Der Donk, W. A. (2010). "5.08 – biosynthesis and mode of action of lantibiotics," in *Comprehensive Natural Products II*, eds H.-W. Liu and L. Mander (Amsterdam: Elsevier), 217–256. doi: 10.1016/B978-008045382-8.00116-7

Dahlhoff, G., Niederer, J. P., and Hoelderich, W. F. (2001). ε-Caprolactam: new by-product free synthesis routes. *Catal. Rev.* 43, 381–441. doi: 10.1081/CR-120001808

de Jesus Sousa, J. A., and Olivares, F. L. (2016). Plant growth promotion by *Streptomycetes*: ecophysiology, mechanisms and applications. *Chem. Biol. Technol. Agric.* 3:24. doi: 10.1186/s40538-016-0073-5

Delves-Broughton, J., Blackburn, P., Evans, R. J., and Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek* 69, 193–202. doi: 10.1007/BF00399424

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., et al. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr. Rev.* 30, 293–342. doi: 10.1210/er.2009-0002

Dushnicky, L. G., Ballance, G. M., Sumuer, M. J., and Macgregor, A. W. (1998). The role of lignification as a resistance mechanism in wheat to a toxin-producing isolate of *Pyrenophora tritici*-repentis. *Can. J. Plant Pathol.* 20, 35–47. doi: 10.1080/07060669809500443

Ehret, D. L., Edwards, D., Helmer, T., Lin, W., Jones, G., Dorais, M., et al. (2010). Effects of oxygen-enriched nutrient solution on greenhouse cucumber and pepper production. *Sci. Hortic.* 125, 602–607. doi: 10.1016/j.scienta.2010.05.009

Eisenman, H. C., and Casadevall, A. (2012). Synthesis and assembly of fungal melanin. *Appl. Microbiol. Biotechnol.* 93, 931–940. doi: 10.1007/s00253-011-3777-2

El-Tarabily, K. A., Nassar, A. H., Hardy, G. S. J., and Sivasithamparam, K. (2010). Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *J. Appl. Microbiol.* 106, 13–26. doi: 10.1111/j.1365-2672.2008.03926.x

Everaert, K., and Baeyens, J. (2002). The formation and emission of dioxins in large scale thermal processes. *Chemosphere* 46, 439–448. doi: 10.1016/S0045-6535(01)00143-6

Fan, T. W. M., Lane, A. N., Shenker, M., Bartley, J. P., Crowley, D., and Higashi, R. M. (2001). Comprehensive chemical profiling of gramieous plant root exudates using high-resolution NMR and MS. *Phytochemistry* 57, 209–221. doi: 10.1016/S0031-9422(01)00007-3

Fan, X., and Song, F. (2014). Bioremediation of atrazine: recent advances and promises. *J. Soils Sediments* 14, 1727–1737. doi: 10.1007/s11368-014-0921-5

Fenizia, S., Thume, K., Wirgenings, M., and Pohnert, G. (2020). Ectoine from bacterial and algal origin is a compatible solute in microalgae. *Mar. Drugs* 18:42. doi: 10.3390/md18010042

Fürnkranz, M., Müller, H., and Berg, G. (2009). Characterization of plant growth promoting bacteria from crops in Bolivia. *J. Plant Dis. Prot.* 116, 149–155. doi: 10.1007/BF03356303

Gallucci, M. N., Oliva, M., Casero, C., Dambolena, J., Luna, A., Zygadlo, J., et al. (2009). Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. *Flavour Fragr. J.* 24, 348–354. doi: 10.1002/ffj.1948

Gobran, G. R., and Clegg, S. (1996). A conceptual model for nutrient availability in the mineral soil-root system. *Can. J. Soilence* 76, 125–131. doi: 10.4141/cjss96-019

Gordon, S. A., and Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiol.* 26, 192–195. doi: 10.1104/pp.26.1.192

Hamdali, H., Hafidi, M., Virolle, M. J. L., and Ouhdouch, Y. (2008). Growth promotion and protection against damping-off of wheat by two rock phosphate solubilizing actinomycetes in a P-deficient soil under greenhouse conditions. *Appl. Soil Ecol.* 40, 510–517. doi: 10.1016/j.apsoil.2008.08.001

Hammerschmidt, R., and Kuć, J. W. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. *Physiol. Plant Pathol.* 20, 61–71. doi: 10.1016/0048-4059(82)90024-8

Hansen, V. M., Winding, A., and Madsen, A. M. (2010). Exposure to bioaerosols during the growth season of tomatoes in an organic greenhouse using Supresivit (*Trichoderma harzianum*) and Mycostop (*Streptomyces griseoviridis*). *Appl. Environ. Microbiol.* 76, 5874–5881. doi: 10.1128/AEM.00446-10

Hassan, A. A., and Sorial, G. (2009). Biological treatment of benzene in a controlled trickle bed air biofilter. *Chemosphere* 75, 1315–1321. doi: 10.1016/j.chemosphere.2009.03.008

Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G., and Vom Saal, F. S. (1999). Exposure to bisphenol A advances puberty. *Nature* 401, 763–764. doi: 10.1038/44517

Ippolito, A., El Ghaouth, A., Wilson, C. L., and Wisniewski, M. (2000). Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. *Postharvest Biol. Technol.* 19, 265–272. doi: 10.1016/S0925-5214(00)00104-6

Islam, S., Akanda, A. M., Prova, A., Islam, M. T., and Hossain, M. M. (2016). Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. *Front. Microbiol.* 6:1360. doi: 10.3389/fmicb.2015.01360

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K. F., Itoh, M., Kawashima, S., et al. (2006). From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res.* 34, D354–D357. doi: 10.1093/nar/gkj102

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277–D280. doi: 10.1093/nar/gkh063

Kannan, V., and Sureendar, R. (2009). Synergistic effect of beneficial rhizosphere microflora in biocontrol and plant growth promotion. *J. Basic Microbiol.* 49, 158–164. doi: 10.1002/jobm.200800011

Kersten, R. D., Yang, Y. L., Xu, Y., Cimermancic, P., Nam, S. J., Fenical, W., et al. (2011). A mass spectrometry-guided genome mining approach for natural product peptidogenomics. *Nat. Chem. Biol.* 7, 794–802. doi: 10.1038/nchembio.684

Kirschning, A., Bechthold, A., and Rohr, J. (1997). Chemical and biochemical aspects of deoxysugars and deoxysugar oligosaccharides. *Top. Curr. Chem.* 188, 1–84. doi: 10.1007/BFb0119234

Knerr, P. J., and van der Donk, W. A. (2012). Discovery, biosynthesis, and engineering of lantipeptides. *Annu. Rev. Biochem.* 81, 479–505. doi: 10.1146/annurev-biochem-060110-113521

Koren, S., and Phillippy, A. M. (2015). One chromosome, one contig: complete microbial genomes from long-read sequencing and assembly. *Curr. Opin. Microbiol.* 23, 110–120. doi: 10.1016/j.mib.2014.11.014

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., and Phillippy, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res.* 27, 722–736. doi: 10.1101/gr.215087.116

Kortemaa, H., Pennanen, T., Smolander, A., and Haahtela, K. (1997). Distribution of antagonistic *Streptomyces griseoviridis* in rhizosphere and non-rhizosphere sand. *J. Phytopathol.* 145, 137–143. doi: 10.1111/j.1439-0434.1997.tb00376.x

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., et al. (2009). Circos: an information aesthetic for comparative genomics. *Genome Res.* 19, 1639–1645. doi: 10.1101/gr.092759.109

Lambais, M. R., and Mehdy, M. C. (1995). Differential expression of defense-related genes in arbuscular mycorrhiza. *Can. J. Bot.* 73, 533–540. doi: 10.1139/b95-293

Lapworth, D. J., Baran, N., Stuart, M. E., and Ward, R. S. (2012). Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. *Environ. Pollut.* 163, 287–303. doi: 10.1016/j.envpol.2011.12.034

Lee, M. Y., Myeong, J. S., Park, H. J., Han, K., and Kim, E. S. (2006). Isolation and partial characterization of a cryptic polyene gene cluster in *Pseudonocardia autotrophica*. *J. Ind. Microbiol. Biotechnol.* 33, 84–87. doi: 10.1007/s10295-005-0018-7

Li, W., Jaroszewski, L., and Godzik, A. (2002). Tolerating some redundancy significantly speeds up clustering of large protein databases. *Bioinformatics* 18, 77–82. doi: 10.1093/bioinformatics/18.1.77

Liu, K., Mcinroy, J. A., Hu, C.-H., and Kloepper, J. W. (2018). Mixtures of plant-growth-promoting rhizobacteria enhance biological control of multiple plant diseases and plant-growth promotion in the presence of pathogens. *Plant Dis.* 102, 67–72. doi: 10.1094/PDIS-04-17-0478-RE

Lohmann, R., Breivik, K., Dachs, J., and Muir, D. (2007). Global fate of POPs: current and future research directions. *Environ. Pollut.* 150, 150–165. doi: 10.1016/j.envpol.2007.06.051

Luo, Y., Zeng, K., and Ming, J. (2012). Control of blue and green mold decay of citrus fruit by *Pichia membranefaciens* and induction of defense responses. *Sci. Hortic.* 135, 120–127. doi: 10.1016/j.scienta.2011.11.031

Maansson, M., Vynne, N. G., Klitgaard, A., Nybo, J. L., Melchiorsen, J., Nguyen, D. D., et al. (2016). An integrated metabolomic and genomic mining workflow to uncover the biosynthetic potential of bacteria. *Msystems* 1:e00028-15. doi: 10.1128/mSystems.00028-15

Maffini, M. V., Rubin, B. S., Sonnenschein, C., and Soto, A. M. (2006). Endocrine disruptors and reproductive health: the case of bisphenol-A. *Mol. Cell. Endocrinol.* 25, 179–186. doi: 10.1016/j.mce.2006.04.033

Magrane, M., and Consortium, U. P. (2011). UniProt Knowledgebase: a hub of integrated protein data. *Database* 2011:bar009. doi: 10.1093/database/bar009

Mahadevan, B., and Crawford, D. L. (1997). Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. *Enzyme Microb. Technol.* 20, 489–493. doi: 10.1016/S0141-0229(96)00175-5

Marczynski, B., Peel, M., and Baur, X. (2000). New aspects in genotoxic risk assessment of styrene exposure–a working hypothesis. *Med. Hypotheses* 54, 619–623. doi: 10.1054/mehy.1999.0907

Minuto, A., Spadaro, D., Garibaldi, A., and Gullino, M. L. (2006). Control of soilborne pathogens of tomato using a commercial formulation of *Streptomyces griseoviridis* and solarization. *Crop Prot.* 25, 468–475. doi: 10.1016/j.cropro.2005.08.001

Mishra, S. K., Taft, W. H., Putnam, A. R., and Ries, S. K. (1987). Plant growth regulatory metabolites from novel Actinomycetes. *J. Plant Growth Regul.* 6, 75–83. doi: 10.1007/BF02026457

Misiek, M., and Hoffmeister, D. (2007). Fungal genetics, genomics, and secondary metabolites in pharmaceutical sciences. *Planta Med.* 73, 103–115. doi: 10.1055/s-2007-967104

Miyanaga, A. (2017). Structure and function of polyketide biosynthetic enzymes: various strategies for production of structurally diverse polyketides. *Biosci. Biotechnol. Biochem.* 81, 2227–2236. doi: 10.1080/09168451.2017.1391687

Montesinos, E., Bonaterra, A., Badosa, E., Francés, J., Alemany, J., Llorente, I., et al. (2002). Plant-microbe interactions and the new biotechnological methods of plant disease control. *Int. Microbiol.* 5, 169–175.

Morehead, N. R., Eadie, B. J., Lake, B., Landrum, P. F., and Berner, D. (1986). The sorption of PAH onto dissolved organic matter in Lake Michigan waters. *Chemosphere* 15, 403–412. doi: 10.1016/0045-6535(86)90534-5

Nicholson, R. L., and Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* 30, 369–389. doi: 10.1146/annurev.py.30.090192.002101

Nimnoi, P., Pongsilp, N., and Lumyong, S. (2010). Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production. *World J. Microbiol. Biotechnol.* 26, 193–203. doi: 10.1007/s11274-009-0159-3

Nishiya, Y., and Imanaka, T. (1998). Purification and characterization of a novel glycine oxidase from *Bacillus subtilis*. *FEBS Lett.* 438, 263–266. doi: 10.1016/S0014-5793(98)01313-1

Ntalli, N. G., Ferrari, F., Giannakou, I., and Menkissoglu-Spiroudi, U. (2011). Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. *Pest Manag. Sci.* 67, 341–351. doi: 10.1002/ps.2070

Olanrewaju, O. S., and Babalola, O. O. (2019). *Streptomyces*: implications and interactions in plant growth promotion. *Appl. Microbiol. Biotechnol.* 103, 1179–1188. doi: 10.1007/s00253-018-09577-y

Ortíz Castro, R., Contreras Cornejo, H. A., Macías Rodríguez, L., and López Bucio, J. (2009). The role of microbial signals in plant growth and development. *Plant Signal. Behav.* 4, 701–712. doi: 10.4161/psb.4.8.9047

Palaniyandi, S. A., Yang, S. H., Zhang, L., and Suh, J.-W. (2013). Effects of actinobacteria on plant disease suppression and growth promotion. *Appl. Microbiol. Biotechnol.* 97, 9621–9636. doi: 10.1007/s00253-013-5206-1

Pellegrini, L., Rohfritsch, O., Fritig, B., and Legrand, M. (1994). Phenylalanine ammonia-lyase in tobacco. Molecular cloning and gene expression during the hypersensitive reaction to tobacco mosaic virus and the response to a fungal elicitor. *Plant Physiol.* 106, 877–886. doi: 10.1104/pp.106.3.877

Penrose, D. M., and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant.* 118, 10–15. doi: 10.1034/j.1399-3054.2003.00086.x

Poulsen, S. M., Kofoed, C., and Vester, B. (2000). Inhibition of the ribosomal Peptidyl transferase reaction by the mycarose moiety of the antibiotics carbomycin, spiramycin and tylosin. *J. Mol. Biol.* 304, 471–481. doi: 10.1006/jmbi.2000.4229

Prashar, P., Kapoor, N., and Sachdeva, S. (2013). "Biocontrol of plant pathogens using plant growth promoting bacteria," in *Sustainable Agriculture Reviews*, ed. E. Lichtfouse (Dordrecht: Springer), 319–360. doi: 10.1007/978-94-007-5961-9_10

Quintero, J. C., Moreira, M. T., Feijoo, G., and Lema, J. M. (2008). Screening of white rot fungal species for their capacity to degrade lindane and other isomers of hexachlorocyclohexane (HCH). *Cien. Inv. Agr.* 35, 159–167. doi: 10.4067/S0718-16202008000200005

Ray, H., Douches, D. S., and Hammerschmidt, R. (1998). Transformation of potato with cucumber peroxidase: expression and disease response. *Physiol. Mol. Plant Pathol.* 53, 93–103. doi: 10.1006/pmpp.1998.0164

Rene, E. R., Špačková, R., Veiga, M. C., and Kennes, C. (2010). Biofiltration of mixtures of gas-phase styrene and acetone with the fungus *Sporothrix variecibatus*. *J. Hazard. Mater.* 184, 204–214. doi: 10.1016/j.jhazmat.2010.08.024

Saier, M. H., Reddy, V. S., Tamang, D. G., and Västermark, A. (2014). The transporter classification database. *Nucleic Acids Res.* 42, D251–D258. doi: 10.1093/nar/gkt1097

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sakharov, I. Y., and Ardila, G. B. (1999). Variations of peroxidase activity in cocoa (*Theobroma cacao* L.) beans during their ripening, fermentation and drying. *Food Chem.* 65, 51–54. doi: 10.1016/S0308-8146(98)00160-5

Samac, D. A., Willert, A. M., Mcbride, M. J., and Kinkel, L. L. (2003). Effects of antibiotic-producing *Streptomyces* on nodulation and leaf spot in alfalfa. *Appl. Soil Ecol.* 22, 55–66. doi: 10.1016/S0929-1393(02)00109-9

Shahidi Bonjar, G. H., Rashid Farrokhi, P., Aghighi, S., Shahidi Bonjar, L., and Aghelizadeh, A. (2005). Antifungal characterization of actinomycetes isolated from Kerman, Iran and their future prospects in biological control strategies in greenhouse and field conditions. *Plant Pathol. J.* 4, 78–84. doi: 10.3923/ppj.2005.78.84

Shen, T., Wang, C., Yang, H., Deng, Z., Wang, S., Shen, B., et al. (2016). Identification, solid-state fermentation and biocontrol effects of *Streptomyces hygroscopicus* B04 on strawberry root rot. *Appl. Soil Ecol.* 103, 36–43. doi: 10.1016/j.apsoil.2016.02.016

Shilpashree, K., Patil, M. R., Savitha, M. H., and Uma, M. S. (2013). Estimation of total phenols and peroxidase isozyme in plants infected with blackeye cowpea mosaic viral disease. *Mysore J. Agric. Sci.* 47, 519–522.

Shokrollahzadeh, S., Azizmohseni, F., Golmohammad, F., Shokouhi, H., and Khademhaghighat, F. (2008). Biodegradation potential and bacterial diversity of a petrochemical wastewater treatment plant in Iran. *Bioresour. Technol.* 99, 6127–6133. doi: 10.1016/j.biortech.2007.12.034

Southerton, S. G., and Deverall, B. J. (1990). Histochemical and chemical evidence for lignin accumulation during the expression of resistance to leaf rust fungi in wheat. *Physiol. Mol. Plant Pathol.* 36, 483–494. doi: 10.1016/0885-5765(90)90021-O

Storrs, S. I., and Kiesecker, J. M. (2004). Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. *Environ. Health Perspect.* 112, 1054–1057. doi: 10.1289/ehp.6821

Strap, J. L. (2011). "Actinobacteria–plant interactions: a boon to agriculture," in *Bacteria in Agrobiology: Plant Growth Responses*, ed. D. K. Maheshwari (Berlin: Springer), 285–307. doi: 10.1007/978-3-642-20332-9_13

Strobel, G. A. (2003). Endophytes as sources of bioactive products. *Microbes Infect.* 5, 535–544. doi: 10.1016/S1286-4579(03)00073-X

Sun, C., Yang, Z., Zhang, C., Liu, Z., He, J., Liu, Q., et al. (2019). Genome mining of *Streptomyces atratus* SCSIO ZH16: discovery of atratumycin and identification of its biosynthetic gene cluster. *Org. Lett.* 21, 1453–1457. doi: 10.1021/acs.orglett.9b00208

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., et al. (2003). The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* 4:41. doi: 10.1186/1471-2105-4-41

Theobald, S., Vesth, T. C., and Andersen, M. R. (2019). Genus level analysis of PKS-NRPS and NRPS-PKS hybrids reveals their origin in *Aspergilli*. *BMC Genomics* 20:847. doi: 10.1186/s12864-019-6114-2

Tran, N. H., Hu, J., and Ong, S. L. (2013). Simultaneous determination of PPCPs, EDCs, and artificial sweeteners in environmental water samples using a single-step SPE coupled with HPLC-MS/MS and isotope dilution. *Talanta* 113, 82–92. doi: 10.1016/j.talanta.2013.03.072

Udwary, D. W., Zeigler, L., Asolkar, R. N., Singan, V., Lapidus, A., Fenical, W., et al. (2007). Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10376–10381. doi: 10.1073/pnas.0700962104

Van Pee, K. (2001). Microbial biosynthesis of halometabolites. *Arch. Microbiol.* 175, 250–258. doi: 10.1007/s002030100263

Van Pee, K., and Patallo, E. P. (2006). Flavin-dependent halogenases involved in secondary metabolism in bacteria. *Appl. Microbiol. Biotechnol.* 70, 631–641. doi: 10.1007/s00253-005-0232-2

Van Wees, S. C., Van Der Ent, S., and Pieterse, C. M. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448. doi: 10.1016/j.pbi.2008.05.005

Vance, C., Kirk, T., and Sherwood, R. (1980). Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopathol.* 18, 259–288. doi: 10.1146/annurev.py.18.090180.001355

Verbon, E. H., Trapet, P. L., Stringlis, I. A., Kruijs, S., Bakker, P. A., and Pieterse, C. M. (2017). Iron and immunity. *Annu. Rev. Phytopathol.* 55, 355–375. doi: 10.1146/annurev-phyto-080516-035537

Viaene, T., Langendries, S., Beirinckx, S., Maes, M., and Goormachtig, S. (2016). Streptomyces as a plant's best friend? *FEMS Microbiol. Ecol.* 92:fiw119. doi: 10.1093/femsec/fiw119

Volpin, H., Phillips, D. A., Okon, Y., and Kapulnik, Y. (1995). Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. *Plant Physiol.* 108, 1449–1454. doi: 10.1104/pp.108.4.1449

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963. doi: 10.1371/journal.pone.0112963

Wang, B., Huang, J., Deng, S., Yang, X., and Yu, G. (2012). Addressing the environmental risk of persistent organic pollutants in China. *Front. Environ. Sci. Eng.* 6:2–16. doi: 10.1007/s11783-011-0370-y

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., et al. (2013). Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiol. Lett.* 341, 45–51. doi: 10.1111/1574-6968.12088

Wang, Z., Solanki, M. K., Pang, F., Singh, R. K., Yang, L. T., Li, Y. R., et al. (2016). Identification and efficiency of a nitrogen-fixing endophytic actinobacterial strain from sugarcane. *Sugar Tech* 19, 492–500. doi: 10.1007/s12355-016-0498-y

Wang, Z., Solanki, M. K., Yu, Z. X., Yang, L. T., An, Q. L., Dong, D. F., et al. (2018). Draft genome analysis offers insights into the mechanism by which *Streptomyces chartreusis* WZS021 increases drought tolerance in sugarcane. *Front. Microbiol.* 9:3262. doi: 10.3389/fmicb.2018.03262

Wang, Z., Wang, C., Li, F., Li, Z., Chen, M., Wang, Y., et al. (2013). Fumigant activity of volatiles from *Streptomyces alboflavus* TD-1 against *Fusarium moniliforme* Sheldon. *J. Microbiol.* 51, 477–483. doi: 10.1007/s12275-013-2586-y

Willis, K. J., and Ling, N. (2003). The toxicity of emamectin benzoate, an aquaculture pesticide, to planktonic marine copepods. *Aquaculture* 221, 289–297. doi: 10.1016/S0044-8486(03)00066-8

Yu, D., Li, T., Wang, Y. G., and Xia, H. (2004). Identification and functional analysis of dTDP-Glucose-4,6-dehydratase gene and its linked gene cluster in an aminoglycoside antibiotics producer of *Streptomyces tenebrarius* H6. *Curr. Microbiol.* 49, 99–107. doi: 10.1007/s00284-004-4212-z

Yuan, M., Yu, Y., Li, H. R., Dong, N., and Zhang, X. H. (2014). Phylogenetic diversity and biological activity of Actinobacteria isolated from the Chukchi shelf marine sediments in the arctic ocean. *Mar. Drugs* 12, 1281–1297. doi: 10.3390/md12031281

Yuan, W. M., and Crawford, D. L. (1995). Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl. Environ. Microbiol.* 61, 3119–3128. doi: 10.1128/aem.61.8.3119-3128.1995

Zhao, Y., Tu, K., Shao, X., Jing, W., and Su, Z. (2008). Effects of the yeast *Pichia guilliermondii* against *Rhizopus nigricans* on tomato fruit. *Postharvest Biol. Technol.* 49, 113–120. doi: 10.1016/j.postharvbio.2008.01.001

Zhu, Q., Dabi, T., Beeche, A., Yamamoto, R., Lawton, M. A., and Lamb, C. (1995). Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. *Plant Mol. Biol.* 29, 535–550. doi: 10.1007/BF00020983

Ziemert, N., Podell, S., Penn, K., Badger, J. H., Allen, E., and Jensen, P. R. (2012). The natural product domain seeker NaPDoS: a phylogeny based bioinformatic tool to classify secondary metabolite gene diversity. *PLoS One* 7:e34064. doi: 10.1371/journal.pone.0034064

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frontiers in Microbiology

REVIEW
published: 14 January 2022
doi: 10.3389/fmicb.2021.829099

The Potential Role of Microbial Biostimulants in the Amelioration of Climate Change-Associated Abiotic Stresses on Crops

Ayomide Emmanuel Fadiji[1], Olubukola Oluranti Babalola[1], Gustavo Santoyo[2] and Michele Perazzolli[3,4]*

[1] Food Security and Safety Niche, Faculty of Natural and Agricultural Sciences, North-West University, Potchefstroom, South Africa, [2] Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mexico, [3] Center Agriculture Food Environment (C3A), University of Trento, San Michele all'Adige, Italy, [4] Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy

Crop plants are more often exposed to abiotic stresses in the current age of fast-evolving climate change. This includes exposure to extreme and unpredictable changes in climatic conditions, phytosanitary hazards, and cultivation conditions, which results in drastic losses in worldwide agricultural productions. Plants coexist with microbial symbionts, some of which play key roles in the ecosystem and plant processes. The application of microbial biostimulants, which take advantage of symbiotic relationships, is a long-term strategy for improving plant productivity and performance, even in the face of climate change-associated stresses. Beneficial filamentous fungi, yeasts, and bacteria are examples of microbial biostimulants, which can boost the growth, yield, nutrition and stress tolerance in plants. This paper highlights recent information about the role of microbial biostimulants and their potential application in mitigating the abiotic stresses occurring on crop plants due to climate change. A critical evaluation for their efficient use under diverse climatic conditions is also made. Currently, accessible products generally improve cultural conditions, but their action mechanisms are mostly unknown, and their benefits are frequently inconsistent. Thus, further studies that could lead to the more precisely targeted products are discussed.

Keywords: arbuscular mycorrhizal fungi, plant-microbe interaction, PGPR, sustainable agriculture, climate change

OPEN ACCESS

Edited by:
Mahaveer P. Sharma, ICAR Indian Institute of Soybean Research, India

Reviewed by:
Puneet Singh Chauhan, National Botanical Research Institute (CSIR), India
Ajar Nath Yadav, Eternal University, India

****Correspondence:***
Olubukola Oluranti Babalola
olubukola.babalola@nwu.ac.za

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *04 December 2021*
Accepted: *29 December 2021*
Published: *14 January 2022*

Citation:
Fadiji AE, Babalola OO, Santoyo G and Perazzolli M (2022) The Potential Role of Microbial Biostimulants in the Amelioration of Climate Change-Associated Abiotic Stresses on Crops. Front. Microbiol. 12:829099. doi: 10.3389/fmicb.2021.829099

INTRODUCTION

Global climate records in the last decades have revealed a rise in global temperature alongside changes in rainfalls, resulting in various serious implications on environmental and agricultural aspects (Füssel et al., 2012). Crop plants are more frequently exposed to abiotic stresses caused by climate change because, aside from direct implications of abiotic stresses on plants, climate change could increase the number of pests and diseases, as well as increase the severity and frequency of the outbreak of diseases (De Wolf and Isard, 2007; Garrett et al., 2016). According to recent estimates, abiotic stresses are anticipated to cause up to 50% losses, or higher, in worldwide agricultural productivity, depending on the region (Kumar and Verma, 2018). These losses, coupled

with the continual rise in the human population, revealed that about 60% boosting of agricultural production is needed to meet the world food needs (Wild, 2003), with a concrete risk of dramatic deforestation increment and loss of natural ecosystems (Byerlee et al., 2014). Increased plant resilience to mitigate climate change-associated stresses is a sustainable method for ensuring food security with a restricted increase in agricultural surface, and the use of microbial biostimulants is one of the best options to achieve this goal (Calvo et al., 2014; Yakhin et al., 2017).

Plants are associated with a diverse group of microorganisms (the microbiome) in their endosphere (internal compartments), rhizosphere (attached soil to roots), and phyllosphere (aboveground parts), making microbial biostimulants particularly fascinating (Compant et al., 2019; Babalola et al., 2020). Crop plants coexist with microbial symbionts, which play key roles in plant production, performance, nutrition, and tolerance to abiotic stress (Vandenkoornhuyse et al., 2015; Enebe and Babalola, 2018; Ojuederie et al., 2019). For instance, geological evidence indicates that the relationship between microbes and plants predates the emergence from the water, suggesting that symbiosis involving arbuscular mycorrhizal was important in the process of terrestrialization (Selosse and Le Tacon, 1998). Moreover, microorganisms are involved in multiple biogeochemical cycles, such as nitrogen and carbon cycling, nitrogen fixation, soil formation and plant nutrition acquisition in the ecosystems (Wagg et al., 2014; Igiehon et al., 2019). As a result, many microbial symbionts can be used as a biofertilizer, releasing additional nutrients to the plant through synergistic mechanisms, which include nitrogen fixation (e.g., *Mesorhizobium loti*, *Rhizobium etli*, *Azotobacter vinelandii*, and *Azospirillum brasilense*), phosphate solubilization (e.g., Arbuscular mycorrhizal fungi, *Azospirillum* spp., *Bacillus* spp., and *Pseudomonas* spp.), cellulolytic activity (*Aspergillus* spp., *Trichoderma* spp., *Bacillus* spp., and *Penicillium* spp.), soil acidification (*Bacillus* spp. and *Arthrobacter* spp.), and production of siderophores (e.g., *Pseudomonas* spp.) (Bhattacharyya and Jha, 2012; Orozco-Mosqueda et al., 2021). Also, thanks to its abilities to boost plant development, defenses, antibacterial compounds, combat pathogen infections and feed on nematodes, *Trichoderma* spp. is a well-studied symbiotic fungal genus (Adnan et al., 2019; Szczałba et al., 2019). Despite the beneficial effects exhibited on their hosts, some of which include increased protection from abiotic stresses and nutritional efficiency, some weaknesses may limit the use of *Trichoderma* spp. as commercial biostimulant products, such as difficulties of *in vitro* cultivation and escalating of bioproduction, the lack of understanding on host specificity and population dynamics in the agroecosystem (Du Jardin, 2015). Other types of fungi can form part of the beneficial microbiome associated with plants, such as yeasts belonging to *Brettanomyces naardensis*, *Candida oleophila*, *Aureobasidium pullulans*, *Metschnikowia fructicola*, *Cryptococcus albidus*, and *Saccharomyces cerevisiae* (Freimoser et al., 2019; Nafady et al., 2019). Foliar infections can be controlled by yeasts that could colonize the leaf of a plant using direct antagonism (Preininger et al., 2018) or through the induction of systemic resistance (Lee et al., 2017). Likewise, yeast inhabiting the soil can enhance the growth of the plant through phosphate solubilization, digestion of organic materials, soil aggregation and stimulation of root development, and suppressing root infections (Sarabia et al., 2018). Plant growth-promoting bacteria (PGPB), which includes rhizobacteria or bacterial endophytes, are known to majorly populate the plant rhizosphere and the most studies genera are *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Burkholderia*, *Gluconacetobacter*, *Pseudomonas*, *Bacillus*, *Streptomyces*, and *Serratia* (Kour et al., 2020a). Additional genera are more recently proposed as possible bioinoculants with biocontrol and/or plant growth-promoting activities, such as *Rouxiella badensis* and *Rahnella* spp. (Ulloa-Muñoz et al., 2020; Morales-Cedeño et al., 2021). Physiological, molecular, and biochemical investigations of the interactions that exist between plant and beneficial microorganisms have shown that the presence of microbe-induced plant stress responses (Farrar et al., 2014; Igiehon et al., 2021; Igiehon and Babalola, 2021), which may trigger induced systemic tolerance (IST) against abiotic stressors (Yang et al., 2009; Vacheron et al., 2015).

Microbial biostimulants are a viable alternative for supporting plants exposed to abiotic stresses in the current context of fast-developing climate change (Santoyo et al., 2021b). While recent advancements and laboratory studies have revealed the positive activities of plant-associated microorganisms, the efficacy of microbial biostimulants is yet to be successfully validated in field experiments. As a result, microbial biostimulants are often used as supplemental therapies rather than being used to their full potential in crop management. The goal of this paper is to summarize current information about microbial biostimulants, especially the current commercially available products, examine their applications in enhancing plant tolerance to abiotic stresses caused by climate changes, and forecast the creation of novel products that may be used in adverse conditions. Also, limitations in the application of microbial biostimulants under field conditions alongside further studies required for the better development of targeted products are discussed.

CLIMATE CHANGE AND ITS IMPACT ON AGRICULTURE

Climate change is caused by a variety of factors, including variations in solar radiation, shifts in the Earth orbit, changes in the composition of the atmospheric gases, ocean oscillations, and changes in the surface characteristics of the soil (Meena et al., 2017; Cassia et al., 2018). The impacts of climate change, which often influence not only human existence but also ecological systems, elicit strong conflicts and emotions (Rising and Devineni, 2020). Agriculture is highly vulnerable to climate change because it is so reliant on soil quality, irrigation and weather conditions (Kumar et al., 2021). Floods, droughts, heat stress, rainfall unpredictability, and severe weather occurrences have a significant negative influence on global agricultural practices as a result of climate change (Myers et al., 2017; Rising and Devineni, 2020; Fiodor et al., 2021). It is clear that climate change may have a greater impact in some regions of the world, suggesting that existing farming systems and infrastructure will need to change and rapidly adapt to future

changes (Müller et al., 2011). Plants, as stationary creatures, are compelled to adapt to their surrounding conditions to live and they can respond to diverse abiotic stressors with precise acclimations of physiological, developmental, and metabolic processes (Meena et al., 2017; Myers et al., 2017). Unfortunately, plant tolerance to one abiotic factor may be reduced as a result of exposure to another abiotic or biotic factor, increasing plant sensitivity (Laanisto and Niinemets, 2015). However, a multivariate acclimation in response to multiple abiotic factors is also possible (Laanisto and Niinemets, 2015).

Global Warming

The Intergovernmental Panel on Climate Change (IPCC) defines global warming as an "increase in both sea surface temperature and surface air averaged above 30 years" throughout the planet (Allen et al., 2018). It is also claimed that as the Earth moves from the relatively stable Holocene to the more dynamic Anthropocene, where human activities are now implicated as a major source of climate change and human-caused global warming has now surpassed pre-industrial levels by roughly 1°C (Allen et al., 2018). Subsequently, several parts of the world have experienced a far greater rise in temperature than the worldwide average, leading to a possible rise from 1.5 to 4.8°C by 2100, according to estimates (Fiodor et al., 2021; Jagadish et al., 2021). Furthermore, land-based warming is outpacing the global average and it was estimated 1.5°C in the years 2006–2015 compared to the years 1850–1900 (Fiodor et al., 2021).

Temperature is an important environmental factor that affects physiological processes and plant growth. Temperature increases promote quicker mass growth and shorter culture times (Zhao et al., 2017). However, a sudden increase or decrease in temperature can damage plant cells, resulting in reduced growth and yield overall (Zhao et al., 2017; Seguini et al., 2019). Also, for every 1°C increase in world average temperature, 3.1 to 7.4% yield losses were estimated in rice, maize, soybean and wheat for over 29 years of warming trends (Seguini et al., 2019). Another research revealed a 4 and 6% reduction in maize and wheat yields, respectively (Lobell and Gourdji, 2012). Heat stress is a limiting factor for photosynthesis in C3 crops, like rice and wheat, as well as C4 plants, like maize and sugarcane crops (Crafts-Brandner and Salvucci, 2002; Song et al., 2014; Zhao et al., 2017). Plants are also affected by temperature rise due to changes in humidity since reduced water vapor concentration in the air causes water loss from the plant, forcing stomata to be closed and lowering photosynthetic efficiency and leaf cooling by transpiration (Zhang et al., 2019; Díaz-Barradas et al., 2020). Moreover, water stress in plants is caused by prolonged high temperatures, which results in water shortages (Díaz-Barradas et al., 2020).

The hazard of global warming to crops is not limited to changes in global mean temperature, but also to sea-level rise, desertification, extreme weather conditions and changed precipitation (Russo et al., 2014). For example, due to water mixing, sea-level rise causes salinization of fresh water and land loss (Fiodor et al., 2021). Exacerbation of uncommon weather phenomena, such as floods, heatwaves, and severe precipitation, is being reported when the hydrological cycle accelerates with global warming (Hutchins et al., 2019; Perkins-Kirkpatrick and Lewis, 2020; Hawkings et al., 2021). Thus, global warming might have a slew of unanticipated consequences. For example, the melting of Greenland's ice sheet is supposed to become a possible source of hazardous mercury contamination in the Atlantic seas (Hawkings et al., 2021).

Greenhouse Gas Emission

Greenhouse gases (GHGs) are parts of the troposphere of the Earth, and they may sustain solar energy. Thanks to GHGs, the average temperature in most areas remains below 40°C, allowing plants to flourish freely (Fiodor et al., 2021). Forest fires, seas, earthquakes, permafrost, wetlands, and volcanoes are the natural environmental sources of GHG emissions that are generally absorbed by the environment (Yue and Gao, 2018). However, human activity causes an excess in GHG emission, which upsets the equilibrium (Yue and Gao, 2018). Methane, nitrous oxide and carbon dioxide are examples of the three most common greenhouse gases generated by human activity and more than 90% of human-derived global warming is thought to be caused by these gases (Wu and Mu, 2019). Along with the rise in greenhouse gases release, global warming processes have become more intense as a result of urbanization, globalization, and industrialization (Yusuf et al., 2020), while the ever-increasing human population has increased the need for energy production.

When comparing carbon dioxide levels in the atmosphere before and after the industrial revolution, it is apparent to state that human activities are the primary source of this gas (Meena et al., 2017; Hawkings et al., 2021). Carbon dioxide levels are quickly rising, from 280 ppm before the industrial revolution to 400 ppm now and it is estimated that 540 ppm will be achieved by 2100 (Myers et al., 2017). Deforestation-related increases in land use account for one-fifth of total carbon dioxide emissions (Szczałba et al., 2019). Over the decades, vast amounts of carbon dioxide are introduced into the atmosphere as fossil carbon that has accumulated in the Earth's crust over millions of years (Anwar et al., 2020). Greenhouse gas emissions in the European Union (EU) and United States of America (USA) were 4.4 and 6.5 billion metric tons of carbon dioxide equivalent in 2018–2019, respectively (Fiodor et al., 2021; Gołasa et al., 2021). China topped the list of the biggest polluters in 2014, accounting for 30% of all carbon dioxide emissions, while Japan, Russia, India, European Union and the United States contributed 4, 5, 7, 9, and 15% of carbon dioxide emissions, respectively (Fiodor et al., 2021). Different major economic sectors are associated to GHG emission, such as (i) industry, (ii) heat and electricity production, (iii) forestry, agriculture, and other land use, (iv) building, and (v) transportation (EPA, 2021). In particular, forestry, agriculture, and other land uses were responsible for 24% of worldwide greenhouse gas emissions in the year 2010, while only agriculture showed a proportion that ranged from 1 to 14%, which is similar to the production from the industry (Fiodor et al., 2021). Since 1990, greenhouse gas emissions in agriculture have risen by 12%, due to a 9% increase in nitrous oxide emissions from soil management practices and a 60% rise in nitrous oxide and methane emissions from animal manure management systems (EPA, 2021; Fiodor et al., 2021),

indicating the increasing application of emission-intensive liquid systems over the years. However, agricultural soil management strategies that increase nitrogen availability in the soil and reduce nitrous oxide emissions are available (IPCC, 2014). Nitrous oxide emissions from agricultural areas are caused by the application of organic and chemical fertilizers, the growth of nitrogen-fixing crops, irrigation methods and organic soil draining (EPA, 2021). In particular, excess nitrogen application can result in increased nitrous oxide emission without contributing to the growth and yield of the crop, indicating that more precise nitrogen fertilization for optimum crop production may help lower greenhouse gas emissions (Fiodor et al., 2021).

Changes in nutrient composition and photosynthetic rates in plants are two major effects of GHGs in agriculture (Myers et al., 2017). Reduced transpiration occurs as a result of increment in carbon dioxide concentrations, with a consequent increment in photosynthesis and water use efficiency. Rapid plant growth in high carbon dioxide environments changes the composition of essential elements for animals and humans. For example, legumes and cereals grown under carbon dioxide concentration of 550 ppm have iron and zinc levels that are 3–11% lower compared to a plant grown under normal atmospheric concentration (Gornall et al., 2010; Sangiorgio et al., 2020). Likewise, phosphorus, calcium, potassium, zinc, sulfur, iron, copper, and magnesium concentrations decrease by about 5–13% in various crops when the carbon dioxide concentration is 690 ppm (Loladze, 2014). Under elevated carbon dioxide, a decrease in nitrogen and protein content in edible parts of C3 plants have been widely reported (Jobe et al., 2020), indicating an increased risk of nutritional problems for people living in the underdeveloped parts of the world (Loladze, 2014).

THE IMPACT OF MICROBIAL BIOSTIMULANTS IN AMELIORATING CLIMATE CHANGE-INDUCED STRESSES ON PLANTS

High Temperature

High temperatures affect the physiology of the plant by raising the respiration rates and leaf transpiration and altering photosynthate allocation (Munns, 2002; Malhotra, 2017). Rubisco affinity for carbon dioxide decreases at high temperatures and its affinity for oxygen increases (Jordan and Ogren, 1984; Sangiorgio et al., 2020). Carbon dioxide solubility is reduced more than oxygen when the temperature rises, lowering carbon dioxide concentration in the chloroplast in comparison to oxygen (Ku and Edwards, 1977). Furthermore, plants shut their stomata to decrease evapotranspiration water losses when the temperature rises. When stomata closure occurs, carbon dioxide concentration declines rapidly, but oxygen concentration rises, limiting photosynthesis, increasing the photorespiration (Bhattacharya, 2019).

Higher temperatures in spring would allow many cycles per year for annual crops, such as tomato (Sangiorgio et al., 2020) and lettuce (Pearson et al., 1997), enhancing early flowering in horticultural crops (Wheeler et al., 1993; Bisbis et al., 2018). On the other hand, increasing temperatures may provide difficulty for floral differentiation in several horticultural crops. For example, high temperatures enhance male floral development but reduce feminine flower differentiation in cucumbers (Sangiorgio et al., 2020). In fruit crops, such as apple (Funes et al., 2016), peach, and plum (Hazarika, 2013), difficulty in meeting cold requirements for floral differentiation might reduce crop production (Luedeling, 2012). In the long run, climate change may move fruit cultivation areas to the North (Luedeling, 2012), but moderate winter temperatures are capable of stimulating early blooming, thereby exposing plants to late frost risks (Vitasse and Rebetez, 2018). Finally, increasing temperatures may affect agricultural practice in tropical environments which often result in the extinction of vulnerable crops (Gornall et al., 2010).

Heat stress causes complex molecular, biochemical, and physiological reactions in plants (Sangiorgio et al., 2020), which may result in the synthesis of heat shock proteins, enzymes that degrade reactive oxygen species (ROS), osmoprotectant chemicals, amino acids, sugars, and sulfur compounds (Shulaev et al., 2008). Thus, oxidative stress and ROS production are stimulated by heat stress, which is sensed by histidine kinases and Heat stress factors (Hsfs), then redox-sensitive transcription factors are downstream activated by the MAPK signal pathway, which subsequently activates other transcription factors (e.g., BF1c and Rboh) to turn on the expression of genes involved in the synthesis of antioxidant enzymes (Qu et al., 2013; **Figure 1**).

Hormonal signaling is in charge of heat stress responses. Ethylene is one among them (Byerlee et al., 2014), and it is involved not only in senescence, development, plant physiology, and development but also in plant responses to abiotic stressors, such as heat, salinity and drought (Dubois et al., 2018). Plant responses to heat stress can be boosted by microbial biostimulants. For example, the synthesis of ROS-degrading enzymes (e.g., superoxide, catalase, peroxidases, and dismutase) enhance heat stress tolerance and it can be boosted in plants colonized by beneficial bacteria, such as *Pseudomonas* and *Bacillus*, and mycorrhizal fungi, such as *Septoglomus deserticola* and *Septoglomus constrictum* (Duc et al., 2018). SoilPro® (Liventia, TX, United States) is a microbial biostimulant that helps in improving soil quality and heat stress tolerance and it contains *P. fluorescens* and *P. aeruginosa*, which are commercialized for bioremediation, phytostimulation, and improved capabilities of soil fertility (Sangiorgio et al., 2020). Likewise, *Bacillus* spp. have been developed not only as biopesticides (e.g., *Bacillus amyloliquefaciens*, *Bacillus firmus*, *Bacillus pumilus*, *Bacillus thuringensis*, *Bacillus licheniformis*, *Bacillus sphaericus*, and *Bacillus subtilis*), and some biostimulant products made up of *Bacillus* spp. are available, such as Endox® (Scam, Spa, Italy) and Activate® (Natural resources Group, Inc., United States). While these microorganisms are used in many commercial treatments, whether alone or as a mixture, mitigation from heat stress is not usually stated as one of their benefits.

The use of microorganisms that limit ethylene emissions has a lot of promise because lowering ethylene levels in stressful situations might help plants escape the harmful effects of

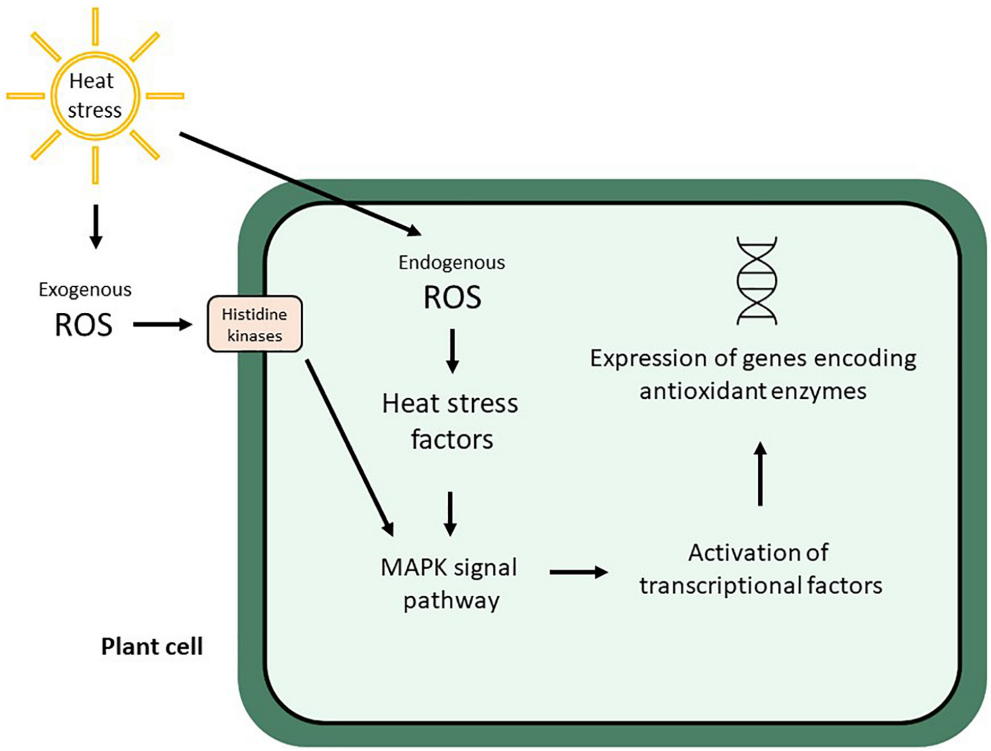


FIGURE 1 | Plant cell response to exogenous and endogenous ROS production stimulated by heat stress (modified from Qu et al., 2013).

heat stress (Glick, 2014). In particular, bacteria that exhibit 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, appear to be highly promising, such as *Bacillus subtilis* BERA 7, *Leclercia adecarboxylata* MO1, *Pseudomonas fluorescens* YsS6, and *Pseudomonas migulae* 8R6 (del Carmen Orozco-Mosqueda et al., 2020). For example, the ACC deaminase-producing *Paraburkholderia phytofirmans* PsJN allows normal tomato development under heat stress (Esmaeel et al., 2018). Despite its apparent beneficial properties, *P. phytofirmans* PsJN is yet to be used as a commercial product (Sangiorgio et al., 2020). The ability of microbial biostimulants able to mitigate heat stress in crops was associated to the reduction of ROS content, the increase in heat shock protein and osmoprotectant (e.g., sugar and proline) content and the modulation of hormonal levels (**Figure 2**), such as ethylene and abscisic acid (**Table 1**). In particular, *Pseudomonas* sp. AKMP6 and *P. putida* AKMP7, *Glomus* sp., *B. aryabhatthai* SRBO2, *B. amyloliquefaciens*, *A. brasilense*, and *P. phytofirmans* (Ali et al., 2009; Ali and Xie, 2020). However, further efforts on the understanding of the mechanism of action of microbial biostimulants against heat stress and on the development of efficient formulations are required to mitigate the impact of heat stress on agricultural and food production by sustainable approaches.

Low Temperatures

Frost events have been reported in several counties in recent years, such as in Italy, Switzerland, Germany, Belgium, USA, and France, resulting in serious crop damage and economic losses (Unterberger et al., 2018; Vitasse and Rebetez, 2018). Low temperatures decrease plant metabolism, leading to reduced photosynthetic activity, reduced foliar growth, and early senescence, when temperatures are above the freezing point (Huner et al., 1993). Bud development can be hampered by temperatures below freezing since rehydrated and growing buds might be destroyed by the cold stress (Mishra et al., 2012). Cold can dehydrate plant tissues, causing a rise in the concentration of osmolytes in the cytoplasm of the cell and leading to the breakdown of the plasma membrane (Mishra et al., 2012).

Microorganisms possessing ice nucleating activity (INA^+), which can survive on fruits, leaves, and roots, can promote the development of ice cores in plants (Lindow, 1983). The extracellular polymeric substances (exopolysaccharides) produced by these microorganisms are made up of proteins that function as ice nucleation centers on their cell wall, promoting the production of ice crystals (Lee et al., 1995). These microbes are mostly bacteria, although ice-nucleating fungi have also been discovered (Pouleur et al., 1992), which may colonize the plant both epiphytically and endophytically. For example, *P. syringae* was the first bacterial strain reported to possess ice nucleating activity (Arny et al., 1976; Fadiji and Babalola, 2020a,b; Galambos et al., 2021). The presence of *P. syringae* strains with ice-nucleating activity enhanced the sensitivity of soybean and tomato to cold damage in experimental conditions (Anderson et al., 1982). Likewise, other species exhibited ice nucleating activity, such as *Erwinia herbicola* (syn: *P. agglomerans*) (Lindow et al., 1978), *Xanthomonas campestris* (Kim et al., 1987), and the

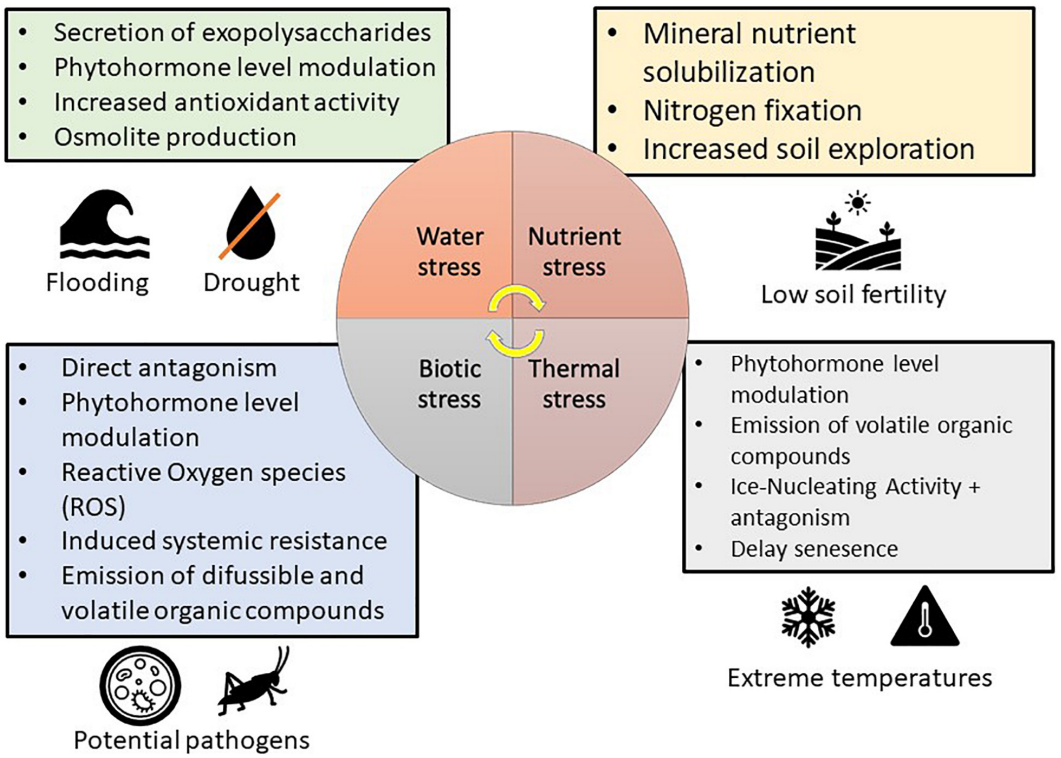


FIGURE 2 | Protective mechanisms used by microbial biostimulants in mitigation of various stresses in plants (modified from Sangiorgio et al., 2020).

TABLE 1 | Microbial biostimulants that mitigate extreme temperature stress in crops.

Crop Plant	Microbes involved	Mechanism of action	References
Protection against cold and freezing stress			
Grapevine	*Paraburkholderia phytofirmans*	Production of 1-Aminocyclopropane-1-carboxylic Acid (ACC)	Theocharis et al., 2012
Pear and apple	*Pseudomonas fluorescens* A506	Competition with bacteria having INA^+	Lindow and Brandl, 2003
Bean plant	*Pseudomonas fragi*, *Pseudomonas fluorescens*, *Pseudomonas proteolytica*, *Brevibacterium frigoritolerans*, and *Pseudomonas chlororaphis*	Reduction of reactive Oxygen Species (ROS) and lipid peroxidation	Tiryaki et al., 2019
Wheat	*Pantoea dispersa* 1A, *Pseudomonas* spp. and *S. marcescens* SRM	Production of ACC and IAA	Mishra et al., 2008; Selvakumar et al., 2008a,b
Protection against heat stress			
Sorghum and wheat	*Pseudomonas* sp. AKMP6 and *Pseudomonas putida* AKMP7	Reduction of reactive Oxygen Species (ROS), increment in content sugar, proline, starch, chlorophyll, protein, and amino acid, phytohormone production.	Ali et al., 2009, 2011
Tomato	*Glomus* sp.	Higher scavenging activity of ROS in the rots and leaves and reduction in peroxidation of lipid.	Duc et al., 2018
Soybean	*Bacillus aryabhatthai* SRBO2	Production of abscisic acid	Park et al., 2017
Wheat	*Bacillus amyloliquefaciens*, and *Azospirillum brasilense*	Reduction of reactive Oxygen Species (ROS) and heat shock proteins pre-activation	Abd El-Daim et al., 2014
Potato	*Paraburkholderia phytofirmans*	Reduction of H_2O_2 and Production of ACC.	Sangiorgio et al., 2020

Gram-positive bacteria *Lysinibacillus* sp. (Failor et al., 2017). On the other hand, frost damage was minimized using *P. syringae* A506 with an inactivated ice-nucleating gene (Skirvin et al., 2000) and the use of microbial biostimulants capable of competing INA^+ bacteria has become a critical strategy for reducing frost damages (**Figure 2**; Selvakumar et al., 2012). For example, Blightban A506® (Nufarm Americas, Inc., TX, United States), which was produced using lyophilized *P. fluorescens* A506 (Lindow and Brandl, 2003), is a commercial product for the prevention of frost damage in USA (**Table 1**) and this formulation is also used to combat fire blight (*E. amylovora*) in pear and apple trees (Lindow and Brandl, 2003).

Microbial biostimulants can also help to reduce the effect of chilling temperatures. Plant growth suppression caused by low temperature can be countered by microbial symbionts that produce growth-related hormones (e.g., auxins or gibberellins) or that reduce ethylene concentration (**Figure 2** and **Table 1**; Sangiorgio et al., 2020). Several bacteria produce indole-3-acetic acid (IAA), which represents an example of auxin (Amara et al., 2015). For instance, IAA is produced by *P. dispersa* 1A and *S. marcescens* SRM strains isolated from the Himalayan northwest (Amara et al., 2015). Thus, wheat seedlings inoculated with these strains and cultivated under cold condition exhibited considerably better nutrient and yield absorption ability competed to control seedlings (Selvakumar et al., 2008a). Likewise, cold tolerance is enhanced in wheat seedlings treated with *Pseudomonas* sp. NARs9 and PGERs (Mishra et al., 2008, 2012). Cold stress causes a rise in ethylene production, which reduces plant productivity (Glick, 2014) and *P. phytofirmans* PsJN, which exhibited ACC deaminase activity, improved cold tolerance in grapevine by decreasing the damage to the cell membrane (Theocharis et al., 2012). Bean plants cultivated in extreme cold conditions and inoculated with psychrophilic ACC deaminase-producing bacteria, such as *P. fragi*, *P. fluorescens*, *P. proteolytica*, *B. frigoritolerans*, and *P. chlororaphis*, revealed less frost damage through reduced ROS production and lipid peroxidation (Tiryaki et al., 2019). Out of all the bacterial species identified, *P. fluorescens* and *P. chlororaphis* are the examples of the few microbial biostimulants found in the market as biopesticides (Cedomon®, BioAgri AB, Uppsala, Sweden) while the other one is formulated in combination with similar PGPR products such as HyperGalaxy® (Holmes Enviro, Llc., Philomath, OR, United States), BFMS® (Tainio, Cheney, WA, United States), SoilBiotic® (SoilBiotics, Reddick, IL, United States) and BioStrain® (Monty's Plant Food Company, Louisville, KY, United States). More studies toward the formulation of microbial biostimulant to combat cold stress in crop plant is advocated.

Salinity and Drought Stress

Drought or excessive salinity can induce water stress, which happens when leaf transpiration losses or surpass root absorption, resulting in a decrease in water content and turgor in plant tissues (Sangiorgio et al., 2020). Drought stress has been increasingly severe and frequent, especially in semi-arid regions (Trenberth et al., 2014). Furthermore, when rainfall is light and irregular, salt buildup in the soil can worsen the effects of drought (Othman et al., 2006), since a rise in the solutes level in the saline soil lowers the osmotic potential of the liquid phase of the soil, limiting the absorption of water by plant roots (Sequi, 2006). For example, soil salinization affects around 30% of total arable land in the Mediterranean regions (Rasool et al., 2013). Drought stress and the resulting soil salinization are also major contributors to desertification in overexploited regions, they affect soil biodiversity and composition, contributing to plant deterioration, sparse soil covering and consequent soil erosion (Vicente-Serrano et al., 2020). Drylands now occupy 46% of the world's land surface, impacting about 250 million persons in developing countries (Huang et al., 2020).

Drought stress influences plant physiology and morphology, causing harmful ROS buildup (Smirnoff, 1993), emission of ethylene (Ali et al., 2014), and decreased mineral nutrient availability, absorption, and transport (Rouphael et al., 2012). Plant tolerance to drought and salinity can be improved by microbial biostimulants through a variety of direct and plant-mediated processes (**Figure 2** and **Table 2**). For example, microbial biostimulants can produce bacterial exopolysaccharides that enhance soil structure by forming micro and macroaggregates (Grover et al., 2011), improving plant development under water stress conditions (Sangiorgio et al., 2020). Furthermore, exopolysaccharides form hydrophilic biofilms and create a microenvironment that promotes the retention of water by shielding microbes from drought stress (Nwodo et al., 2012) and binds Na^+ ions to reduce uptake by the plant (Tewari and Arora, 2014). For example, *Pseudomonas* spp. PF23 (Tewari and Arora, 2014) and *Pseudomonas putida* GAP-P45 (Tallon et al., 2003) exhibit protective potentials by the production of exopolysaccharides. Exploring root capacity can be strengthened by mycorrhizal fungi (Khan et al., 2015), allowing for increased root biomass, improved structure of the soil, increased water retention, and reduced leaching of the mineral nutrients (Cavagnaro et al., 2015; Santoyo et al., 2021a). In particular, *Glomus* sp. generates a glycoprotein called glomalin, which has an aggregating impact on soil structure and improves plant development and drought tolerance (Gong et al., 2013). Likewise, ascomycetes (*P. glomerata* LWL2, *Exophiala* sp. LHL08, *P. formosus* LHL10, and *Penicillium* sp. LWL3) colonize cucumber plants, enhancing leaf development and chlorophyll content under drought stress (Khan et al., 2015; Fadiji et al., 2020). Apart from promoting root development, mycorrhizal fungi can also improve water absorption through aquaporins (Aroca et al., 2007), which is one of the large families of integral membrane transporters that enable the flow of water through the phospholipid bilayer layer of the cell membrane (Bray, 2000). For example, *Glomus intraradices* mycorrhizes common bean plant and mitigate water stress affecting aquaporin activity and improving water conductivity in the roots (Aroca et al., 2007; Omomowo et al., 2018). *Glomus intraradices*, when cultivated in association with carrot, increased the expression of two fungal aquaporins (GintAQPF2 and GintAQPF1), which consequently enhanced water transport between the 2 symbionts and therefore gave the plant higher resilience to water shortage (Li T. et al., 2013). *G. intraradices* is commonly found in commercial products, often formulated in combination with many other

TABLE 2 | Microbial biostimulants that mitigate salinity and drought stress in crops.

Crop Plant	Microbes involved	Mechanism of action	References
Protection against salinity stress			
Tomato	*Leclercia adecarboxylata* MO1	ACC deaminase and IAA production	Khan et al., 2019
French bean	*Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp.	VOCs, ACC deaminase and IAA production	Gupta and Pandey, 2019
Canola	*Pseudomonas* spp.	ACC deaminase and IAA production	Akhgar et al., 2014
Pepper	*Burkholderia and Bacillus*	Enhancement of proline accumulation	Ait Barka et al., 2006
Tomato	*Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6	IAA production	Ali et al., 2014
Soybean	*Pseudomonas simiae*	Quinoline and Glycine max 4-nitroguaiacol promote seed germination	Vaishnav et al., 2016
Tomato	*Pseudomonas fluorescens* UM270	IAA and VOCs production	Hernández-León et al., 2015
Lettuce	*Bacillus subtilis*	Cytokinin signaling and shoot biomass	Arkhipova et al., 2007
Potato	*Bacillus* spp.	IAA production	Tahir et al., 2019
Protection against drought stress			
Wheat	*Bacillus thuringiensis* AZP2	Production of volatile organic compounds	Khan et al., 2018
Sunflower	*Pseudomonas putida* strain GAP-P45 and *Pseudomonas* spp. PF23	Production of exopolysaccharide	Tallon et al., 2003; Tewari and Arora, 2014
Pepper	*Bacillus licheformis* strain K11	Stress-related protein and genes	Lim and Kim, 2013; Ali and Xie, 2020
Maize	*Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD	Reduced H_2O_2 induced damage	Naveed et al., 2014
Wheat	*Streptomyces coelicolor* DE07, *Streptomyces olivaceus* DE10, and *Streptomyces geysiriensis* DE27	Phytohormone (IAA) synthesis and increment in water stress tolerance	Yandigeri et al., 2012
Mung bean	*Staphylococcus* sp. Acb12, *Staphylococcus* sp. Acb13 and *Staphylococcus* sp. Acb14	Production of indole acetic acid, ACC deaminase, and promotion of plant growth	Jayakumar et al., 2020
Pepper	*Bacillus, Achromobacter*, *Klebsiella* sp. and *Citrobacter* sp.	Production of ACC deaminase	Marasco et al., 2012
Cucumber	*Pterolepis glomerata* LWL2, *Exophiala* sp. LHL08, *Paecilomyces formosus* LHL10, and *Penicillium* sp. LWL3	Production of glomalin	Gong et al., 2013; Khan et al., 2015
Beans and carrot	*Glomus intraradices*	Increasement in the expression aquaporins (GintAQPF2 and GintAQPF1)	Aroca et al., 2008; Li T. et al., 2013
Orange	*Funneliformis mosseae*	Production of IAA	Liu et al., 2018
Cucumber	*Acinetobacter*, *Pseudomonas*, and *Burkholderia* spp.	Production of gibberellins	Kang et al., 2015
Soybean	*Pseudomonas putida* H-2-3	Synthesis of ABA	Kang et al., 2014
Basil	*Pseudomonas* sp., *Bacillus lentus*, and *Azospirillum brasilense*	Production of Ascorbate, peroxidase and glutathione peroxidase	Heidari and Golpayegani, 2012
Wheat	*Pseudomonas libanensis* EU-LWNA-33	Adaptive phosphorus solubilization, production of Ammonia, hydrogen cyanide, and ACC deaminase.	Kour et al., 2020c
Foxtail millet	*Acinetobacter calcoaceticus EU- LRNA-72* and *Penicillium* sp. *EU-FTF-6*	Enhancement of the accumulation glycine betaine, proline, sugars, increased chlorophyll content, and decrease in lipid peroxidation	Kour et al., 2020d
Millet	*Streptomyces laurentii EU-LWT3-69* and *Penicillium* sp. *strain EU-DSF-10*	Enhancement of the accumulation of glycine betaine, proline, sugars, and decrease in lipid peroxidation	Kour et al., 2020b

beneficial bacterial and fungal strains, such as OroSoil® (Fomet, Spa, Verona, Italy) and MycoApply® All Purpose (Mycorrhizal Applications, Inc., OR, United States), but it also exists in single formulations, such as Agtiv® (PremierTech, Rivière-du-Loup, Canada) and Groundwork® (GroundWork BioAg, Ltd., Hashahar, Israel).

Microbial biostimulants have a variety of additional effects against drought stress on associated plants, such as the antioxidant defenses, the production of protective osmolytes (e.g., glycine betaine) or phytohormones, and the secretion of volatile organic compounds (VOCs) (**Figure 2** and **Table 2**; Kaushal and Wani, 2016; Sharifi and Ryu, 2018). Since stress condition boosts ethylene synthesis, which limits plant development, microbial biostimulants (e.g., *P. fluorescens*) might subtract ACC, reduce ethylene synthesis and relieve inhibition mediated by ethylene (Glick, 2014). Other microbial species that produce ACC in reducing drought stress include *Staphylococcus* sp. Acb12, *Staphylococcus* sp. Acb13, Staphylococcus sp. Acb14, *Bacillus, Achromobacter*, *Klebsiella*, and *Citrobacter* spp. (Marasco et al., 2012; Jayakumar et al., 2020). Beneficial microorganisms that produce ACC deaminase might help to mitigate these side effects under drought and salt stresses,

such as *A. piechaudii* ARV8 in pepper and tomato plants (Mayak et al., 2004) and *P. fluorescens* TDK1 in the peanut seedlings (Saravanakumar and Samiyappan, 2007). Plant growth promotion and soil improvement are attributed to *Achromobacter* spp. and *Pseudomonas* spp. and these beneficial microorganisms are developed in commercial products, such as SSB® and SOS® (Liventia, Inc., San Antonio, TX, United States) (**Table 3**).

Meanwhile, microbial biostimulants can synthesize IAA, which stimulates plant growth and root branching of the plant under drought stress (Ouyang et al., 2017), for example, species belonging to *Alcaligenes*, *Sinorhizobium*, *Serratia*, *Bacillus*, and *Arthrobacter* (Ouyang et al., 2017; **Table 2**). In particular, when inoculated on salt-stressed cucumber and tomato plants, the bacterium *P. chlororaphis* TSAU13, an IAA producing strain, can enhance plant tolerance to drought and salinity (Egamberdieva, 2012). Likewise, the mycorrhizal fungus *Funneliformis mosseae* improves IAA level in the root, root hair development, and growth of orange plants under drought stress (Liu et al., 2018). The Wettable Mycorrhizae Blend® (BioLogic Crop Solutions, Inc., CA, United States) is a commercial product based on *Funneliformis mosseae* that improves the plant capacity to absorb nutrients and water (Sangiorgio et al., 2020). Similarly, microorganisms that produce cytokinins and gibberellins are effective in reducing water stress damage by promoting stomatal opening and shoot development in low-water circumstances (Liu et al., 2013). PGPR strains of *Acinetobacter*, *Pseudomonas*, and *Burkholderia* spp. have been characterized as being able to produce gibberellins and these bacteria can boost cucumber growth under salt and drought conditions (Kang et al., 2015). Despite their potentially helpful properties, no commercial products based on these species are available, suggesting that novel products will be probably launched in the future.

After exposure to drought stress, the synthesis of ABA increases to promote stomatal closure. When inoculated with *P. putida* H-2-3, ABA content decreases in soybean, reducing the impact of drought stress on plant productivity (Kang et al., 2014). *P. putida* is sold as the commercial product N-Texx® (CXI, Coppell, TX, United States) in conjunction with *B. subtilis* for their roles on soil fertility, but not particularly for drought stress alleviation. Likewise, *G. intraradices* inoculation lowers ABA concentration and reduces sensitivity to salt stress in lettuce (Jahromi et al., 2008). Plants produce more ethylene because of ABA and water shortage and plant development can be reduced by high ethylene levels.

Under water stress, the production of ROS and oxidative damage to lipids, nucleic acids, and proteins are common. Many microbes can mitigate the negative effects of increased ROS by producing antioxidant molecules or increasing the activity of antioxidant plant enzymes, such as peroxidases and catalase (Vurukonda et al., 2016). When inoculated with *Pseudomonas* sp., basil plants exhibited an increment in the activity of catalase under water stress (Vurukonda et al., 2016). Likewise, *Pseudomonas* sp., *Bacillus lentus*, and *Azospirillum brasilense* are an example of microbial stimulants that have been used individually or in the formulation of microbial consortia which can reduce drought stress in crop plants (Sangiorgio et al., 2020). Ascorbate, peroxidase and glutathione peroxidase activities of *Pseudomonas* sp., *Bacillus lentus*, and *Azospirillum brasilense* was used to enhance drought stress (Heidari and Golpayegani, 2012).

Osmocompatible solute accumulation is a type of stress response that allows the accumulation of inorganic and organic solutes in the vacuole and cytosol, respectively, to lower the osmotic potential of the cell and to maintain its turgor under water stress (Sangiorgio et al., 2020). Numerous bacteria produce osmolytes (Paul et al., 2008), which often work together with osmolytes produced by the plant (e.g., proline) to lower the osmotic potential and stabilize the cell wall components (Sanders and Arndt, 2012). The phosphate-solubilizing *B. polymyxa* strain produces proline when inoculated in tomato plants, reducing the effects of water stress (Shintu and Jayaram, 2015). Betaine produced by osmotolerant bacteria from the rhizosphere (e.g., *Streptomyces tendae* F4) often works in tandem with that produced by the host plant in rice, leading to an increased tolerance to water stress (Dimkpa et al., 2009). Even though the results were encouraging, *B. polymixa* is not yet available as a commercial product.

Some bacteria can interact with plants by VOCs, which can trigger stress adaption reactions mediated by mineral uptake, water conservation, and root growth (Bailly et al., 2014). While the importance of hormone signaling cascades has been demonstrated (Bhattacharyya et al., 2015; Tahir et al., 2017), the underlying VOC-mediated mechanisms involved in plant-microbe associations under extreme conditions remain mainly unknown. Beneficial effects of microbial VOCs are known on plants, such as 2,3-butanediol on plant fitness (Sangiorgio et al., 2020), synthesis of osmoprotectants and control of stomata closure (Zhang et al., 2010). Likewise, 1-heptanol, 3-methyl-butanol, and 2-undecanone produced by *Parabulkholderia phytofirmans* enhance salinity tolerance (Ledger et al., 2016), whereas butyrolactone and 1-butanol increase the root growth and the exchange of carbon in plant's rhizosphere (Gutiérrez-Luna et al., 2010). The future of VOC-based plant promotion will almost certainly hinge on the identification of stress-induced signaling pathways. The production of VOCs by *Bacillus thuringiensis* AZP2 helped in the mitigation of drought stress in wheat plant (Khan et al., 2018).

Although there are a large number of microorganisms that are capable of protecting plants from water stress, a limited number of commercial products exist for this vital purpose. The majority of these market products Ryze® (L.Gobbi, Srl, Genova, Italy), Micosat F® (CCS, Srl, Aosta, Italy; Suma), Grolux® (RRR Supply Inc., Munger, MI, United States), HyperGalaxy® (Holmes Enviro, Llc., Philomath, OR, United States, BFMS® (Tainio, Cheney, WA, United States), Environoc® (Biodyne, Llc., Wayne, IN, United States), and SoilBiotics® (SoilBiotics, Reddick, IL, United States) are just a few of the successfully commercialized products based on consortia of *Azospirillum brasilense*, *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Cellulomonas cellasea*, *P. fluorescens*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Streptomyces albidoflavus*, *Glomus* spp., and *Trichoderma* spp., that exhibit water stress capacity, as well as enhancement of plant yield

TABLE 3 | Summary of some currently available commercial products used in the mitigating abiotic stresses.

Commercial name	Composition	Application	Production company	Country of production
Endox®	*Bacillus* spp.	Mitigation of heat stress	Scam, spa	Italy
Activate®	*Bacillus* spp.	Mitigation of heat stress	Natural resources Group, Inc.	United States
SoilPro®	*P. fluorescens* and *P. aeruginosa*	Mitigation of heat stress	Liventia	United States
Blightban A506®	*P. fluorescens* A506	Prevention of frost damage	Nufarm Americas, Inc.	United States
Cedomon®	*P. fluorescens* and *P. chlororaphis*	Reduction of frost damage	BioAgri AB, Uppsala	Sweden
HyperGalaxy®	*P. fluorescens* and *P. chlororaphis*	Reduction of frost damage	Holmes Enviro, Llc., Philomath	United States
BFMS®	–	Reduction of frost damage	Tainio, Cheney	United States
SoilBiotic®	–	Reduction of frost damage	SoilBiotics, Reddick, IL	United States
BioStrain®	–	Reduction of frost damage	Monty's Plant Food Company	United States
OroSoil®		Mitigation of drought stress	Fomet, Spa, Verona	Italy
MycoApply All Purpose®	–	Mitigation of drought stress	Mycorrhizal Applications, Inc.	United States
Agtiv®	–	Mitigation of drought stress	PremierTech, Rivière-du-Loup	Canada
Groundwork®	–	Mitigation of drought stress	GroundWork BioAg, Ltd.	Israel
SSB® and SOS®	*Achromobacter* spp. and *Pseudomonas* spp.	Mitigation of drought and salt stresses	Liventia, Inc.	United States
Wettable Mycorrhizae Blend®	*Funneliformis mosseae*	Mitigation of drought stress	BioLogic Crop Solutions, Inc.	United States
N-Texx®	*P. putida*	Mitigation of drought stress	CXI, Coppell	United States
Ryze®	–	Mitigation of drought stress	L.Gobbi, Srl	Italy
Micosat F®	–	Mitigation of drought stress	CCS, Srl, Aosta	Italy
Grolux®	–	Mitigation of drought stress	RRR Supply Inc.	United States
HyperGalaxy®	–	Mitigation of drought stress	Holmes Enviro, Llc.	United States
Environoc®	–	Mitigation of drought stress	Biodyne, Llc.	United States

through the production of exopolysaccharides alongside the enrichment of nutrients and soil organic matter (**Table 3**). Efforts toward the formulation/production of more universally accepted commercial products, which will be active in most ecosystems will go a long way in the mitigation of water stress in crop plants.

Flooding, Water Stagnation, and Heavy Rainfall

Interannual rainfall and seasonal variability patterns of rain are two of the most significant problems of the current climate change (Foster and Rahmstorf, 2011; Stevenson et al., 2012). Flooding affects 13% of the Earth's surface, and severe rainfall intensity and frequency are expected to rise worldwide in the future (Sangiorgio et al., 2020). Water stagnation, anoxia and hypoxia of the root are caused by heavy floods and rains. The enzyme ACC synthase, noted in the production of ethylene, is produced in significant amounts by roots when they are flooded (Sangiorgio et al., 2020). The enzyme ACC oxidase, requires oxygen to catalyze the final stage of biosynthesis of ethylene and ACC is transported to the aerial portion of the plant via the xylem (Else and Jackson, 1998), where it is transformed to ethylene, resulting in wilting, chlorosis, leaf necrosis, fruit and flower loss, and limited yield (Glick, 2014). Thanks to ACC deaminase activity, which lowers the level of endogenous ethylene, microbial biostimulants can help to alleviate stress related to water stagnation (Glick, 2014; Ali and Kim, 2018). For example, tomato seedlings inoculated with ACC deaminase-producing strains of *Pseudomonas* spp. and *Enterobacter* spp. show a reduction of anoxia stress and an improvement of germination (Grichko and Glick, 2001; Sangiorgio et al., 2020). The commercial product SumaGrow® (RRR Supply Inc., Munger, MI, United States), which contains *Pseudomonas* and *Enterobacter* spp., increases yield and improves stress tolerance, but it does not promise to give particular protection from waterlogging stress. Chlorophyll content, plant elongation, biomass, formation of the adventitious root, and leaf area were promoted, while ethylene levels were reduced, using *Pseudomonas* sp. on cucumber seeds (Li J. et al., 2013) and *Streptomyces* sp. GMKU 336 strain on *Vigna radiata* (Jaemsaeng et al., 2018). The active ingredients of Mycostop® (Verdera Oy, Espoo, Finland) and Actinovate® (Mycorrhizal Applications, Inc., OR, United States) are *S. lydicus* WYEC 108, and *Streptomyces* K61, respectively. Streptomyces spp. is beneficial bacteria frequently utilized against biotic stresses and their application against abiotic stresses is currently understudied. Future studies into the production of more microbial stimulant and the full exploration of the roles of all currently available commercial products for the mitigation of water stress is needed.

GENERAL CONSTRAINS AND PROSPECTS IN THE UTILIZATION OF MICROBIAL BIOSTIMULANTS

The development of a novel microbial biostimulant presents some unique challenges. To begin with, the business registration procedure is often complicated, and there is still a lack of unified international regulation (Backer et al., 2018). Secondly,

it should be noted that product effectiveness highly relies on the type of agricultural crop used, its phenological stage and environmental conditions (Khan et al., 2018). The relationship between microorganisms and host plants must be evaluated while developing microbial biostimulants. To establish the efficacy of microbial biostimulant, favorable and long-lasting colonization of the plant is required, and it could be affected by the indigenous plant-associated microbial communities (Sangiorgio et al., 2020).

Finally, to reduce the effects of cultural and environmental factors on product conservation and efficacy, the optimum formulation should be developed (Bashan et al., 2014). Due to spore formation by bacteria, biostimulants that are formulated using Gram-positive bacteria allow powder formulations to have drying tolerance and long-term stability. Even though there are several types of the effectiveness of microbe utilization in stimulating plant development under adverse conditions, there are relatively few biostimulant products available that particularly address the challenges exacerbated by climate change. Moreover, the high costs associated with microbial biostimulant registration and manufacturing (Nadeem et al., 2014) are key roadblocks to product development, resulting in a small number of products that are commercialized and limited adoption in horticulture. Thus, several factors must be taken into consideration for microbial biostimulant development and use, such as crop and soil characteristics, type of application and microbial strain characteristics. About the first aspect, no microbes can be said to be universally fit for all ecosystems (Adesemoye et al., 2009) or on any plant host (Finkel et al., 2017), indicating that selection of a strain for the production of microbial biostimulant requires consideration of the plant and the soil properties to maximize efficacy under specific conditions (Ipek et al., 2014). Biostimulant efficiency can be reduced when there is competition for space and nutrients between the selected microbial strains and the indigenous microbiota (Ruzzi and Aroca, 2015). Thus, isolation and application of endemic microbial strains, possibly with multiple plant growth promotion traits would be an important issue to observe better results in field trials (Etesami and Maheshwari, 2018). For example, it was well demonstrated that microorganisms extracted from the host plant microbiome are more effective than non-indigenous microbial inoculants (Mazzola and Freilich, 2017). Moreover, it is also important to optimize the mode of microbial biostimulant application (e.g., seed or root inoculation, spore spray, etc.), which should minimize microbe dispersion or mortality caused by abiotic variables, such as temperature and UV light effect (Vejan et al., 2016).

Crop breeding strategies in the future should include the plant ability to form permanent symbiotic partnerships with beneficial microbes as a very important feature, strongly connected to stress tolerance, resilience and production (Msimbira and Smith, 2020). Simultaneously, a better understanding of microbial activities and interaction processes may allow for the selection of appropriate microbial biostimulants for a certain cultivar or crop under a specific cultural situation (Clúa et al., 2018). Likewise, other approaches are required to fully realize the potential of microbial biostimulants, such as a depth characterization of the plant microbial biocoenosis using real-time investigation microbial functional dynamics, next-generation sequencing such as RNA/metagenome/amplicon sequencing optimization and development of the synthetic community of microbes (Fadiji and Babalola, 2020c). As a result, a critical step in the effective selection of microbial biostimulants is the characterization of the indigenous microbiome using high-throughput next-generation sequencing approaches in combination with meta-analysis of the associated population, to identify microorganisms that can persist on plants in harsh conditions (Purahong et al., 2018; Fadiji and Babalola, 2020c). Furthermore, understanding and exploiting plant-microbe interactions requires detailed research of the microbiome using functional markers rather than taxonomic markers (Lemanceau et al., 2017). The use of real-time monitoring tools for important microbial activities might be used to optimize the effectiveness of microbial inoculants. For example, the use of real-time PCR techniques enables for broad-range assessment of the functional genes available in the microbes and might be applied for future monitoring of the agroecological function (Bouffaud et al., 2018).

Finally, the creation of synthetic communities of microbes, i.e., the combination of numerous microbes with specific plant growth-promoting functions, offers an opportunity to improve the effectiveness and reliability of microbial biostimulants (Ahkami et al., 2017). However, to ensure the sustainability of the beneficial community, the intricacy of ecological interactions between microorganisms should be thoroughly explored, such as competition and commensalism.

CONCLUSION AND FUTURE PROSPECTS

Microbial biostimulants have the potential to be a long-term and successful method for reducing the abiotic stressors that climate change has exacerbated. Furthermore, the application of microbial biostimulants may help to maintain the ecological balance of agro-ecosystems, reducing the usage of pesticides and/or heavy metals for agricultural practices. Nonetheless, several concerns should be considered both at the regulatory level and throughout the development and research, to achieve greater efficacy of the product and wider adoption.

Plant biostimulant has a claim-based definition, which means that the function is used to establish the product. Many potent ingredients with various activities and objectives can be found in a single product. As a result, the inherent heterogeneity of microbial biostimulants could elude regulatory categorization (e.g., fungicide, fertilizer, and amendment). Products may be subjected to lengthy and costly trial procedures depending on the country of registration. The lack of a consistent worldwide regulatory framework creates a barrier to product marketing and may deter the development of novel products. In agroecological and biological research, the adoption of microbial biostimulants still has certain drawbacks, mostly due to their lesser efficacy and greater environmental sensitivity when compared to synthetic products (e.g., pesticides, fertilizers, and growth regulators). Furthermore, the efficacy of

microbial biostimulants varied widely according to the crop and environmental conditions. Future research should focus on developing better-targeted products, such as delving deeper into interactions of the microbial biostimulant with indigenous plant-associated microbiomes.

The application of microbial biostimulants might provide a long-term and cost-effective solution to plant productivity losses caused by changing climatic factors, as well as aid in the optimization of human inputs in agro-ecosystem. Results from preliminary experiments involving microbial biostimulants should be disseminated by all relevant policymakers and stakeholders, such as extension services and growers, to ensure that this methodology can be largely applied to a variety of crops, regions, and under different environmental conditions.

AUTHOR CONTRIBUTIONS

AF and OB conceived the ideas, collected the data, and developed the manuscript. GS and MP provided professional input and critiqued the work. All authors have carefully read the final manuscript and have agreed that the manuscript be published.

FUNDING

This study was funded by the National Research Foundation, South Africa (UID123634 and UID132595).

ACKNOWLEDGMENTS

AF gratefully acknowledged the National Research Foundation, South Africa/The World Academy of Science African Renaissance Ph.D. Scholarship (Ref: UID116107), which supported his studies. OB appreciates the National Research Foundation, South Africa, for the grant (UID123634 and UID132595) that have supported this research.

REFERENCES

Abd El-Daim, I. A., Bejai, S., and Meijer, J. (2014). Improved heat stress tolerance of wheat seedlings by bacterial seed treatment. *Plant Soil* 379, 337–350. doi: 10.1007/s11104-014-2063-3

Adesemoye, A., Torbert, H., and Kloepper, J. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microb. Ecol.* 58, 921–929. doi: 10.1007/s00248-009-9531-y

Adnan, M., Islam, W., Shabbir, A., Khan, K. A., Ghramh, H. A., Huang, Z., et al. (2019). Plant defense against fungal pathogens by antagonistic fungi with Trichoderma in focus. *Microb. Pathog.* 129, 7–18. doi: 10.1016/j.micpath.2019.01.042

Ahkami, A. H., White Iii, R. A., Handakumbura, P. P., and Jansson, C. (2017). Rhizosphere engineering: enhancing sustainable plant ecosystem productivity. *Rhizosphere* 3, 233–243.

Ait Barka, E., Nowak, J., and Clément, C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. *Appl. Environ. Microbiol.* 72, 7246–7252. doi: 10.1128/AEM.01047-06

Akhgar, A., Arzanlou, M., Bakker, P., and Hamidpour, M. (2014). Characterization of 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing *Pseudomonas* spp. in the rhizosphere of salt-stressed canola. *Pedosphere* 24, 461–468.

Ali, S. Z., Sandhya, V., Grover, M., Kishore, N., Rao, L. V., and Venkateswarlu, B. (2009). *Pseudomonas* sp. strain AKM-P6 enhances tolerance of sorghum seedlings to elevated temperatures. *Biol. Fertil. Soils* 46, 45–55. doi: 10.1007/s00374-009-0404-9

Ali, S. Z., Sandhya, V., Grover, M., Linga, V. R., and Bandi, V. (2011). Effect of inoculation with a thermotolerant plant growth promoting *Pseudomonas putida* strain AKMP7 on growth of wheat (*Triticum* spp.) under heat stress. *J. Plant Interact.* 6, 239–246.

Ali, S., and Kim, W.-C. (2018). Plant growth promotion under water: decrease of waterlogging-induced ACC and ethylene levels by ACC deaminase-producing bacteria. *Front. Microbiol.* 9:1096. doi: 10.3389/fmicb.2018.01096

Ali, S., and Xie, L. (2020). Plant growth promoting and stress mitigating abilities of soil born microorganisms. *Recent Pat. Food Nutr. Agric.* 11, 96–104. doi: 10.2174/2212798410666190515115548

Ali, S., Charles, T. C., and Glick, B. R. (2014). Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. *Plant Physiol. Biochem.* 80, 160–167. doi: 10.1016/j.plaphy.2014.04.003

Allen, M. R., Dube, O. P., Solecki, W., Aragón-Durand, F., Cramer, W., Humphreys, S., et al. (2018). "Framing and context," in *Global Warming of 1.5 C: An IPCC Special Report on the Impacts of Global Warming of 1.5 C Above pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat Of Climate Change, Sustainable Development, and Efforts to eradicate poverty. Sustainable development, and Efforts to Eradicate Poverty'*, eds V. Masson-Delmotte, P. Zhai, H. O. Pörtner, D. Roberts, J. Skea, P. R. Shukla, et al. (Geneva: IPCC), 41–91.

Amara, U., Khalid, R., and Hayat, R. (2015). "Soil bacteria and phytohormones for sustainable crop production," in *Bacterial Metabolites in Sustainable Agroecosystem*, ed. D. K. Maheshwari (Cham: Springer), 87–103.

Anderson, J., Buchanan, D., Stall, R., and Hall, C. (1982). Frost injury of tender plants increased by *Pseudomonas syringae* van Hall. *J. Am. Soc. Hortic. Sci.* 107, 123–125.

Anwar, A., Younis, M., and Ullah, I. (2020). Impact of urbanization and economic growth on CO2 emission: a case of far east Asian countries. *Int. J. Environ. Res. Public Health* 17:2531. doi: 10.3390/ijerph17072531

Arkhipova, T., Prinsen, E., Veselov, S., Martinenko, E., Melentiev, A., and Kudoyarova, G. (2007). Cytokinin producing bacteria enhance plant growth in drying soil. *Plant Soil Biol.* 292, 305–315. doi: 10.1007/s11104-007-9233-5

Arny, D., Lindow, S., and Upper, C. (1976). Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*. *Nature* 262, 282–284.

Aroca, R., Porcel, R., and Ruiz-Lozano, J. M. (2007). How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytol.* 173, 808–816. doi: 10.1111/j.1469-8137.2006.01961.x

Aroca, R., Vernieri, P., and Ruiz-Lozano, J. M. (2008). Mycorrhizal and non-mycorrhizal Lactuca sativa plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. *J. Exp. Bot.* 59, 2029–2041. doi: 10.1093/jxb/ern057

Babalola, O. O., Fadiji, A. E., Enagbonma, B. J., Alori, E. T., Ayilara, M. S., and Ayangbenro, A. S. (2020). The nexus between plant and plant microbiome: revelation of the networking strategies. *Front. Microbiol.* 11:2128. doi: 10.3389/fmicb.2020.548037

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Bailly, A., Groenhagen, U., Schulz, S., Geisler, M., Eberl, L., and Weisskopf, L. (2014). The inter-kingdom volatile signal indole promotes root development by interfering with auxin signalling. *Plant J.* 80, 758–771. doi: 10.1111/tpj.12666

Bashan, Y., De-Bashan, L. E., Prabhu, S., and Hernandez, J.-P. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant Soil* 378, 1–33. doi: 10.1007/s11104-013-1956-x

Bhattacharya, A. (2019). "Effect of high temperature on carbohydratemetabolism in plants," in *Effect of High Temperature on Crop Productivity and Metabolism of Macro Molecules*, ed. A. Bhattacharya (Cambridge, MA: Academic Press), 115–216.

Bhattacharyya, D., Garladinne, M., and Lee, Y. H. (2015). Volatile indole produced by rhizobacterium Proteus vulgaris JBLS202 stimulates growth of *Arabidopsis thaliana* through auxin, cytokinin, and brassinosteroid pathways. *J. Plant Growth Regul.* 34, 158–168. doi: 10.1007/s00344-014-9453-x

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bisbis, M. B., Gruda, N., and Blanke, M. (2018). Potential impacts of climate change on vegetable production and product quality–A review. *J. Clean. Product.* 170, 1602–1620. doi: 10.1016/j.jclepro.2017.09.224

Bouffaud, M.-L., Renoud, S., Dubost, A., Moënne-Loccoz, Y., and Muller, D. (2018). 1-Aminocyclopropane-1-carboxylate deaminase producers associated to maize and other *Poaceae* species. *Microbiome* 6:114. doi: 10.1186/s40168-018-0503-7

Bray, E. A. (2000). "Response to abiotic stress," in *Biochemistry and Molecular Biology of Plants*, eds B. B. Buchanan, W. Gruissem, and R. L. Jones (Rockville, MD: ASPP), 1158–1203.

Byerlee, D., Stevenson, J., and Villoria, N. (2014). Does intensification slow crop land expansion or encourage deforestation? *Glob. Food Secur.* 3, 92–98.

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41.

Cassia, R., Nocioni, M., Correa-Aragunde, N., and Lamattina, L. (2018). Climate change and the impact of greenhouse gasses: CO2 and NO, friends and foes of plant oxidative stress. *Front. Plant Sci.* 9:273. doi: 10.3389/fpls.2018.00273

Cavagnaro, T. R., Bender, S. F., Asghari, H. R., and Van Der Heijden, M. G. (2015). The role of arbuscular mycorrhizas in reducing soil nutrient loss. *Trends Plant Sci.* 20, 283–290. doi: 10.1016/j.tplants.2015.03.004

Clúa, J., Roda, C., Zanetti, M. E., and Blanco, F. A. (2018). Compatibility between legumes and rhizobia for the establishment of a successful nitrogen-fixing symbiosis. *Genes* 9:125. doi: 10.3390/genes9030125

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Crafts-Brandner, S. J., and Salvucci, M. E. (2002). Sensitivity of photosynthesis in a C4 plant, maize, to heat stress. *Plant Physiol.* 129, 1773–1780. doi: 10.1104/pp.002170

De Wolf, E. D., and Isard, S. A. (2007). Disease cycle approach to plant disease prediction. *Annu. Rev. Phytopathol.* 45, 203–220. doi: 10.1146/annurev.phyto.44.070505.143329

del Carmen Orozco-Mosqueda, M., Glick, B. R., and Santoyo, G. (2020). ACC deaminase in plant growth-promoting bacteria (PGPB): an efficient mechanism to counter salt stress in crops. *Microbiol. Res.* 235:126439. doi: 10.1016/j.micres.2020.126439

Díaz-Barradas, M., Gallego-Fernández, J., and Zunzunegui, M. (2020). Plant response to water stress of native and non-native Oenothera drummondii populations. *Plant Physiol. Biochem.* 154, 219–228. doi: 10.1016/j.plaphy.2020.06.001

Dimkpa, C., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2009). Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. *J. Appl. Microbiol.* 107, 1687–1696. doi: 10.1111/j.1365-2672.2009.04355.x

Du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Dubois, M., Van Den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant growth. *Trends Plant Sci.* 23, 311–323. doi: 10.1016/j.tplants.2018.01.003

Duc, N. H., Csintalan, Z., and Posta, K. (2018). Arbuscular mycorrhizal fungi mitigate negative effects of combined drought and heat stress on tomato plants. *Plant Physiol. Biochem.* 132, 297–307. doi: 10.1016/j.plaphy.2018.09.011

Egamberdieva, D. (2012). *Pseudomonas chlororaphis*: a salt-tolerant bacterial inoculant for plant growth stimulation under saline soil conditions. *Acta Physiol. Plant.* 34, 751–756. doi: 10.1007/s11738-011-0875-9

Else, M. A., and Jackson, M. B. (1998). Transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in the transpiration stream of tomato (Lycopersicon esculentum) in relation to foliar ethylene production and petiole epinasty. *Funct. Plant Biol.* 25, 453–458. doi: 10.1071/pp97105

Enebe, M. C., and Babalola, O. O. (2018). The influence of plant growth-promoting rhizobacteria in plant tolerance to abiotic stress: a survival strategy. *Appl. Microbiol. Biotechnol.* 102, 7821–7835. doi: 10.1007/s00253-018-9214-z

EPA (2021). *Greenhouse Gas (GHG) Emissions. Sources of Greenhouse Gas Emissions*. Available online at: https://www.epa.gov/ghgemissions/sources-greenhouse-gas-emissions#agriculture (accessed June 8, 2021).

Esmaeel, Q., Miotto, L., Rondeau, M., Leclère, V., Clément, C., Jacquard, C., et al. (2018). *Paraburkholderia phytofirmans* PsJN-plants interaction: from perception to the induced mechanisms. *Front. Microbiol.* 9:2093. doi: 10.3389/fmicb.2018.02093

Etesami, H., and Maheshwari, D. K. (2018). Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects. *Ecotoxicol. Environ. Saf* 156, 225–246. doi: 10.1016/j.ecoenv.2018.03.013

Fadiji, A. E., and Babalola, O. O. (2020b). Exploring the potentialities of beneficial endophytes for improved plant growth. *Saudi J. Biol. Sci.* 27, 3622–3633. doi: 10.1016/j.sjbs.2020.08.002

Fadiji, A. E., and Babalola, O. O. (2020a). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Front. Bioeng. Biotechnol.* 8:467. doi: 10.3389/fbioe.2020.00467

Fadiji, A. E., and Babalola, O. O. (2020c). Metagenomics methods for the study of plant-associated microbial communities: a review. *J. Microbiol. Methods* 170:105860. doi: 10.1016/j.mimet.2020.105860

Fadiji, A. E., Ayangbenro, A. S., and Babalola, O. O. (2020). Organic Farming enhances the diversity and community structure of endophytic archaea and fungi in maize plant: a shotgun approach. *J. Soil Sci. Plant Nutr.* 20, 2587–2599. doi: 10.1007/s42729-020-00324-9

Failor, K., Schmale, D. G., Vinatzer, B. A., and Monteil, C. L. (2017). Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms. *ISME J.* 11, 2740–2753. doi: 10.1038/ismej.2017.124

Farrar, K., Bryant, D., and Cope-Selby, N. (2014). Understanding and engineering beneficial plant–microbe interactions: plant growth promotion in energy crops. *Plant Biotechnol. J.* 12, 1193–1206. doi: 10.1111/pbi.12279

Finkel, O. M., Castrillo, G., Paredes, S. H., González, I. S., and Dangl, J. L. (2017). Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* 38, 155–163. doi: 10.1016/j.pbi.2017.04.018

Fiodor, A., Singh, S., and Pranaw, K. (2021). The contrivance of plant growth promoting microbes to mitigate climate change impact in agriculture. *Microorganisms* 9:1841. doi: 10.3390/microorganisms9091841

Foster, G., and Rahmstorf, S. (2011). Global temperature evolution 1979–2010. *Environ. Res. Lett.* 6:044022. doi: 10.1088/1748-9326/6/4/044022

Freimoser, F. M., Rueda-Mejia, M. P., Tilocca, B., and Migheli, Q. (2019). Biocontrol yeasts: mechanisms and applications. *World J. Microbiol. Biotechnol.* 35:154. doi: 10.1007/s11274-019-2728-4

Funes, I., Aranda, X., Biel, C., Carbó, J., Camps, F., Molina, A. J., et al. (2016). Future climate change impacts on apple flowering date in a Mediterranean subbasin. *Agric. Water Manage.* 164, 19–27. doi: 10.1016/j.agwat.2015.06.013

Füssel, H.-M., Heinke, J., Popp, A., and Gerten, D. (2012). "Food security in a changing climate," in *Climate Change, Justice and Sustainability: Linking Climate and Development Policy*, eds O. Edenhofer, J. Wallacher, H. Lotze-Campen, M. Reder, B. Knopf, and J. Müller (Dordrecht: Springer), 33–43.

Galambos, N., Compant, S., Wäckers, F., Sessitsch, A., Anfora, G., Mazzoni, V., et al. (2021). Beneficial insects deliver plant growth-promoting bacterial endophytes between tomato plants. *Microorganisms* 9:1294. doi: 10.3390/microorganisms9061294

Garrett, K. A., Nita, M., De Wolf, E., Esker, P. D., Gomez-Montano, L., and Sparks, A. H. (2016). "Plant pathogens as indicators of climate change," in *Climate Change*, 2nd Edn, ed. T. M. Letcher (Amsterdam: Elsevier), 325–338. doi: 10.1016/b978-0-444-63524-2.00021-x

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Gołasa, P., Wysokiński, M., Bieńkowska-Gołasa, W., Gradziuk, P., Golonko, M., Gradziuk, B., et al. (2021). Sources of greenhouse gas emissions in agriculture, with particular emphasis on emissions from energy used. *Energies* 14:3784. doi: 10.3390/en14133784

Gong, M., Tang, M., Chen, H., Zhang, Q., and Feng, X. (2013). Effects of two Glomus species on the growth and physiological performance of Sophora davidii seedlings under water stress. *New For.* 44, 399–408. doi: 10.1007/s11056-012-9349-1

Gornall, J., Betts, R., Burke, E., Clark, R., Camp, J., Willett, K., et al. (2010). Implications of climate change for agricultural productivity in the early twenty-first century. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 2973–2989. doi: 10.1098/rstb.2010.0158

Grichko, V. P., and Glick, B. R. (2001). Amelioration of flooding stress by ACC deaminase-containingplant growth-promoting bacteria. *Plant Physiol. Biochem.* 39, 11–17. doi: 10.1016/s0981-9428(00)01212-2

Grover, M., Ali, S. Z., Sandhya, V., Rasul, A., and Venkateswarlu, B. (2011). Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World J. Microbiol. Biotechnol.* 27, 1231–1240. doi: 10.1007/s11274-010-0572-7

Gupta, S., and Pandey, S. (2019). ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (Phaseolus vulgaris) plants. *Front. Microbiol.* 10:1506. doi: 10.3389/fmicb.2019.01506

Gutiérrez-Luna, F. M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., De La Cruz, H. R., and Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in Arabidopsis thaliana through volatile organic compound emission. *Symbiosis* 51, 75–83. doi: 10.1007/s13199-010-0066-2

Hawkings, J. R., Linhoff, B. S., Wadham, J. L., Stibal, M., Lamborg, C. H., Carling, G. T., et al. (2021). Large subglacial source of mercury from the southwestern margin of the Greenland Ice Sheet. *Nat. Geosci.* 14, 496–502. doi: 10.1038/s41561-021-00753-w

Hazarika, T. (2013). Climate change and Indian horticulture: opportunities, challenges and mitigation strategies. *Int. J. Environ. Eng. Manage.* 4, 629–630.

Heidari, M., and Golpayegani, A. (2012). Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil (*Ocimum basilicum* L.). *J. Saudi Soc. Agric. Sci.* 11, 57–61. doi: 10.1016/j.jssas.2011.09.001

Hernández-León, R., Rojas-Solís, D., Contreras-Pérez, M., Del Carmen Orozco-Mosqueda, M., Macías-Rodríguez, L. I., Reyes-De, et al. (2015). Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains. *Biol. Control* 81, 83–92. doi: 10.1016/j.biocontrol.2014.11.011

Huang, J., Zhang, G., Zhang, Y., Guan, X., Wei, Y., and Guo, R. (2020). Global desertification vulnerability to climate change and human activities. *Land Degrad. Dev.* 31, 1380–1391. doi: 10.1002/ldr.3556

Huner, N. P., Öquist, G., Hurry, V. M., Krol, M., Falk, S., and Griffith, M. (1993). Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynth. Res.* 37, 19–39. doi: 10.1007/BF02185436

Hutchins, D. A., Jansson, J. K., Remais, J. V., Rich, V. I., Singh, B. K., and Trivedi, P. (2019). Climate change microbiology—problems and perspectives. *Nat. Rev. Microbiol.* 17, 391–396. doi: 10.1038/s41579-019-0178-5

Igiehon, N. O., Babalola, O. O., and Aremu, B. R. (2019). Genomic insights into plant growth promoting rhizobia capable of enhancing soybean germination under drought stress. *BMC Microbiol.* 19:159. doi: 10.1186/s12866-019-1536-1

Igiehon, N. O., Babalola, O. O., Cheseto, X., and Torto, B. (2021). Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress. *Microbiol. Res.* 242:126640. doi: 10.1016/j.micres.2020.126640

Igiehon, O. N., and Babalola, O. O. (2021). Rhizobium and mycorrhizal fungal species improved soybean yield under drought stress conditions. *Curr. Microbiol.* 78, 1615–1627. doi: 10.1007/s00284-021-02432-w

IPCC (2014). "Mitigation of climate change," in *Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds O. Edenhofer, R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, et al. (Cambridge: Cambridge University Press).

Ipek, M., Pirlak, L., Esitken, A., Figen Dönmez, M., Turan, M., and Sahin, F. (2014). Plant growth-promoting rhizobacteria (PGPR) increase yield, growth and nutrition of strawberry under high-calcareous soil conditions. *J. Plant Nutr.* 37, 990–1001. doi: 10.1080/01904167.2014.881857

Jaemsaeng, R., Jantasuriyarat, C., and Thamchaipenet, A. (2018). Positive role of 1-aminocyclopropane-1-carboxylate deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean. *Agric. Nat. Resour.* 52, 330–334. doi: 10.1016/j.anres.2018.09.008

Jagadish, S. K., Way, D. A., and Sharkey, T. D. (2021). Plant heat stress: concepts directing future research. *Plant Cell Environ.* 44, 1992–2005. doi: 10.1111/pce.14050

Jahromi, F., Aroca, R., Porcel, R., and Ruiz-Lozano, J. M. (2008). Influence of salinity on the in vitro development of *Glomus intraradices* and on the *in vivo* physiological and molecular responses of mycorrhizal lettuce plants. *Microb. Ecol.* 55, 45–53. doi: 10.1007/s00248-007-9249-7

Jayakumar, A., Padmakumar, P., Nair, I. C., and Radhakrishnan, E. (2020). Drought tolerant bacterial endophytes with potential plant probiotic effects from Ananas comosus. *Biologia* 75, 1769–1778. doi: 10.2478/s11756-020-00483-1

Jobe, T. O., Rahimzadeh Karvansara, P., Zenzen, I., and Kopriva, S. (2020). Ensuring nutritious food under elevated CO2 conditions: a case for improved C4 crops. *Front. Plant Sci.* 11:1267. doi: 10.3389/fpls.2020.01267

Jordan, D. B., and Ogren, W. L. (1984). The CO2/O2 specificity of ribulose 1, 5-bisphosphate carboxylase/oxygenase. *Planta* 161, 308–313. doi: 10.1007/bf00398720

Kang, S.-M., Radhakrishnan, R., Khan, A. L., Kim, M.-J., Park, J.-M., Kim, B.-R., et al. (2014). Gibberellin secreting rhizobacterium, *Pseudomonas* putida H-2-3 modulates the hormonal and stress physiology of soybean to improve the plant growth under saline and drought conditions. *Plant Physiol. Biochem.* 84, 115–124. doi: 10.1016/j.plaphy.2014.09.001

Kang, S.-M., Radhakrishnan, R., You, Y.-H., Khan, A. L., Park, J.-M., Lee, S.-M., et al. (2015). Cucumber performance is improved by inoculation with plant growth-promoting microorganisms. *Acta Agric. Scand. Sect. B Soil Plant* 65, 36–44. doi: 10.1080/09064710.2014.960889

Kaushal, M., and Wani, S. P. (2016). Plant-growth-promoting rhizobacteria: drought stress alleviators to ameliorate crop production in drylands. *Ann. Microbiol.* 66, 35–42. doi: 10.1007/s13213-015-1112-3

Khan, A. L., Hussain, J., Al-Harrasi, A., Al-Rawahi, A., and Lee, I.-J. (2015). Endophytic fungi: resource for gibberellins and crop abiotic stress resistance. *Crit. Rev. Biotechnol.* 35, 62–74. doi: 10.3109/07388551.2013.800018

Khan, M. A., Asaf, S., Khan, A. L., Ullah, I., Ali, S., Kang, S.-M., et al. (2019). Alleviation of salt stress response in soybean plants with the endophytic bacterial isolate *Curtobacterium* sp. SAK1. *Ann. Microbiol.* 69, 797–800. doi: 10.1007/s13213-019-01470-x

Khan, N., Bano, A., and Zandi, P. (2018). Effects of exogenously applied plant growth regulators in combination with PGPR on the physiology and root growth of chickpea (*Cicer arietinum*) and their role in drought tolerance. *J. Plant Interact.* 13, 239–247. doi: 10.1080/17429145.2018.1471527

Kim, H., Orser, C., Lindow, S., and Sands, D. (1987). *Xanthomonas campestris* pv. translucens strains active in ice nucleation. *Plant Dis.* 71, 994–997.

Kour, D., Kaur, T., Devi, R., Rana, K. L., Yadav, N., Rastegari, A. A., et al. (2020a). "Kour, Biotechnological applications of beneficial microbiomes for evergreen agriculture and human health," in *New and Future Developments in Microbial Biotechnology and Bioengineering*, eds A. A. Rastegari, A. N. Yadav, and N. Yadav (Elsevier), 255–279. doi: 10.1016/b978-0-12-820528-0.00019-3

Kour, D., Rana, K. L., Sheikh, I., Kumar, V., Yadav, A. N., Dhaliwal, H. S., et al. (2020c). Alleviation of drought stress and plant growth promotion by *Pseudomonas* libanensis EU-LWNA-33, a drought-adaptive phosphorus-solubilizing bacterium. *Proc. Natl. Acad. Sci. India B Biol. Sci.* 90, 785–795. doi: 10.1007/s40011-019-01151-4

Kour, D., Rana, K. L., Yadav, A. N., Sheikh, I., Kumar, V., Dhaliwal, H. S., et al. (2020d). Amelioration of drought stress in Foxtail millet (*Setaria italica* L.) by P-solubilizing drought-tolerant microbes with multifarious plant growth promoting attributes. *Environ. Sustain.* 3, 23–34. doi: 10.1007/s42398-020-00094-1

Kour, D., Rana, K. L., Kaur, T., Sheikh, I., Yadav, A. N., Kumar, V., et al. (2020b). Microbe-mediated alleviation of drought stress and acquisition of phosphorus

in great millet (*Sorghum bicolour* L.) by drought-adaptive and phosphorus-solubilizing microbes. *Biocatal. Agric. Biotechnol.* 23:101501.

Ku, S.-B., and Edwards, G. E. (1977). Oxygen inhibition of photosynthesis: I. Temperature dependence and relation to O2/CO2 solubility ratio. *Plant Physiol.* 59, 986–990. doi: 10.1007/BF00389372

Kumar, A., and Verma, J. P. (2018). Does plant—microbe interaction confer stress tolerance in plants: a review? *Microbiol. Res.* 207, 41–52. doi: 10.1016/j.micres.2017.11.004

Kumar, N., Khamzina, A., Knöfel, P., Lamers, J., and Tischbein, B. (2021). Afforestation of degraded croplands as a water-saving option in irrigated region of the aral sea basin. *Water* 13:1433. doi: 10.3390/w13101433

Laanisto, L., and Niinemets, Ü. (2015). Polytolerance to abiotic stresses: How universal is the shade–drought tolerance trade-off in woody species? *Glob. Ecol. Biogeogr.* 24, 571–580. doi: 10.1111/geb.12288

Ledger, T., Rojas, S., Timmermann, T., Pinedo, I., Poupin, M. J., Garrido, T., et al. (2016). Volatile-mediated effects predominate in *Paraburkholderia phytofirmans* growth promotion and salt stress tolerance of *Arabidopsis thaliana*. *Front. Microbiol.* 7:1838. doi: 10.3389/fmicb.2016.01838

Lee, G., Lee, S.-H., Kim, K. M., and Ryu, C.-M. (2017). Foliar application of the leaf-colonizing yeast *Pseudozyma churashimaensis* elicits systemic defense of pepper against bacterial and viral pathogens. *Sci. Rep.* 7:39432. doi: 10.1038/srep39432

Lee, M. R., Lee, R. E. Jr., Strong-Gunderson, J. M., and Minges, S. R. (1995). Isolation of ice-nucleating active bacteria from the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* 32, 358–365. doi: 10.1006/cryo.1995.1036

Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017). Let the core microbiota be functional. *Trends Plant Sci.* 22, 583–595. doi: 10.1016/j.tplants.2017.04.008

Li, J., Mcconkey, B. J., Cheng, Z., Guo, S., and Glick, B. R. (2013). Identification of plant growth-promoting bacteria-responsive proteins in cucumber roots under hypoxic stress using a proteomic approach. *J. Proteomics* 84, 119–131. doi: 10.1016/j.jprot.2013.03.011

Li, T., Hu, Y.-J., Hao, Z.-P., Li, H., and Chen, B.-D. (2013). Aquaporin genes GintAQPF1 and GintAQPF2 from Glomus intraradices contribute to plant drought tolerance. *Plant Signal. Behav.* 8:e24030. doi: 10.4161/psb.24030

Lim, J.-H., and Kim, S.-D. (2013). Induction of drought stress resistance by multi-functional PGPR Bacillus licheniformis K11 in pepper. *Plant Pathol. J.* 29, 201–208. doi: 10.5423/PPJ.SI.02.2013.0021

Lindow, S. (1983). The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* 21, 363–384. doi: 10.1146/annurev.py.21.090183.002051

Lindow, S. E., and Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69, 1875–1883. doi: 10.1128/aem.69.4.1875-1883.2003

Lindow, S., Arny, D., and Upper, C. (1978). Erwinia herbicola: a bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* 68, 523–527.

Liu, C.-Y., Zhang, F., Zhang, D.-J., Srivastava, A., Wu, Q.-S., and Zou, Y.-N. (2018). Mycorrhiza stimulates root-hair growth and IAA synthesis and transport in trifoliate orange under drought stress. *Sci. Rep.* 8:1978. doi: 10.1038/s41598-018-20456-4

Liu, F., Xing, S., Ma, H., Du, Z., and Ma, B. (2013). Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. *Appl. Microbiol. Biotechnol.* 97, 9155–9164. doi: 10.1007/s00253-013-5193-2

Lobell, D. B., and Gourdji, S. M. (2012). The influence of climate change on global crop productivity. *Plant Physiol.* 160, 1686–1697. doi: 10.1104/pp.112.208298

Loladze, I. (2014). Hidden shift of the ionome of plants exposed to elevated CO2 depletes minerals at the base of human nutrition. *eLife* 3:e02245. doi: 10.7554/eLife.02245

Luedeling, E. (2012). Climate change impacts on winter chill for temperate fruit and nut production: a review. *Sci. Hortic.* 144, 218–229.

Malhotra, S. (2017). Horticultural crops and climate change: a review. *Indian J. Agric. Sci.* 87, 12–22.

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., et al. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PLoS One* 7:e48479. doi: 10.1371/journal.pone.0048479

Mayak, S., Tirosh, T., and Glick, B. R. (2004). Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Sci.* 166, 525–530. doi: 10.1016/j.plantsci.2003.10.025

Mazzola, M., and Freilich, S. (2017). Prospects for biological soilborne disease control: application of indigenous versus synthetic microbiomes. *Phytopathology* 107, 256–263. doi: 10.1094/PHYTO-09-16-0330-RVW

Meena, K. K., Sorty, A. M., Bitla, U. M., Choudhary, K., Gupta, P., Pareek, A., et al. (2017). Abiotic stress responses and microbe-mediated mitigation in plants: the omics strategies. *Front. Plant Sci.* 8:172. doi: 10.3389/fpls.2017.00172

Mishra, P. K., Bisht, S. C., Bisht, J. K., and Bhatt, J. C. (2012). "Cold-tolerant PGPRs as bioinoculants for stress management," in *Bacteria in Agrobiology: Stress Management*, ed. D. K. Maheshwari (Berlin: Springer), 95–118.

Mishra, P. K., Mishra, S., Selvakumar, G., Bisht, S. C., Bisht, J. K., Kundu, S., et al. (2008). Characterisation of a psychrotolerant plant growth promoting *Pseudomonas* sp. strain PGERs17 (MTCC 9000) isolated from North Western Indian Himalayas. *Ann. Microbiol.* 58, 561–568.

Morales-Cedeño, L. R., De Los Santos-Villalobos, S., and Santoyo, G. (2021). Functional and genomic analysis of *Rouxiella badensis* SER3 as a novel biocontrol agent of fungal pathogens. *Front. Microbiol.* 12:709855. doi: 10.3389/fmicb.2021.709855

Msimbira, L. A., and Smith, D. L. (2020). The roles of plant growth promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. *Front. Sustain. Food Syst.* 4:106. doi: 10.3389/fsufs.2020.00106

Müller, C., Cramer, W., Hare, W. L., and Lotze-Campen, H. (2011). Climate change risks for African agriculture. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4313–4315.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant Cell Environ.* 25, 239–250. doi: 10.1046/j.0016-8025.2001.00808.x

Myers, S. S., Smith, M. R., Guth, S., Golden, C. D., Vaitla, B., Mueller, N. D., et al. (2017). Climate change and global food systems: potential impacts on food security and undernutrition. *Annu. Rev. Public Health* 38, 259–277. doi: 10.1146/annurev-publhealth-031816-044356

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., and Ashraf, M. (2014). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnol. Adv.* 32, 429–448. doi: 10.1016/j.biotechadv.2013.12.005

Nafady, N. A., Hashem, M., Hassan, E. A., Ahmed, H. A., and Alamri, S. A. (2019). The combined effect of arbuscular mycorrhizae and plant-growth-promoting yeast improves sunflower defense against Macrophomina phaseolina diseases. *Biol. Control* 138:104049. doi: 10.1016/j.biocontrol.2019.104049

Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., and Sessitsch, A. (2014). Increased drought stress resilience of maize through endophytic colonization by Burkholderia phytofirmans PsJN and *Enterobacter* sp. FD17. *Environ. Exp. Bot.* 97, 30–39. doi: 10.1016/j.envexpbot.2013.09.014

Nwodo, U. U., Green, E., and Okoh, A. I. (2012). Bacterial exopolysaccharides: functionality and prospects. *Int. J. Mol. Sci.* 13, 14002–14015. doi: 10.3390/ijms131114002

Ojuederie, O. B., Olanrewaju, O. S., and Babalola, O. O. (2019). Plant growth promoting rhizobacterial mitigation of drought stress in crop plants: implications for sustainable agriculture. *Agronomy* 9:712. doi: 10.3390/agronomy9110712

Omomowo, I. O., Fadiji, A. E., and Omomowo, O. I. (2018). Assessment of bio-efficacy of *Glomus versiforme* and *Trichoderma harzianum* in inhibiting powdery mildew disease and enhancing the growth of cowpea. *Ann. Agric. Sci.* 63, 9–17. doi: 10.1016/j.aoas.2018.03.001

Orozco-Mosqueda, M., Flores, A., Rojas-Sánchez, B., Urtis-Flores, C. A., Morales-Cedeño, L. R., Valencia-Marin, M. F., et al. (2021). Plant growth-promoting bacteria as bioinoculants: attributes and challenges for sustainable crop improvement. *Agronomy* 11:1167. doi: 10.1002/jobm.202000370

Othman, Y., Al-Karaki, G., Al-Tawaha, A., and Al-Horani, A. (2006). Variation in germination and ion uptake in barley genotypes under salinity conditions. *World J. Agric. Sci.* 2, 11–15.

Ouyang, L., Pei, H., and Xu, Z. (2017). Low nitrogen stress stimulating the indole-3-acetic acid biosynthesis of *Serratia* sp. ZM is vital for the survival of the bacterium and its plant growth-promoting characteristic. *Arch. Microbiol.* 199, 425–432. doi: 10.1007/s00203-016-1312-7

Park, Y.-G., Mun, B.-G., Kang, S.-M., Hussain, A., Shahzad, R., Seo, C.-W., et al. (2017). Bacillus aryabhattai SRB02 tolerates oxidative and nitrosative stress and promotes the growth of soybean by modulating the production of phytohormones. *PLoS One* 12:e0173203. doi: 10.1371/journal.pone.0173203

Paul, M. J., Primavesi, L. F., Jhurreea, D., and Zhang, Y. (2008). Trehalose metabolism and signaling. *Annu. Rev. Plant Biol.* 59, 417–441. doi: 10.1146/annurev.arplant.59.032607.092945

Pearson, S., Wheeler, T., Hadley, P., and Wheldon, A. (1997). A validated model to predict the effects of environment on the growth of lettuce (*Lactuca sativa* L.): implications for climate change. *J. Hortic. Sci.* 72, 503–517. doi: 10.1080/14620316.1997.11515538

Perkins-Kirkpatrick, S., and Lewis, S. (2020). Increasing trends in regional heatwaves. *Nat. Commun.* 11:3357. doi: 10.1038/s41467-020-16970-7

Pouleur, S., Richard, C., Martin, J.-G., and Antoun, H. (1992). Ice nucleation activity in Fusarium acuminatum and Fusarium avenaceum. *Appl. Environ. Microbiol.* 58, 2960–2964. doi: 10.1128/aem.58.9.2960-2964.1992

Preininger, C., Sauer, U., Bejarano, A., and Berninger, T. (2018). Concepts and applications of foliar spray for microbial inoculants. *Appl. Microbiol. Biotechnol.* 102, 7265–7282. doi: 10.1007/s00253-018-9173-4

Purahong, W., Orrù, L., Donati, I., Perpetuini, G., Cellini, A., Lamontanara, A., et al. (2018). Plant microbiome and its link to plant health: host species, organs and *Pseudomonas syringae* pv. actinidiae infection shaping bacterial phyllosphere communities of kiwifruit plants. *Front. Plant Sci.* 9:1563. doi: 10.3389/fpls.2018.01563

Qu, A.-L., Ding, Y.-F., Jiang, Q., and Zhu, C. (2013). Molecular mechanisms of the plant heat stress response. *Biochem. Biophys. Res. Commun.* 432, 203–207. doi: 10.1016/j.bbrc.2013.01.104

Rasool, S., Hameed, A., Azooz, M., Siddiqi, T., and Ahmad, P. (2013). "Salt stress: Causes, types and responses of plants," in *Ecophysiology and Responses of Plants Under Salt Stress*, eds M. N. V. Prasad and P. Ahmad (New York, NY: Springer), 1–24. doi: 10.1007/978-1-4614-4747-4_1

Rising, J., and Devineni, N. (2020). Crop switching reduces agricultural losses from climate change in the United States by half under RCP 8.5. *Nat. Commun.* 11:4991. doi: 10.1038/s41467-020-18725-w

Rouphael, Y., Cardarelli, M., Schwarz, D., Franken, P., and Colla, G. (2012). "Effects of drought on nutrient uptake and assimilation in vegetable crops," in *Plant Responses to Drought Stress*, ed. R. Aroca (Springer: Berlin), 171–195. doi: 10.1111/plb.13304

Russo, S., Dosio, A., Graversen, R. G., Sillmann, J., Carrao, H., Dunbar, M. B., et al. (2014). Magnitude of extreme heat waves in present climate and their projection in a warming world. *J. Geophys. Res. Atmos.* 119, 12500–12512.

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Sanders, G. J., and Arndt, S. K. (2012). "Osmotic adjustment under drought conditions," in *Plant Responses to Drought Stress*, ed. R. A. Hernandez-Ros (Berlin: Springer), 199–229. doi: 10.1007/978-3-642-32653-0_8

Sangiorgio, D., Cellini, A., Donati, I., Pastore, C., Onofrietti, C., and Spinelli, F. (2020). Facing climate change: application of microbial biostimulants to mitigate stress in horticultural crops. *Agronomy* 10:794. doi: 10.3390/agronomy10060794

Santoyo, G., Guzmán-Guzmán, P., Parra-Cota, F. I., Santos-Villalobos, S. D. L., Orozco-Mosqueda, M., and Glick, B. R. (2021b). Plant growth stimulation by microbial consortia. *Agronomy* 11:219. doi: 10.3390/agronomy11020219

Santoyo, G., Gamalero, E., and Glick, B. R. (2021a). Mycorrhizal-bacterial amelioration of plant abiotic and biotic stress. *Front. Sustain. Food Syst.* 5:139. doi: 10.3389/fsufs.2021.672881

Sarabia, M., Cazares, S., González-Rodríguez, A., Mora, F., Carreón-Abud, Y., and Larsen, J. (2018). Plant growth promotion traits of rhizosphere yeasts and their response to soil characteristics and crop cycle in maize agroecosystems. *Rhizosphere* 6, 67–73. doi: 10.1016/j.rhisph.2018.04.002

Saravanakumar, D., and Samiyappan, R. (2007). ACC deaminase from *Pseudomonas* fluorescens mediated saline resistance in groundnut (*Arachis hypogea*) plants. *J. Appl. Microbiol.* 102, 1283–1292. doi: 10.1111/j.1365-2672.2006.03179.x

Seguini, L., Bussay, A., and Baruth, B. (2019). From extreme weather to impacts: the role of the areas of concern maps in the JRC MARS bulletin. *Agric. Syst.* 168, 213–223. doi: 10.1016/j.agsy.2018.07.003

Selosse, M.-A., and Le Tacon, F. (1998). The land flora: A phototroph-fungus partnership? *Trends Ecol. Evol.* 13, 15–20. doi: 10.1016/s0169-5347(97)01230-5

Selvakumar, G., Kundu, S., Joshi, P., Nazim, S., Gupta, A., Mishra, P., et al. (2008a). Characterization of a cold-tolerant plant growth-promoting bacterium *Pantoea dispersa* 1A isolated from a sub-alpine soil in the North Western Indian Himalayas. *World J. Microbiol. Biotechnol.* 24, 955–960. doi: 10.1007/s11274-007-9558-5

Selvakumar, G., Mohan, M., Kundu, S., Gupta, A., Joshi, P., Nazim, S., et al. (2008b). Cold tolerance and plant growth promotion potential of *Serratia marcescens* strain SRM (MTCC 8708) isolated from flowers of summer squash (*Cucurbita pepo*). *Lett. Appl. Microbiol.* 46, 171–175. doi: 10.1111/j.1472-765X.2007.02282.x

Selvakumar, G., Panneerselvam, P., and Ganeshamurthy, A. N. (2012). "Bacterial mediated alleviation of abiotic stress in crops," in *Bacteria in Agrobiology: Stress Management*, ed. D. K. Maheshwari (Berlin: Springer), 205–224. doi: 10.1007/978-3-662-45795-5_10

Sequi, P. (2006). *Fondamenti di Chimica del Suolo*, 2nd Edn. Bologna: Pàtron Editore.

Sharifi, R., and Ryu, C.-M. (2018). Sniffing bacterial volatile compounds for healthier plants. *Curr. Opin. Plant Biol.* 44, 88–97. doi: 10.1016/j.pbi.2018.03.004

Shintu, P., and Jayaram, K. (2015). Phosphate solubilising bacteria (*Bacillus polymyxa*)-An effective approach to mitigate drought in tomato (*Lycopersicon esculentum* Mill.). *Trop. Plant Res.* 2, 17–22.

Shulaev, V., Cortes, D., Miller, G., and Mittler, R. (2008). Metabolomics for plant stress response. *Physiol. Plant.* 132, 199–208. doi: 10.1111/j.1399-3054.2007.01025.x

Skirvin, R., Kohler, E., Steiner, H., Ayers, D., Laughnan, A., Norton, M., et al. (2000). The use of genetically engineered bacteria to control frost on strawberries and potatoes. Whatever happened to all of that research? *Sci. Hortic.* 84, 179–189. doi: 10.1016/s0304-4238(99)00097-7

Smirnoff, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol.* 125, 27–58. doi: 10.1111/j.1469-8137.1993.tb03863.x

Song, Y., Chen, Q., Ci, D., Shao, X., and Zhang, D. (2014). Effects of high temperature on photosynthesis and related gene expression in poplar. *BMC Plant Biol.* 14:111. doi: 10.1186/1471-2229-14-111

Stevenson, S., Fox-Kemper, B., Jochum, M., Neale, R., Deser, C., and Meehl, G. (2012). Will there be a significant change to El Niño in the twenty-first century? *J. Clim.* 25, 2129–2145.

Szczałba, M., Kopta, T., Gąstoł, M., and Sękara, A. (2019). Comprehensive insight into arbuscular mycorrhizal fungi, *Trichoderma* spp. and plant multilevel interactions with emphasis on biostimulation of horticultural crops. *J. Appl. Microbiol.* 127, 630–647. doi: 10.1111/jam.14247

Tahir, H. A., Gu, Q., Wu, H., Raza, W., Hanif, A., Wu, L., et al. (2017). Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. *Front. Microbiol.* 8:171. doi: 10.3389/fmicb.2017.00171

Tahir, M., Ahmad, I., Shahid, M., Shah, G. M., Farooq, A. B. U., Akram, M., et al. (2019). Regulation of antioxidant production, ion uptake and productivity in potato (*Solanum tuberosum* L.) plant inoculated with growth promoting salt tolerant Bacillus strains. *Ecotoxicol. Environ. Saf.* 178, 33–42. doi: 10.1016/j.ecoenv.2019.04.027

Tallon, R., Bressollier, P., and Urdaci, M. C. (2003). Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. *Res. Microbiol.* 154, 705–712. doi: 10.1016/j.resmic.2003.09.006

Tewari, S., and Arora, N. K. (2014). Multifunctional exopolysaccharides from *Pseudomonas aeruginosa* PF23 involved in plant growth stimulation, biocontrol and stress amelioration in sunflower under saline conditions. *Curr. Microbiol.* 69, 484–494. doi: 10.1007/s00284-014-0612-x

Theocharis, A., Bordiec, S., Fernandez, O., Paquis, S., Dhondt-Cordelier, S., Baillieul, F., et al. (2012). Burkholderia phytofirmans PsJN primes *Vitis vinifera* L. and confers a better tolerance to low nonfreezing temperatures. *Mol. Plant Microbe Interact.* 25, 241–249. doi: 10.1094/MPMI-05-11-0124

Tiryaki, D., Aydın, İ., and Atıcı, Ö. (2019). Psychrotolerant bacteria isolated from the leaf apoplast of cold-adapted wild plants improve the cold resistance of bean (*Phaseolus vulgaris* L.) under low temperature. *Cryobiology* 86, 111–119. doi: 10.1016/j.cryobiol.2018.11.001

Trenberth, K. E., Dai, A., Van Der Schrier, G., Jones, P. D., Barichivich, J., Briffa, K. R., et al. (2014). Global warming and changes in drought. *Nat. Clim. Change* 4, 17–22.

Ulloa-Muñoz, R., Olivera-Gonzales, P., Castañeda-Barreto, A., Villena, G. K., and Tamariz-Angeles, C. (2020). Diversity of endophytic plant-growth microorganisms from *Gentianella weberbaueri* and *Valeriana pycnantha*, highland Peruvian medicinal plants. *Microbiol. Res.* 233:126413. doi: 10.1016/j.micres.2020.126413

Unterberger, C., Brunner, L., Nabernegg, S., Steininger, K. W., Steiner, A. K., Stabentheiner, E., et al. (2018). Spring frost risk for regional apple production under a warmer climate. *PLoS One* 13:e0200201. doi: 10.1371/journal.pone.0200201

Vacheron, J., Renoud, S., Muller, D., Babalola, O. O., and Prigent-Combaret, C. (2015). "Handbook for Azospirillum. Technical Issues and Protocols. Everything practical you always wanted to know about Azospirillum sp. but were afraid to ask," in *Alleviation of Abiotic and Biotic Stresses in Plants by Azospirillum*, eds F. D. Cassan, Y. Okon, M. Cecilia, and M. C. Creus (Cham: Springer), 333–365.

Vaishnav, A., Kumari, S., Jain, S., Varma, A., Tuteja, N., and Choudhary, D. K. (2016). PGPR-mediated expression of salt tolerance gene in soybean through volatiles under sodium nitroprusside. *J. Basic Microbiol.* 56, 1274–1288. doi: 10.1002/jobm.201600188

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., and Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules* 21:573. doi: 10.3390/molecules21050573

Vicente-Serrano, S. M., Quiring, S. M., Pena-Gallardo, M., Yuan, S., and Dominguez-Castro, F. (2020). A review of environmental droughts: Increased risk under global warming? *Earth Sci. Rev.* 201:102953. doi: 10.1080/15287390490492313

Vitasse, Y., and Rebetez, M. (2018). Unprecedented risk of spring frost damage in Switzerland and Germany in 2017. *Clim. Change* 149, 233–246. doi: 10.1007/s10584-018-2234-y

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., and Skz, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* 184, 13–24. doi: 10.1016/j.micres.2015.12.003

Wagg, C., Bender, S. F., Widmer, F., and Van Der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5266–5270. doi: 10.1073/pnas.1320054111

Wheeler, T., Hadley, P., Ellis, R., and Morison, J. (1993). Changes in growth and radiation use by lettuce crops in relation to temperature and ontogeny. *Agric. For. Meteorol.* 66, 173–186. doi: 10.1016/0168-1923(93)90069-t

Wild, A. (2003). *Soils, Land and Food: Managing the Land During the Twenty-First Century*. Cambridge: Cambridge University Press.

Wu, B., and Mu, C. (2019). Effects on greenhouse gas (CH4, CO2, N2O) emissions of conversion from over-mature forest to secondary forest and Korean pine plantation in northeast China. *Forests* 10:788. doi: 10.3390/f10090788

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Yandigeri, M. S., Meena, K. K., Singh, D., Malviya, N., Singh, D. P., Solanki, M. K., et al. (2012). Drought-tolerant endophytic actinobacteria promote growth of wheat (*Triticum aestivum*) under water stress conditions. *Plant Growth Regul.* 68, 411–420. doi: 10.1007/s10725-012-9730-2

Yang, J., Kloepper, J. W., and Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14, 1–4. doi: 10.1016/j.tplants.2008.10.004

Yue, X., and Gao, Q. (2018). Contributions of natural systems and human activity to greenhouse gas emissions. *Adv. Clim. Change Res.* 9, 243–252. doi: 10.1016/j.accre.2018.12.003

Yusuf, A. M., Abubakar, A. B., and Mamman, S. O. (2020). Relationship between greenhouse gas emission, energy consumption, and economic growth: evidence from some selected oil-producing African countries. *Environ. Sci. Pollut. Res. Int.* 27, 15815–15823. doi: 10.1007/s11356-020-08065-z

Zhang, H., Murzello, C., Sun, Y., Kim, M.-S., Xie, X., Jeter, R. M., et al. (2010). Choline and osmotic-stress tolerance induced in Arabidopsis by the soil microbe Bacillus subtilis (GB03). *Mol. Plant Microbe Interact.* 23, 1097–1104. doi: 10.1094/MPMI-23-8-1097

Zhang, H., Pan, C., Gu, S., Ma, Q., Zhang, Y., Li, X., et al. (2019). Stomatal movements are involved in elevated CO2-mitigated high temperature stress in tomato. *Physiol. Plant.* 165, 569–583. doi: 10.1111/ppl.12752

Zhao, C., Liu, B., Piao, S., Wang, X., Lobell, D. B., Huang, Y., et al. (2017). Temperature increase reduces global yields of major crops in four independent estimates. *Proc. Natl. Acad. Sci. U.S.A.* 114, 9326–9331. doi: 10.1073/pnas.1701762114

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 January 2022
doi: 10.3389/fmicb.2021.788709

Hyunsoonleella sp. HU1-3 Increased the Biomass of *Ulva fasciata*

*Han Wang[1†], Ali Mohamed Elyamine[1†], Yuchun Liu[1], Wei Liu[1], Qixuan Chen[1], Yan Xu[1,2], Tao Peng[1] and Zhong Hu[1,3]**

[1] Key Laboratory of Resources and Environmental Microbiology, Department of Biology, Shantou University, Shantou, China, [2] Heyuan Polytechnic, Heyuan, China, [3] Southern Marine Science and Engineering Guangdong Laboratory, Guangzhou, China

Green algae are photosynthetic organisms and play an important role in coastal environment. The microbial community on the surface of green algae has an effect on the health and nutrition of the host. However, few species of epiphytic microbiota have been reported to play a role in promoting the growth of algae. In this study, 16S rDNA sequencing was used to study the changes of microbial composition on the surface of *Ulva fasciata* at different growth stages. Some growth promoting bacteria were identified. The possible growth-promoting behavior of the strains were verified by co-culture of pure bacteria obtained from the surface of *U. fasciata* with its sterile host. Among the identified species, a new bacterial species, *Hyunsoonleella* sp. HU1-3 (belonging to the family Flavobacteriaceae) significantly promoted the growth of *U. fasciata*. The results also showed that there were many genes involved in the synthesis of growth hormone and cytokinin in the genome of *Hyunsoonleella* sp. HU1-3. This study identified the bacterium *Hyunsoonleella* sp. HU1-3 for the first time, in which this bacterium has strong growth-promoting effects on *U. fasciata*. Our findings not only provide insights on the establishment of the surface microbiota of *U. fasciata*, but also indicate that *Hyunsoonleella* sp. HU1-3 is one of the important species to promote the growth of *U. fasciata*.

Keywords: *Ulva fasciata*, growth stage, *Hyunsoonleella* sp. HU1-3, bacterial treatment, promoting-plant-growth-bacteria

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Tal Luzzatto-Knaan, University of Haifa, Israel
Matthew Agler, Friedrich Schiller University Jena, Germany

***Correspondence:**
Zhong Hu
hzh@stu.edu.cn

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *03 October 2021*
Accepted: *09 December 2021*
Published: *31 January 2022*

Citation:
Wang H, Elyamine AM, Liu Y, Liu W, Chen Q, Xu Y, Peng T and Hu Z (2022) Hyunsoonleella sp. HU1-3 Increased the Biomass of Ulva fasciata. Front. Microbiol. 12:788709. doi: 10.3389/fmicb.2021.788709

INTRODUCTION

Epiphytic microbiota refers to a specific community harboring the surface of plants and does not parasitize or interfere with the growth and development of its host (Gopal and Gupta, 2016). The microbiota can be found on terrestrial plants, marine macroalgae (Short et al., 2015) and intertidal macroalgae (Hovel et al., 2016). For example, in marine ecosystems, microorganisms such as epiphytic bacteria, fungi and archaea gather on the surface of algae, making this micro-habitat an extremely active interface between host and microbes (Wahl et al., 2012).

Long-term coexistence enables microorganisms to evolve adaptive characteristics closely relating to plants (Vorholt, 2012). Many epiphytes may have a mensal, commensal or parasitic relationships with plants, including macroalgae (Gopal and Gupta, 2016). Microbiota can help plants better absorb nutrients necessary for growth and development (Doty et al., 2016). Recently, the significant role of epiphytic microbiota in assisting plants maintain normal growth and

metabolism has attracted scientists attention (Barott et al., 2011). Host-adapted microorganisms are more resistant to abiotic stresses such as harmful ultraviolet radiation (Kamo et al., 2018), oxidative stress and desiccation (Vorholt, 2012), and affect plant health by reducing biotic or abiotic stresses.

The surface of seaweed also provides habitat for microbial communities (Wiese et al., 2009). The continuous colonization of epiphytic microbiota on healthy algae indicates that there is a positive interaction between them and the host. For example, the growth and development of some green algae depend on specific bacteria (Provasoli and Pintner, 1980). Some microorganisms can provide vitamins (Droop, 2007), fatty acids (Karthick and Mohanraju, 2018), and other active substances to regulate the growth of algae. Furthermore, microorganisms can even produce antimicrobial agents for algae to enable the hosts to adapt to environmental stresses (Miranda et al., 2013; El Shafay et al., 2016). Studies have shown that their abundance is also related to availability of nutrients on the host surface (Paix et al., 2020).

Marine green macroalgae *Ulva* is widely distributed in coastal areas all over the world. It is commonly used as a model to study the environmental adaptability of biological life cycle (Juhmani et al., 2020). It has been reported that high nutrient content in seawater leads to an increase in the number of *Ulva*, which may lead to green tide (Zhang et al., 2019). The development of high-throughput sequencing technology makes it possible to characterize the composition of *Ulva* epiphytic microbiota. To our knowledge, the current research on the epiphytic microbiota of *Ulva* mainly focuses on the functional characteristics of microorganisms that responsible for *Ulva* morphogenesis and the different species composition that responsible for *Ulva* surface morphogenesis during the green tide (Nakanishi et al., 1996; Dobretsov and Qian, 2002; Spoerner et al., 2012; Ghaderiardakani et al., 2017, 2019; Weiss et al., 2017; Alsufyani et al., 2020; Chen et al., 2020; Gianmaria et al., 2020; Inaba et al., 2020; Juhmani et al., 2020; Liu et al., 2020; Qu et al., 2020). However, the biodiversity and functional relationship of epiphytic microbiota harbored by *Ulva* have not been fully explored. Previous studies have shown that bacterial communities located in different genotypes of the same algae usually have small differences. In addition to the influences of genotype, the composition of epiphytic bacterial community is also affected by seasons, environment and nutrients (El-Said and El-Sikaily, 2013).

Most studies show that α-Proteobacteria and γ-Proteobacteria are the main bacterial phyla colonizing the surface of algae (Bondoso et al., 2014). Different plants, such as rice (Edwards et al., 2015), grape (Zarraonaindia et al., 2015), sugarcane (De Souza et al., 2016), and citrus (Xu et al., 2018), have the same core microbiota (at the genus level), among which *Pseudomonas*, *Methylobacterium*, and *Sphingomonas* are the most common. This suggests that the core microbiome is selectively recruited and usually has the necessary functions to co-existence with the host. The identification methods and techniques of core microorganisms constitute the basis for studying how to construct a synthetic microbiota, revealing plant-microbiome interaction and possibly promoting plant growth.

Ulva fasciata are widely distributed in the rocky intertidal zone along the coast of China. Bacteria on algae vary with season and the life cycle of host (Lachnit et al., 2011). The purpose of this study was to determine (1) whether the epiphytic microbial community on the surface of *U. fasciata* varies at different growth stages, (2) whether the epiphytic microbiota is related to the growth stage of *U. fasciata*, and (3) whether the selected bacteria can promote the growth of *U. fasciata*.

The application of the bacterial treatment method in a large number of studies showed that the bacterial treatment method not only reveals how plants affect their microbial communities and how microbial communities affect plant growth and health but also has high operability and application value for agricultural production such as promoting plant growth and resisting plant diseases. It is believed that the application of the bacterial treatment method will also bring light to the solution of ecological problems such as green tide. Future studies will focus on the detailed identification of the nutrient fixation and promotion abilities of these isolates from the surface of *U. fasciata*.

MATERIALS AND METHODS

Sampling and Determination of Environmental Parameters

Ulva fasciata was randomly sampled from Nanao Island, Shantou, Guangdong, China (about 100 m from the coast) (116.53°E 23.11°N). In the seedling stage, *U. fasciata* were sampled once a week on November 9th, 16th, and 23th, that corresponding to seedling stages 1, 2, and 3, respectively. During the growth stage, *U. fasciata* were sampled on December 27, February 2, and March 28, which corresponding to growth stages 1, 2, and 3, respectively. At maturity, *U. fasciata* were collected on April 2, May 3, and June 3, corresponding to maturity stages 1, 2, and 3, respectively (**Figure 1A**). The samples were collected in three replicates, washed with sterile seawater, and stored in 50-mL sterilized centrifuge tubes.

During nine sampling, the *in situ* environmental parameters (temperature, salinity, pH, and dissolved oxygen concentration) were recorded (**Supplementary Table 1**). At the same time, 1 L seawater samples were collected, filter through 0.45 and 0.22 μm membranes to remove impurities and collect bacteria. The filtrates (0.22 μm filter membrane) were stored in sterile sampling bags and the filtered water was cryopreserved for the determination of physical and chemical parameters, including dissolved organic carbon (TOC), inorganic carbon (TIC), total nitrogen (TN), and total carbon (TC) according to the methods described by Urgun-Demirtas et al. (2008). All samples were placed in a foam box with ice cubes and transferred to the laboratory within 4 h. In the laboratory, the microbiota harboring the surface of *U. fasciata* was then immediately collected. The *U. fasciata* samples in a 50-mL sterilized centrifuge tube was mixed with an appropriate amount of sterile seawater and treated with ultrasonic (1 min, 50 W, 5s/5s). Algae were removed, and then the suspension was centrifuged at 4,000 × *g* for 10 min to collect the pallet, which was then used for bacterial DNA extraction.

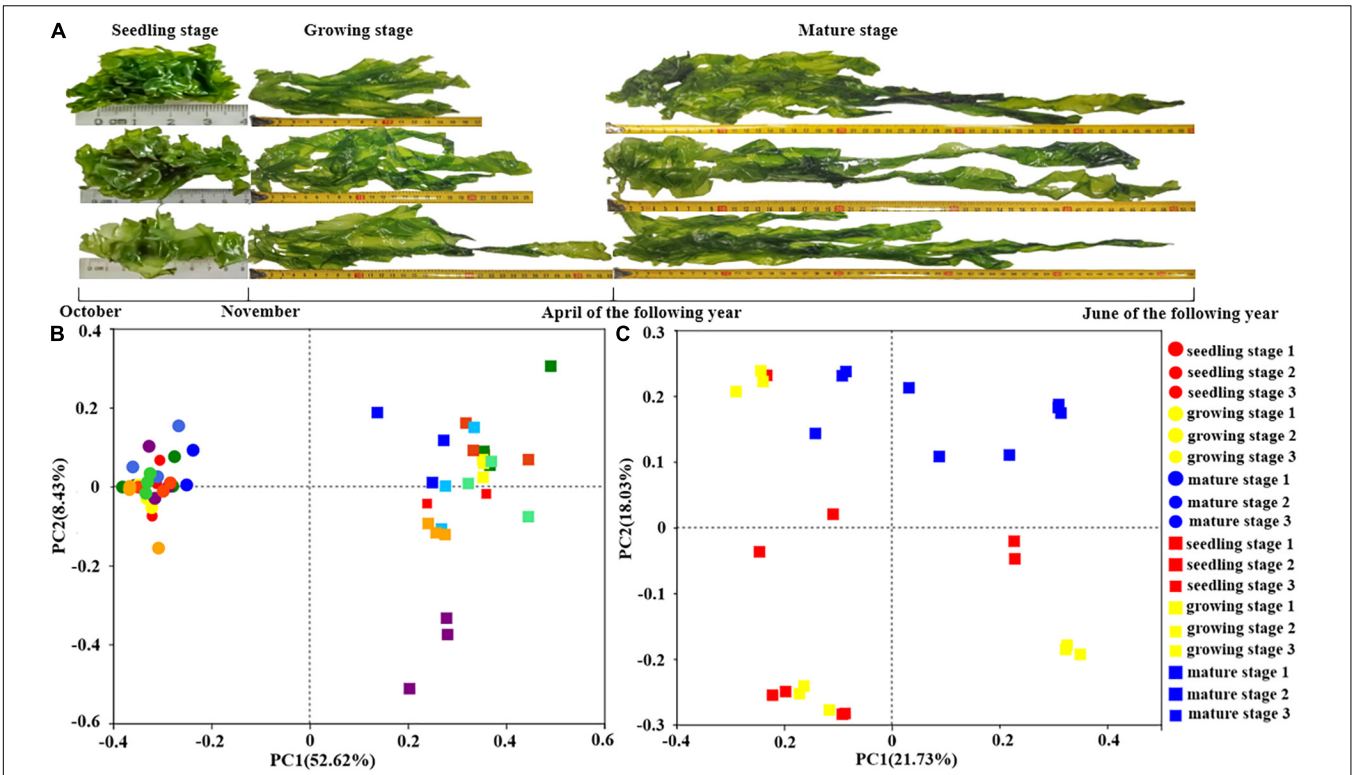


FIGURE 1 | The surface microbiota of *Ulva fasciata* variation at different growth stages. **(A)** Photographs of *U. fasciata* samples in different life cycles **(B)** Principal Coordinate Analysis of microbiota changes on the surface of *U. fasciata* and seawater samples of different growth stages at Genus Level **(C)** Principal coordinate analysis of microbiota changes on the surface of *U. fasciata* in different growth stages at Genus Level (the circles represent the seawater sample and the squares represent the surface of the *U. fasciata* sample).

DNA Extracting and 16S rDNA Sequencing

Bacterial DNA was extracted using the CTAB method (Zhou et al., 1996). DNA quality and integrity were evaluated by gel electrophoresis. Samples with high-quality DNA were stored at −80°C until further use. The DNA of all samples was sent to Shanghai Majorbio Bio-Pharm Technology Company for sequencing and data analyses.

Sequence Data and Statistical Analysis

To determine the species and abundance of bacteria, the V3–V4 region of the 16S rRNA gene was amplified using primers 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and 338F (5′-ACTCCTACGGGAGGCAGCAG-3′), and the PCR products were sequenced using Illumina MiSeq sequencing platform. The sequencing data were stitched by Flash V1.2.11, and then the optimized Fastq files were obtained after quality control by Fastp V0.19.6. The sequences were preprocess using the software Usearch 7 (parameter setting: minsize 2), and the sequences were grouped into operational classification units (OTUs) for species level classification using 97% sequence similarity. After obtaining the OTU classification table, those OTUs assigned to chloroplasts or mitochondria and eukaryotes were excluded from the data set. OTU counts were normalized, the original count was divided by the total number of each sample, and then multiplied by the median to calculate the relative abundance. Using QIIME V1.9.1, the taxonomic information was assigned to each representative OTU sequence and filtered OTUs were used to calculate alpha diversity indexes, including Shannon, Chao1, and Simpson. Calculate the Bray-Curtis distance for all pairs of samples to determine the amount of diversity shared between communities (β-diversity) (Liu et al., 2021). Principal Co-ordinates Analysis (PCoA) sorting was performed according to Bray-Curtis distance. Adonis based on Bray distance was used to calculated the significant difference between bacterial community and the null hypothesis (Anderson, 2001). Dispersion of microbial communities was evaluated using the pairwise permutation tests with Tukey's HSD. The Kruskal–Wallis test was used to determine whether there were significant differences in the abundance of bacterial classes. Bacterial taxonomic biomarkers for bacterial classification were obtained by referring to the random forest algorithm (Zhang et al., 2018). The Cytoscape software package was used for visualization. All of the above software used default parameters.

Construction of Bacterial Treatment and *U. fasciata* (WSB) Growth Promotion Test and Plant Growth Promotion Experiment

Samples of *U. fasciata* were collected at different times, rinsed and immediately transferred to the laboratory. The blade surface

was gently scraped with a sterile knife, and the scraped materials were gradually diluted and spread on the marine agar plate 2216E, and placed in an incubator at 25°C for 3–4 days. Single colonies were selected, streaked, purified, and inoculated in marine broth 2216E. The 16S rRNA gene of bacteria was amplified with universal primers 27F and 1492R, and the samples were sent to Bioengineering (Shanghai) for sequencing. The phylogenetic names of the isolates were identified by EzBioCloud database. A total of 228 pure strains were obtained. These strains were cultured on marine broth 2216E to logarithmic growth phase. The bacterial suspension was mixed with 40% sterilized glycerol in the ratio of 1:1. These strains were selected as candidate strains for bacterial treatment. To test the promoting effect of bacterial treatment on plant growth, *U. fasciata* without surface bacteria (WSB) was cultured with isolates.

Ulva fasciata without surface bacteria was prepared as follow:

The surface of *U. fasciata* was washed with sterile seawater and ultrasonic waves (1 min, 50 KHz, 5s/5s) for three times to remove bacteria. The mixture of antibiotics (including 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin, 100 μg/mL ampicillin, and 0.1 μg/mL imidazole) was added to the culture tube and incubated for 2 days; the same dose of antibiotics was added on the third day. The surface of *U. fasciata* was quickly scrubbed with a sterile cotton swab dipped in a multi-enzyme cleaning solution (3M), and then rinsed with sterile seawater. The obtained SBR was evaluated through three methods: (1) PCR detection of seawater collected from the last rinsing of *U. fasciata* and algal abrasive solution in the range of about 1500 bp using primers 27F and 1492R, (2) the seawater collected from the last time rinsing of *U. fasciata* was used to culture on marine agar 2216E to confirm the growth of bacteria in 7 days, (3) the sterile surface of *U. fasciata* was stained with 1% (v/v) of DAPI for 20 min, the excess DAPI was removed with sterile water, and then the confocal laser scanning microscopy (CLSM) was used to check whether there were bright blue bacterial spots (bacteria) in the sample. Samples were imaged with CLSM (LSM 700, Zeiss, Germany) and x100 oil immersion objective. Select DAPI channel 400–630 nm. Four random locations were scanned on each sample.

The experiment consisted of three bacterial treatment groups, sterile seawater (C group), *Bacillus cereus* U5-30 (P group) and *Hyunsoonleella* sp. HU1-3 (H group). The bacteria were incubated in marine broth 2216E at 25°C for 2 days, centrifuged, washed twice with sterile seawater, and then resuspended to a density of 10^7 cells per mL. The volume of bacterial liquid added to each bacterial treatment was 5 mL. The mixed bacteria were added to a bottle connected to filtered air and contained 800 mL of sterile seawater, and then co-cultured with *U. fasciata* (WSB) for 7 days. At the end of the experiment, the co-cultured *U. fasciata* was washed with sterile seawater, and the surface bacterial strains of *U. fasciata* were obtained by ultrasonic shock. The 2216E marine agar plate was used to test whether the bacterial treatment strains survived after 7 days.

To determine the growth rate, at the beginning of the co-culture experiment, young *U. fasciata* with similar growth trend and size were selected, weighed (set up as W_t) and sterilized. The vase was placed in a light incubator for 7 days (temperature, light intensity, photoperiod and air supply time were 20°C, 4000 LX, 12 h:12 h, and 24 h, respectively). After 7 days of co-culture, *U. fasciata* was taken out, the surface seawater was removed and weighed (W_0). Growth rate was calculated by the formula of $[(W_t/W_0)^{1/t}\text{-}1] \times 100\%$ (t is days of culture) (Yong et al., 2013). During the experiment, each bacterial treatment group was guaranteed at least three biological replicates. The contents of soluble protein, soluble sugar, phycocyanin, and chlorophyll-a (Schwenzfeier et al., 2011) were determined to characterize the biomass of *U. fasciata.*

The bacteria strains were incubated in 2216E liquid medium at 25°C for 3 days, with or without 100 μg/mL of L-tryptophan, and then centrifuged. After that, the supernatant was diluted to OD600 = 0.8 with sterile seawater, and 10 mL of culture were added to a bottle containing 800 mL of sterile seawater with *U. fasciata* (WSB) and inoculated for 7 days. In the experiments, six supernatant were used, including sterile seawater (A group), 2216E liquid medium (B group), supernatant of *B. cereus* strain U5-30 living in 2216E liquid medium (C group), supernatant of *Hyunsoonleella* sp. strain HU1-3 living in 2216E liquid medium (D group), supernatant of *B. cereus* strain U5-30 living in 2216E liquid medium and L-tryptophan (E group), supernatant of *Hyunsoonleella* sp. strain HU1-3 living in 2216E and L-tryptophan (F group). Six groups of supernatants were separately cocultivated with *U. fasciata* (WSB), and the growth rate of *U. fasciata* was measured by the same method as above.

Genome Sequencing and Assembly

In order to illustrate how *Hyunsoonleella* strain HU1-3 promotes the growth of *U. fasciata*, the extraction of its genome was sent to BGI Corporation for genome test, and double-terminal sequencing was performed based on Illumina PE150 platform. Raw data is processed by low-quality filtering. Low quality bases of more than 40 bp were removed (quality value 38 or less), N bases were up to 10 bp, and the overlap between junctions was more than 15 bp. The repeated contamination is then removed to obtain valid data. The genome size was estimated by K-mer statistical analysis, and the raw sequencing reads were trimmed and assembled by SOAPdenovo v2.04 software[1]. The FastQC software was used to analyze sequence quality (Brown et al., 2017). GeneMark software (V.4.17) (Besemer et al., 2001) was used for comparison and genomic protein-coding genes were obtained. These gene sequences were compared with the known Nr database (non-redundant protein database) (Li et al., 2002). For the comparison results of each sequence, the highest score (identity $\geq$40%, coverage $\geq$40%) was selected for gene function annotation.

Strains Indole-3-Acetic Acid Production and Solubilization of Phosphate

The bacteria strains were incubated in 2216E liquid medium at 25°C for 3 days. 1-mL incubation fluid was centrifuged, the cell precipitates were washed twice with PBS, and then resuspended to 107 cells/mL. 1 mL of the suspension was mixed with 10 mL of liquid medium (with and without 100 μg/mL of tryptophan).

[1]http://soapdenovo2.sourceforge.net/

After 3 days, 50 μL of supernatant was collected and mixed with 50 μL of Salkowski reagent (50 mL 35% $HClO_4$ + 1 mL 0.5 mol/L $FeCl_3$). After 25 min, the absorbance value was read at 530 nm (Bric et al., 1991) to measure the production of bacterial IAA. Bacteria were inoculated in 50 mL sterilized liquid NBRIP medium at a dose of 0.2% and cultured in a flask at 25°C for 7 days. The culture was centrifuged at 5,000 RPM for 20 min, and the precipitation of P_2O_5 was determined by molybdenum blue method according to Galhardo and Masini (2000) to measure the solubility of phosphate. All measurements were conducted in triplicate.

RESULTS

Succession of *U. fasciata* Surface Bacterial Communities and Model of Correlation Between Surface Bacterial Taxonomic Biomarkers

In order to investigate the variations of microbes harboring the surface of *U. fasciata* under natural conditions, seawater samples and *U. fasciata* samples, a total of 2,969,125 high-quality sequences were obtained from all samples. The bacterial community structure over time in different samples is illustrated in **Figures 1B,C**. There was a significant difference ($P < 0.01$) between the microbial community living in seawater and the *U. fasciata* surface community (**Figure 1B**). PCoA analysis also showed that there were different microbiota harboring the surface of *U. fasciata* at different growth stages ($R^2 = 0.68$, $P < 0.01$, adonis) (**Figure 1C**). This indicated that the growth stage plays a significant role in the establishment of microbiota inhabiting the surface of *U. fasciata*. Mature stage samples were close to one another, while seedling stage samples were relatively far away from one another (**Supplementary Figure 1**). The microbial community in mature stage overlapped with that in seedling stage and growth stage, indicating that the microbial community is in a transitional state between mature and seedling/growth stages. Similar results can be obtained by calculating the community diversity index (Shannon) and community richness index (Chao and ace) (**Table 1**), in which the alpha-diversity index of surface bacteria was significantly lower than that of seawater bacteria, while the diversity index of *U. fasciata* surface bacteria remained relatively stable.

The relative abundance of epiphytic microbiota of *U. fasciata* and seawater is illustrated in **Supplementary Figures 2A,B**. Seawater samples were dominated by marine bacteria, such as HIMB11 and NS5 marine groups (**Supplementary Figure 2A**). Microbial community abundance were significant differences in the surface of *U. fasciata* with the seawater (**Supplementary Figure 2B**). The relative abundance of Proteobacteria and Bacteroidetes on *U. fasciata* surface was significantly higher that in seawater. The results of Kruskal-Wallis analysis (**Supplementary Figure 3A**) showed that although the relative abundance of these species increased or decreased throughout the growth period, there was no significant difference. Actinobacteria and Firmicutes changed greatly throughout the growth stage. At the genus level, there were significant differences in the relative abundance of some species (**Supplementary Figure 2C**). For instance, at the seedling stage, the relative abundances of Rhodobacteraceae (13.0%, $P < 0.01$, Kruskal–Wallis), *Acinetobacter* (6.2%, $P < 0.01$, Kruskal–Wallis), and *Vibrio* (1.2%, $P < 0.01$, Kruskal–Wallis) was significantly lower, while the relative abundance of *Ralstonia* (2.3%, $P < 0.01$, Kruskal–Wallis), *Erythrobacter* (2.8%, $P < 0.01$, Kruskal–Wallis), and *Rhodococcus* (2.4%, $P < 0.01$, Kruskal–Wallis) were significantly higher. The composition of microbiota on the surface of *U. fasciata* varied

TABLE 1 | Microbiodiversity indices of *U. fasciata* surface and seawater at different growth stages.

Sample	Stage	Shannon	Ace	Chao
Seawater	Seedling stage 1	4.15 ± 0.30	2153.21 ± 438.36	1122.85 ± 126.96
	Seedling stage 2	3.67 ± 0.50	1185.38 ± 466.28	711.35 ± 204.09
	Seedling stage 3	4.31 ± 0.36	1880.29 ± 212.66	1115.01 ± 116.76
	Growing stage 1	4.25 ± 0.07	2245.17 ± 225.93	1252.29 ± 161.64
	Growing stage 2	4.14 ± 0.11	1774.78 ± 383.26	986.01 ± 141.13
	Growing stage 3	4.20 ± 0.29	2151.60 ± 384.07	1280.32 ± 109.10
	Mature stage 1	3.88 ± 0.65	1948.03 ± 930.45	1110.24 ± 328.99
	Mature stage 2	4.23 ± 0.23	1826.69 ± 613.97	1161.50 ± 300.89
	Mature stage 3	4.22 ± 0.14	2082.02 ± 487.13	1129.71 ± 173.52
Ulva	Seedling stage 1	4.30 ± 0.12	463.64 ± 210.61	436.64 ± 143.87
	Seedling stage 2	3.88 ± 0.15	332.09 ± 139.52	292.25 ± 67.94
	Seedling stage 3	3.07 ± 1.69	337.85 ± 212.87	274.22 ± 138.34
	Growing stage 1	4.22 ± 0.10	565.49 ± 137.80	466.52 ± 41.52
	Growing stage 2	4.48 ± 0.09	477.85 ± 90.53	422.89 ± 68.10
	growing stage 3	4.51 ± 0.18	541.79 ± 80.81	522.55 ± 91.61
	Mature stage 1	4.02 ± 0.44	396.08 ± 103.21	394.94 ± 118.55
	Mature stage 2	4.18 ± 0.13	734.79 ± 167.47	574.22 ± 87.80
	Mature stage 3	4.40 ± 0.05	792.73 ± 149.49	574.09 ± 66.85

greatly throughout the growth, but the dominant species of microbiota were Rhodobacteraceae, *Ralstonia, Erythrobacter, Rhodococcus, Acinetobacter, Alteromonas, Vibrio, Halomonas,* and *Granulosicoccus.*

The regression analysis between the relative abundance of bacteria on the surface of *U. fasciata* species and the growth stage was established and revealed that the model explained 56% of the surface of *U. fasciata* microbiota variance related to *U. fasciata* growth stage (**Supplementary Figure 4**). At the class level, the class number (n = 30) was stable and relative to the cross-validation error curve. Therefore, these 30 bacterial classes, especially *Rhodococcus* and *Hyunsoonleella*, were defined as biomarker groups in the model.

Effects of Environmental Factors and Physiological Parameters of *U. fasciata* on Bacterial Community

The relationship between environmental factors and the abundance of different phyla is shown in **Figure 2**. At the phylum level, the relative abundance of Patescibacteria, Firmicutes and Bacteroidetes was positively correlated with the physiological parameters of different carbon forms. As mentioned above, the *U. fasciata* growth stage is the best explanation for the variation of microbial community. However, the environmental and physiological parameters of *U. fasciata* at different growth stages have different explanations for the variation of microbial community (**Table 2**). Dissolved oxygen (DO) and Phycocyanin had no effect on the microbial community on the surface of *U. fasciata* (R^2 = 0.04 and 0.05, respectively, $P > 0.05$, Adonis). TOC, pH, soluble protein and TN had moderate effects on the microbial community on the surface of *U. fasciata*. IC and TC had the greatest effect on the microbial community on the surface of *U. fasciata* (R^2 = 0.13, 0.14, respectively, $P < 0.05$, Adonis). The results showed that environmental factors and physiological parameters of *U. fasciata* also affected the structure of surface bacteria.

Hyunsoonleella sp. HU1-3 Strains Promote Plant Growth

A total of 228 bacterial strains were isolated from the surface of *U. fasciata* at different growth stages (**Supplementary Figure 5**). These strains were classified into three phyla, including Firmicutes, Bacteroidetes, and Proteobacteria. Although bacteria were still present in the *U. fasciata* (WSB) lapped samples (**Supplementary Figure 6C**) and intercellular bacteria were detected by CLSM (**Supplementary Figure 6P**), the quality of *U. fasciata* (WSB) was comparable to that of wild *U. fasciata* (**Supplementary Figure 7**) ($P > 0.05$, ANOVA).

The prediction results of machine forest model suggest that members of Rhodobacteraceae and Flavobacteriaceae may play an important role in the growth of *U. fasciata*. Therefore, bacterial treatment experiments were carried out to investigate the growth-promoting effect of Rhodobacteraceae and Flavobacteriaceae on *U. fasciata*. The role of endophytic bacteria was ignored in the experiment bacterial treatments. Among the isolates,

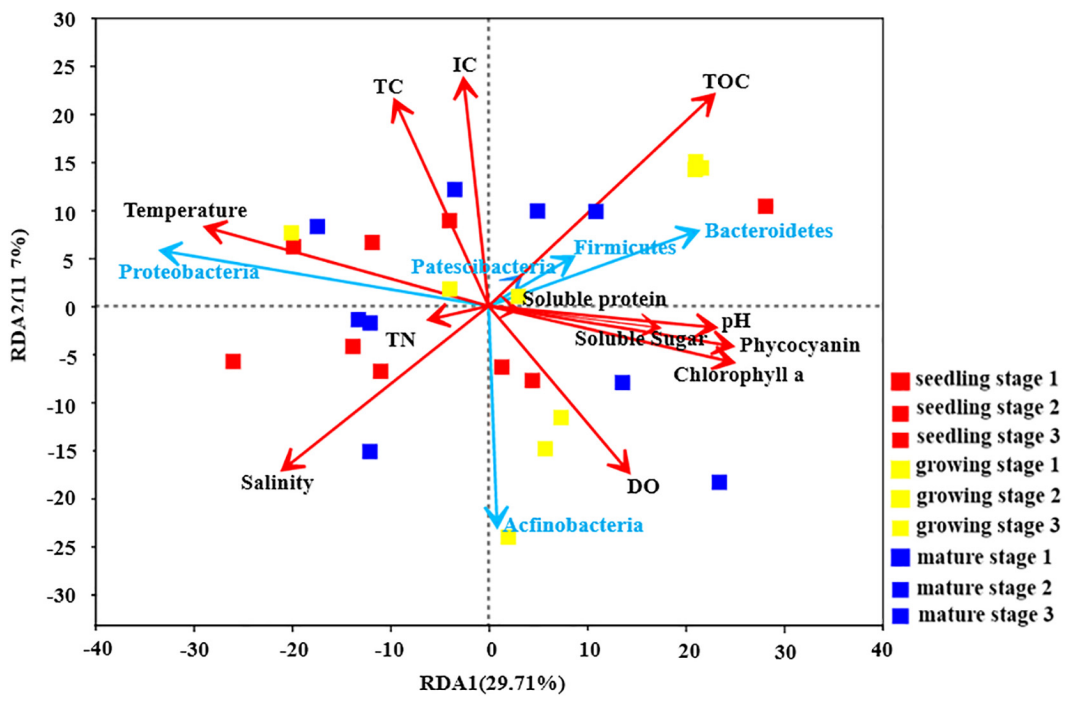


FIGURE 2 | Redundancy Analysis of *U. fasciata* surface microorganisms with environmental factors and plant physiological indexes at *Phylum* level (The red arrows represented different environmental parameters and physiological indicators of *U. fasciata* and the blue arrows represented the top five groups of bacteria in abundance).

TABLE 2 | Effects of growth stage, environmental factors, and physiological parameters of *U. fasciata* on *U. fasciata* surface microbiota.

Characteristics	Sums of sqs	Mean sqs	*F* model	R^2	*P*-value	*P*.adjust
Growth stages	4.11	0.51	4.82	0.68	0.001	0.004
IC (mg/L)	0.81	0.81	3.88	0.13	0.001	0.004
TC (mg/L)	0.83	0.83	3.99	0.14	0.001	0.004
TOC (mg/L)	0.60	0.60	2.76	0.10	0.003	0.009
pH	0.57	0.57	2.62	0.10	0.004	0.009
Soluble protein (mg/g)	0.64	0.64	2.95	0.11	0.004	0.009
TN (mg/L)	0.59	0.59	2.69	0.10	0.005	0.009
Salinity (‰)	0.48	0.48	2.15	0.08	0.014	0.023
Soluble sugar (mg/g)	0.44	0.44	1.99	0.07	0.025	0.036
Chlorophyll A (mg/g)	0.44	0.44	1.99	0.07	0.038	0.049
Temperature (°C)	0.36	0.36	1.57	0.06	0.108	0.128
Phycocyanin (mg/g)	0.30	0.30	1.32	0.05	0.217	0.235
DO(ppm)	0.27	0.27	1.17	0.04	0.319	0.319

The first column of the table represents grouping factors or environmental factors. R^2 represents the degree of explanation of grouping factors to sample differences. The larger R^2 is, the higher the degree of explanation of grouping factors to sample differences. P-value less than 0.05 indicates high reliability of this test.

Hyunsoonleella sp. HU1-3 demonstrated the greatest growth promoting potential on *U. fasciata*. Compared with other bacteria of *Hyunsoonleella*, its 16S rRNA gene sequence similarity and average nucleotide identity values were less than 98.7 and 95%, respectively (Chun et al., 2018), so it is considered as a new species of the genus.

The four physiological indices and growth rate of *U. fasciata* of group H [co-culture of *U. fasciata* (WSB) and *Hyunsoonleella* sp. HU1-3] were significantly higher than those of group C (only seawater was added as the negative control) and group P [co-culture of *U. fasciata* (WSB) and *B. cereus* U5-30 as the positive control] ($P < 0.05$, ANOVA) (**Figure 3**). In fact, compared with group P, the positive effect of *Hyunsoonleella* HU1-3 on soluble protein, soluble sugar and phycocyanin of *U. fasciata* showed that the strain significantly increased the biomass of sterile *U. fasciata* ($P < 0.05$, ANOVA) (**Figures 3A–D**). The growth rate of group H (5.49% day^{-1}) and group P (4.05% day^{-1}) was significantly higher than that of group C (1.10% day^{-1}) (**Figure 3E**).

Six different groups of bacterial supernatants were cocultured with *U. fasciata* (WSB) (**Figure 4**). There was no significant difference in the growth rate of *U. fasciata* between group A (sterile seawater) and group B (sterile 2216E liquid medium) ($P > 0.05$, ANOVA). Interestingly, the supernatant of group C (*B. cereus* strain U5-30) and group D (*Hyunsoonleella* sp. HU1-3) significantly increased the growth rate of *U. fasciata* (1.52% day^{-1} and 2.45% day^{-1}, respectively; $P < 0.05$, ANOVA). Surprisingly, the *U. fasciata* growth rate of group E and group F (2.77% day^{-1} and 3.95% day^{-1}, respectively; $P < 0.05$, ANOVA) was significantly higher than that of group C.

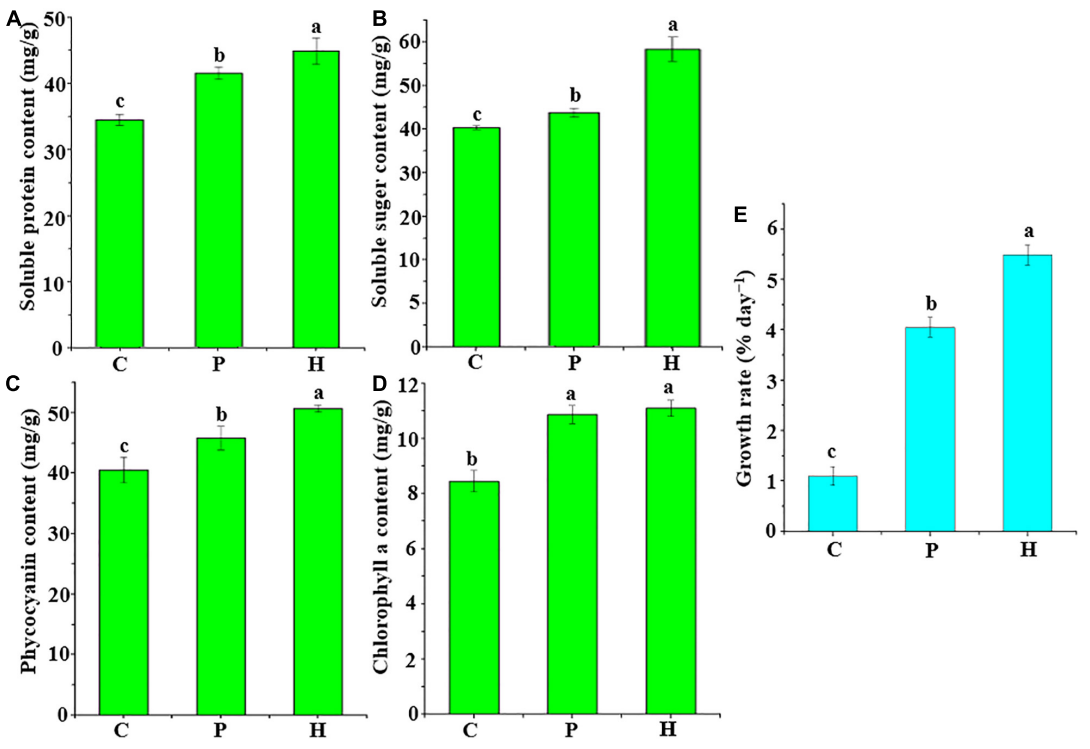


FIGURE 3 | Changes of growth rate and four physiological indices of bacterial treatment and *U. fasciata* (WSB). **(A)** Soluble protein content, **(B)** Soluble sugar content, **(C)** Phycocyanin content, **(D)** Chlorophyll *a* content, and **(E)** Growth rate. C group used sterile seawater for the negative control, P group only contains *Bacillus cereus* U5-30 strain was used to be the positive control, H group contained only one strain of *Hyunsoonleella* sp. HU1-3, the difference coefficient represents the difference between C, P, and H group.

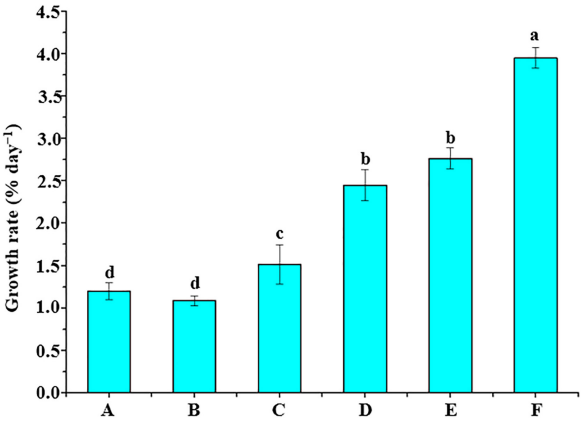


FIGURE 4 | Supernatant of bacterial culture and *U. fasciata* (WSB) growth promotion test. A group added sterile seawater, B group added sterile 2216E liquid medium, C group added the supernatant of the strain *B. cereus* U5-30 living in 2216E, D group added the supernatant of the strain *Hyunsoonleella* sp. HU1-3 living in 2216E, E group added the supernatant of the strain *B. cereus* U5-30 living in 2216E + L-tryptophan, F group added the supernatant of the strain *Hyunsoonleella* sp. HU1-3 living in 2216E + L-tryptophan. The difference coefficient represents the difference between A and F group.

Genetic Elements Involved in *Hyunsoonleella* sp. HU1-3 Plant Growth Promotion

Based on the genome of *Hyunsoonleella* HU1-3 (**Figure 5** and **Supplementary Table 2**), several amidases (such as N-acetylmuramoyl-L-alanine amidase LytC and 2-oxoglutaramate amidase) and tryptophan related enzymes (such as tryptophan 2,3-dioxygenase and tryptophan-tRNA ligase) were identified, indicating that *Hyunsoonleella* sp. HU1-3 may produce IAA through IpyA and IAM pathways. Genes involved in the synthesis of cytokinins (CK) have also been annotated, such as tRNA-dimethylallyltransferase, tRNA-2-methylthio-N6-dimethylallyladenosine, and cytokinin-nucleoside 5-monophosphate ribose hydrolase. These results indicate that *Hyunsoonleella* sp. HU1-3 has the potential to synthesize a variety of CKs. Furthermore, seven phosphate synthases genes were found in the genome of *Hyunsoonleella* sp. HU1-3. Interestingly, *Hyunsoonleella* sp. HU1-3 contains genes involved in vitamin synthesis (thiamine-phosphate synthase and thiamine-monophosphate kinase). Other genes annotated as iron carrier enzymes (such as iron-sulfur cluster carrier protein and iron-dependent repressor IdeR) were also found in the genome.

Indole-3-Acetic Acid Production and Phosphate Solubility Capacity of Strain

Plant growth is known to depends on several transaminase proteins, especially growth hormones, such as IAA. In the absence of tryptophan, the IAA yield of *B. cereus* U5-30 was 209.70 ± 3.64 μg/mL. The addition of tryptophan increased the IAA production to 375.16 ± 3.59 μg/mL. The phosphate solubility of *B. cereus* U5-30 was 191.18 ± 2.87 μg/mL. On the other hand, the IAA yield and phosphate solubility of *Hyunsoonleella* sp. HU1-3 without tryptophan were 227.64 ± 0.82 μg/mL, and 213.98 ± 1.15 μg/mL, respectively. It is speculated that the IAA yield and phosphate solubility of *Hyunsoonleella* sp. HU1-3 are better than *B. cereus* U5-30 (**Table 3**).

DISCUSSION

Epiphyte studies during plant growth are usually carried out under greenhouse conditions (Edwards et al., 2015). We conducted field sampling to represent the life cycle of *U. fasciata*. *U. fasciata*'s growth slowly in November (seedling stage) and reaches maturity in April of the following year. In June, with the increase of seawater temperature, the *U. fasciata* will bleached, rotten and immediately washed away by seawater, which makes sampling difficult. Therefore, we only focus on *U. fasciata* that grows well from seedling stage to decay stage. Roth-Schulze et al. (2018) demonstrated that the bacterial diversity on the surface of *U. fasciata* is different from the surrounding seawater. The PCoA analysis in this study revealed that the composition of epiphytic microbiota of *U. fasciata* was significantly different from that of surrounding seawater. In a previous study, Lachnit et al. (2011) reported that the microbial composition on the surface of *Ulva infantis* varied with the growth stage. Similarly, it was reported that the dynamic change of surface microbiota through the plant life cycle depends on the growth stage of plant (Chaparro et al., 2014; Dombrowski et al., 2016). The dominant bacteria on the surface of *U. fasciata* were Proteobacteria and Bacteroidota. This finding is consistent with Burke et al. (2011).

The core microbiome is defined as a group of shared members in microbial communities from similar habitats (Shade and Handelsman, 2012). Thus, the discovery of core microbiome is very important to understand the stable components of complex microbial assemblages. In this study, the core microbiome was consistent at different growth stages. Some Rhodobacteraceae, *Ralstonia*, *Erythrobacter*, *Rhodococcus*, *Acinetobacter*, *Alteromonas*, *Vibrio*, *Halomonas,* and *Granulosicoccus* were the dominant genera on the surface of *U. fasciata*. It is reported that *Ulva australis* are mainly composed of Alphaproteobacteria, Bacteroidetes, Planctomycetes, and unclassified Gammaproteobacteria (Burke et al., 2011), while the bacterial community associated with green seaweeds is mainly composed of Proteobacteria, Bacteroidetes, Verrucomicrobia, Cyanobacteria, Planctomycetes, Actinobacteria, and Firmicutes (Selvarajan et al., 2019). The species of core microbial communities on *Ulva* surface were similar. However, the differences of green algae epiphytic communities in different environments may be explained by compounds secreted by green algae surface that regulate the composition of microbial communities.

In addition, most of the genera level biomarker groups showed high relative abundance at the corresponding growth stages (e.g., Rhodobacteraceae and Flavobacteriaceae). It is reported that Flavobacteriaceae bacteria are beneficial to the growth of *Ulva*. For example, Spoerner et al. (2012) reported that the addition

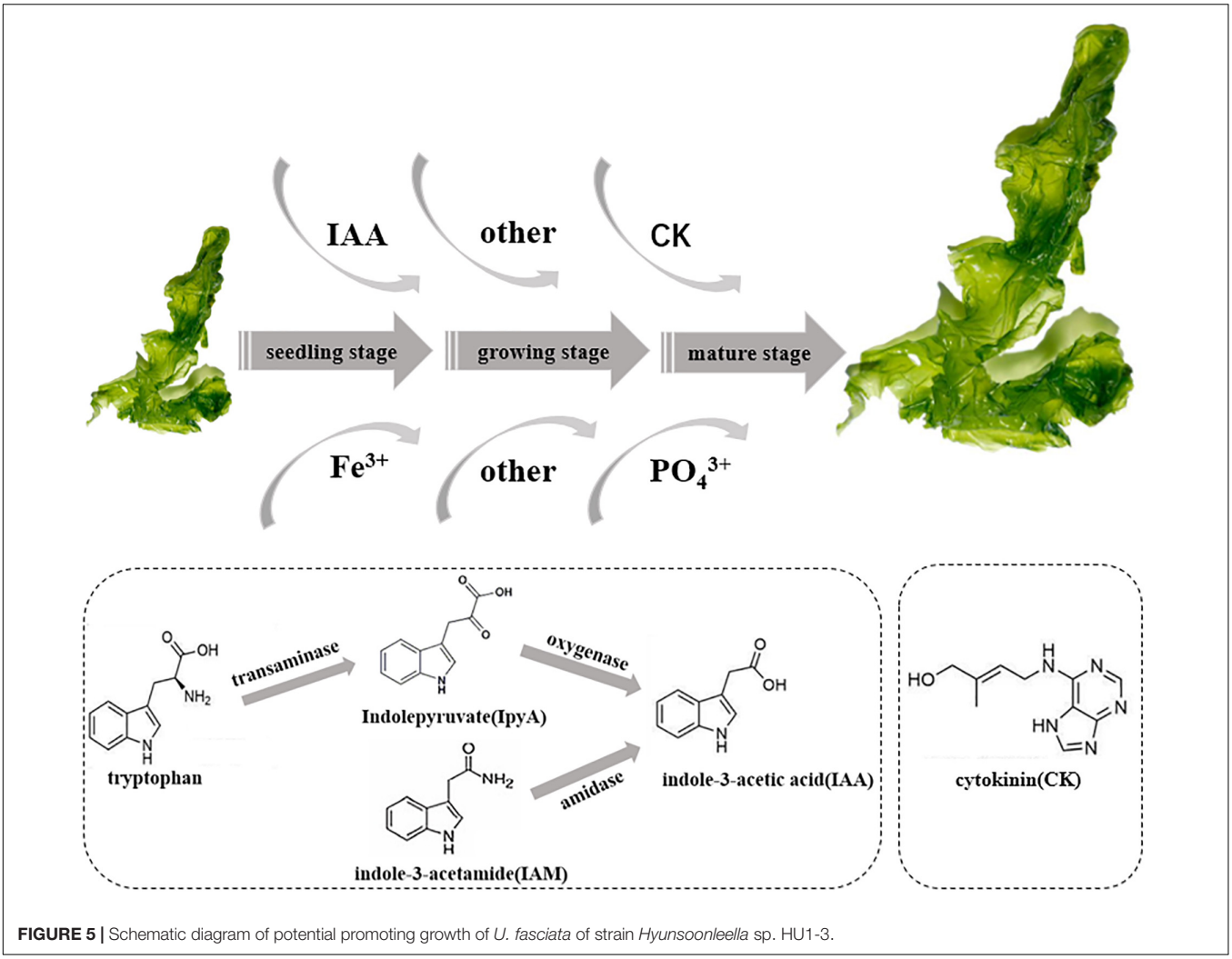


FIGURE 5 | Schematic diagram of potential promoting growth of *U. fasciata* of strain *Hyunsoonleella* sp. HU1-3.

of MS6 from *Maribacter* sp. strain restored the morphogenesis of *Ulva mutabilis*. MS6 is similar to auxin it induces cell wall formation and promotes the growth of basal stem cells. Many other studies also have shown that Rhodobacteraceae promote plant growth (Stevens et al., 2017; Abraham and Silambarasan, 2018; Kuhl et al., 2019; Vergani et al., 2019).

Ambient environmental parameters are another important factor affecting the abundance and diversity of microbial community on the surface of *U. fasciata*. McDonough and Watmough (2015) reported that epiphytic microbiota has a better positive correlation with environmental factors, such as temperature and pH. The authors also emphasized that the of the specificity, abundance and diversity indices of epiphytes were negatively correlated with the concentration of ambient nutrients (McDonough and Watmough, 2015). Moreover, Stratil et al. (2014) revealed that temperature, salinity and DO are the main environmental factors affecting the structure of microbial community. However, these findings contradicted our results and those of Pei et al. (2021), which showed that temperature, salinity and DO have no significantly effects on algal epiphytic microbiota. In this study, IC and TC had a great influence on the surface microbiota of *U. fasciata*. It also reported that algae are more associated with nutrients in the surrounding environment (Aires et al., 2016). Therefore, the authors conclude that the species of algal epiphytic bacteria in different environments are the result the synergistic effects of sampling methods, growth indexes of algae and environmental factors (Selvarajan et al., 2019).

The results of this study revealed that the growth promoting effect of strain HU1-3 (belonging to Flavobacteriaceae) was the best on *U. fasciata*. *B. cereus* U5-30 has the closest genetic

TABLE 3 | The production of IAA and the solubilization capacity of phosphate in the strain.

Stains	Without tryptophan (μg/ml)	With tryptophan (μg/ml)	Soluble phosphate (μg/ml)
Bacillus cereus U5-30	209.70 ± 3.64	375.16 ± 3.59	191.18 ± 2.87
Hyunsoonleella sp. HU1-3	227.64 ± 0.82	395.95 ± 3.11	213.98 ± 1.15

relationship with *Bacillus cereus* AR156, with 100% 16S rRNA gene sequence similarity. *B. cereus* AR156 was isolated from soil and is reported to promote plant growth (Jiang et al., 2017). In this study, *B. cereus* U5-30 can well promote the growth of *U. fasciata*, so it can be used as a positive control of bacterial treatment experiment. Compared with *B. cereus* U5-30, the biomass, phycocyanin, soluble sugar, soluble protein and growth rate of *U. fasciata* were greatly improved after co-culture with *Hyunsoonleella*, indicating that *Hyunsoonleella* sp. HU1-3 was a better growth promoter for *U. fasciata*. To our knowledge, this is the first study of *Hyunsoonleella* sp. as a growth promoter in plants.

The co-culture of different bacterial supernatants with *U. fasciata* (WSB) also indicated that *Hyunsoonleella* sp. HU1-3 increased the growth rate of *U. fasciata*. The genomic sequencing of *Hyunsoonleella* sp. HU1-3 revealed that the strain contained auxin related genes (an important phytohormone for the plant growth), involved in the production of IAA and CK, and several other genes encoding different enzymes (phosphate synthases genes and iron carrier enzymes genes). Plant growth depends on specific growth hormones, such as IAA and CK. These endogenous compounds present in plant tissues are considered to be signals to coordinate plant development (Kudo et al., 2010). This is consistent with previous reports that many Flavobacteriaceae produce auxin and play an important role in promoting plant growth (Elena et al., 2007; Kaul et al., 2018; Vukanti, 2020; Kalyanasundaram et al., 2021). Various phosphatases in the strain not only improve the phosphorus absorption capacity of plant, but also coordinate the transformation of insoluble, inorganic and organic forms of phosphorus into bioavailable forms of phosphate in the growing environment. Soluble phosphorus microorganisms can also improve the efficiency of nitrogen fixation, accelerate the accessibility of other trace elements, and promote the synthesis of iron chelating compounds (Kour et al., 2019). It is reported that inoculating different crops such as corn, tomato and mung bean with phosphorus-solubilizing bacteria can promote plant growth (Sandhya et al., 2010). The presence of genes encoding iron carrier and phosphatase further demonstrated that *Hyunsoonleella* sp. HU1-3 has the potential function of promoting the growth of *U. fasciata.* Further studies are highly recommended in the near future to determine which substances contribute to the growth of *U. fasciata* biomass.

Although *U. fasciata* grows in seawater, the microbial community on the surface of *U. fasciata* seems to be some specific algae-related communities, which are essentially different from the microbial community in the surrounding seawater (at the phylum level). This may be caused by different living environments, such as the nutrient concentration in seawater is relatively low, while the surface of algae emits organic carbon and nutrients (Pregnall, 1983). Previous studies have shown that although the community is variable, there is always a core population on the surface of the algae (Tujula et al., 2010). The results of this study also confirmed that the same bacteria were detected on the surface of *U. fasciata* samples at different growth stages, indicating that these bacteria can be colonized on the surface of algae as a core community. Besides, there were significant differences in epiphytic bacterial community in different *U. fasciata* samples, indicating the functional redundancy of *U. fasciata* epiphytic bacterial communities. This conclusion is consistent with the redundancy hypothesis (Naeem, 1998), which assumes that more than one species can play a specific role in an ecosystem, so as to endow the ecosystem with a certain degree of anti-disturbance ability. This hypothesis has also been confirmed to be applicable to laboratory microbial communities (Leflaive et al., 2008) and soil microbial communities (Yin et al., 2000; Persiani et al., 2008). Although functional redundancy can explain the variability of the epiphytic bacterial community of *U. fasciata*, it cannot explain the significant difference between this algae-associated community and the planktonic bacterial community living in the surrounding seawater. This suggests that there may be a selective mechanism that determines the environment in which bacterial communities exist.

The lottery hypothesis asserts that species with similar nutritional abilities will randomly replenish the ecosystem and occupy space (Sale, 1976). This hypothesis was proposed to explain the coexistence of corals and fish occupying the same ecological niche, but it is also consistent with the observations described in this study. It is assumed that various bacteria in algae-associated communities have the necessary metabolic capacity and colonize in the niches on the surface of algae. Due to functional redundancy, any bacterial species in the community will first colonized when they happen to encounter and occupy the surface of algae. This can explain the community variability between different *U. fasciata* samples. Bacterial species from the surface of the *U. fasciata* may not be able to adapt to plankton habitat in seawater, which is why the bacterial species found on the surface of *U. fasciata* are rarely detected in the surrounding seawater. The lottery hypothesis was conceived for dozens of species of fish that living in corals. It will be interesting to study whether this hypothesis also applies to communities containing thousands of bacterial species. It will also be of great interest to determine whether the isolated bacterial community is unique to the *U. fasciata* or is simply indicative of a surface-associated lifestyle.

CONCLUSION

In conclusion, a new bacterial species, *Hyunsoonleella* sp. HU1-3 was isolated from the surface of *U. fasciata*. This isolate significantly promoted the growth of *U. fasciata*. Genomic analysis revealed that the isolate process genes encoding the synthesis of growth hormones, phosphate synthases genes vitamins and iron carrier enzymes. The combination of high-throughput sequencing and bacterial treatment approaches helps to reveal the interaction between plants and microorganisms under the condition of reducing unknown variables.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW527409; https://www.ncbi.nlm.nih.gov/genbank/, JAEPJQ000000000; https://www.ncbi.nlm.nih.gov/genbank/, PRJNA766413.

AUTHOR CONTRIBUTIONS

HW and ZH designed the study, wrote the manuscript, and contributed to the data analysis. AE, YL, WL, QC, YX, and TP performed the experiments and data acquisition. HW, TP, YL, WL, and ZH contributed to the treatment of the raw data. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Major Project of Talent Team introduction for Guangdong Provincial Laboratory of Southern Marine Science and Engineering (Guangzhou, GML2019ZD0606), Innovation Team Project of Guangdong Normal University (2018KCXTD012), Shantou University Scientific Research Foundation for Talents (NTF19013), and Guangdong Natural Science Foundation General Project (2021A1515010803).

ACKNOWLEDGMENTS

We acknowledge Tran Ngoc Tuan, Shantou University for the work of polishing the article. We gratefully acknowledge the laboratory staff members for their remarkable support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.788709/full#supplementary-material

REFERENCES

Abraham, J., and Silambarasan, S. (2018). Biodegradation of carbendazim by *Rhodococcus erythropolis* and its plant growth-promoting traits. *Biol. Environ. Proc. R. Irish Acad.* 118B, 69–80. doi: 10.3318/bioe.2018.07

Aires, T., Serrão, E. A., and Engelen, A. H. (2016). Host and environmental specificity in bacterial communities associateds to two highly invasive marine species (genus *Asparagopsis*). *Front. Microbiol.* 7:559. doi: 10.3389/fmicb.2016.00559

Alsufyani, T., Califano, G., Deicke, M., Grueneberg, J., Weiss, A., Engelen, A. H., et al. (2020). Macroalgal–bacterial interactions: identification and role of Thallusin in morphogenesis of the seaweed *Ulva* (Chlorophyta). *J. Exp. Bot.* 71, 3340–3349. doi: 10.1093/jxb/eraa066

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Aust. Ecol.* 26, 32–46. doi: 10.1046/j.1442-9993.2001.01070.x

Barott, K. L., Rodriguez-Brito, B., Janouškovec, J., Marhaver, K. L., Smith, J. E., Keeling, P., et al. (2011). Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral *Montastraea annularis*. *Environ. Microbiol.* 13, 1192–1204. doi: 10.3389/fmars.2021.627724

Besemer, J., Lamsadze, A., and Borodovsky, M. (2001). GeneMarkS: a self- training method for prediction of gene starts in microbial genomes: implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res.* 29, 2607–2618. doi: 10.1093/nar/29.12.2607

Bondoso, J., Balagué, V., Gasol, J. M., and Lage, O. M. (2014). Community composition of the *Planctomycetes* associated with different macroalgae. *FEMS Microbiol. Ecol.* 88, 445–456. doi: 10.1111/1574-6941.12258

Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid *in situ* assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol.* 57, 535–538. doi: 10.1128/aem.57.2.535-538.199

Brown, J., Pirrung, M., and McCue, L. A. (2017). FQC Dashboard: integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool. *Bioinformatics* 33, 3137–3139. doi: 10.1093/bioinformatics/btx373

Burke, C., Thomas, T., Lewis, M., Steinberg, P., and Kjelleberg, S. (2011). Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. *ISME J.* 5, 590–600. doi: 10.1038/ismej.2010.164

Chaparro, J. M., Badri, D. V., and Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. *ISME J.* 8, 790–803. doi: 10.1038/ismej.2013.196

Chen, J., Li, H., Zhang, Z., He, C., Shi, Q., Jiao, N., et al. (2020). DOC dynamics and bacterial community succession during long-term degradation of *Ulva prolifera* and their implications for the legacy effect of green tides on refractory DOC pool in seawater. *Water Res.* 185:116268. doi: 10.1016/j.watres.2020.116268

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D. R., da Costa, M. S., et al. (2018). Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 68, 461–466. doi: 10.1099/ijsem.0.002516

De Souza, R. S. C., Okura, V. K., Armanhi, J. S., Jorrín, B., Lozano, N., da Silva, M. J., et al. (2016). Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci. Rep.* 6:28774. doi: 10.1038/srep28774

Dobretsov, S. V., and Qian, P. Y. (2002). Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro-and macrofouling. *Biofouling* 18, 217–228. doi: 10.1080/08927010290013026

Dombrowski, N., Schlaeppi, K., Agler, M. T., Hacquard, S., Kemen, E., Garrido-Oter, R., et al. (2016). Root microbiota dynamics of perennial *Arabis alpina* are dependent on soil residence time but independent of flowering time. *ISME J.* 11, 43–55. doi: 10.1038/ismej.2016.109

Doty, S. L., Sher, A. W., Fleck, N. D., Khorasani, M., Bumgarner, R. E., Khan, Z., et al. (2016). Variable nitrogen fixation in wild *Populus*. *PLoS One* 11:e0155979. doi: 10.1371/journal.pone.0155979

Droop, M. R. (2007). Vitamins, Phytoplankton and bacteria: symbiosis or scavenging? *J. Plankton Res.* 29, 107–113. doi: 10.1093/plankt/fbm009

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

El Shafay, S. M., Ali, S. S., and El-Sheekh, M. M. (2016). Antimicrobial activity of some seaweeds species from Red sea, against multidrug resistant bacteria. *Egypt. J. Aquat. Res.* 42, 65–74. doi: 10.1016/j.ejar.2015.11.006

Elena, T. A., Cherdyntseva, T. A., Botina, S. G., and Netrusov, A. I. (2007). Bacteria associated with orchid roots and microbial production of auxin. *Microbiol. Res.* 162, 69–76. doi: 10.1016/j.micres.2006.07.014

El-Said, G. F., and El-Sikaily, A. (2013). Chemical composition of some seaweed from Mediterranean Sea coast, Egypt. *Environ. Monit. Assess.* 185, 6089–6099. doi: 10.1007/s10661-012-3009-y

Galhardo, C. X., and Masini, J. C. (2000). Spectrophotometric determination of phosphate and silicate by sequential injection using molybdenum blue chemistry. *Anal. Chim. Acta* 417, 191–200. doi: 10.1016/S0003-2670(00)00933-8

Ghaderiardakani, F., Califano, G., Mohr, J. F., Abreu, M. H., Coates, J. C., and Wichard, T. (2019). Analysis of algal growth- and morphogenesis-promoting

factors in an integrated multi-trophic aquaculture system for farming *Ulva* spp. *Aquac. Environ. Interact.* 11, 375–391. doi: 10.3354/aei00319

Ghaderiardakani, F., Coates, J. C., and Wichard, T. (2017). Bacteria-induced morphogenesis of *Ulva intestinalis* and *Ulva mutabilis* (Chlorophyta): a contribution to the lottery theory. *FEMS Microbiol. Ecol.* 93:fix094. doi: 10.1093/femsec/fix094

Gianmaria, C., Kwantes, M., Abreu, M. H., Costa, R., and Wichard, T. (2020). Cultivating the macroalgal holobiont: effects of integrated multi-trophic aquaculture on the microbiome of *Ulva rigida* (Chlorophyta). *Front. Mar. Sci.* 7:52. doi: 10.3389/fmars.2020.00052

Gopal, M., and Gupta, A. (2016). Microbiome selection could spur next-generation plant breeding strategies. *Front. Microbiol.* 7:1971. doi: 10.3389/fmicb.2016.01971

Hovel, K. A., Warneke, A. M., Virtue-Hilborn, S. P., and Sanchez, A. E. (2016). Mesopredator foraging success in eelgrass (*Zostera marina* L.): relative effects of epiphytes, shoot density, and prey abundance. *J. Exp. Mar. Biol. Ecol.* 474, 142–147. doi: 10.1016/j.jembe.2015.10.014

Inaba, N., Kodama, I., Nagai, S., Shiraishi, T., Matsuno, K., Yamaguchi, A., et al. (2020). Distribution of harmful algal growth-limiting bacteria on artificially introduced *Ulva* and natural Macroalgal beds. *Appl. Sci.* 10:5658. doi: 10.3390/app10165658Song

Jiang, C. H., Chen, Y., Yan, F., Fan, Z. H., and Guo, J. H. (2017). Whole-genome sequence of *Bacillus cereus* AR156, a potential biocontrol agent with high soilborne disease biocontrol efficacy and plant growth promotion. *Genome Announc.* 5:e00886-17. doi: 10.1128/genomeA.00886-17

Juhmani, A.-S., Vezzi, A., Wahsha, M., Buosi, A., Pascale, F. D., Schiavon, R., et al. (2020). Diversity and dynamics of seaweed associated microbial communities inhabiting the lagoon of venice. *Microorganisms* 8:1657. doi: 10.3390/microorganisms8111657

Kalyanasundaram, T. G., Syed, N., and Subburamu, K. (2021). "Chapter 17 – Recent developments in plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture," in *Recent Developments in Applied Microbiology and Biochemistry*, ed. B. Viswanath (Cambridge, MA: Academic Press), 181–192. doi: 10.1016/B978-0-12-821406-0.00017-5

Kamo, T., Hiradate, S., Suzuki, K., Fujita, I., Yamaki, S., Yoneda, T., et al. (2018). Methylobamine, a UVA-absorbing compound from the plant-associated bacteria *Methylobacterium* sp. *Nat. Product Commun.* 13:1934578X1801300208. doi: 10.1177/1934578X1801300208

Karthick, P., and Mohanraju, R. (2018). Antimicrobial potential of epiphytic bacteria associated with seaweeds of little Andaman, India. *Front. Microbiol.* 9:611. doi: 10.3389/fmicb.2018.00611

Kaul, S., Gupta, S., Sharma, T., and Dhar, M. K. (2018). Unfolding the role of rhizomicrobiome toward sustainable agriculture. *Root Biol.* 52, 341–365. doi: 10.1016/j.gfs.2019.01.007

Kour, D., Rana, K. L., Yadav, N., Yadav, A. N., Kumar, A., Meena, V. S., et al. (2019). "Rhizospheric microbiomes: biodiversity, mechanisms of plant growth promotion, and biotechnological applications for sustainable agriculture," in *Plant Growth Promoting Rhizobacteria for Agricultural Sustainability: From Theory to Practices*, eds A. Kumar and V. S. Meena (Singapore: Springer), 19–65. doi: 10.1007/978-981-13-7553-8_2

Kudo, T., Kiba, T., and Sakakibara, H. (2010). Metabolism and long-distance translocation of cytokinins. *J. Integr. Plant Biol.* 52, 53–60. doi: 10.1111/j.1744-7909.2010.00898.x

Kuhl, T., Felder, M., Nussbaumer, T., Fischer, D., Kublik, S., Chowdhury, S. P., et al. (2019). Genome assembly of a plant-associated *Rhodococcus qingshengii* strain (RL1) isolated from *Eruca sativa* mill and showing plant growth-promoting properties. *Microbiol. Resour. Announc.* 8:e01106-19. doi: 10.1128/MRA.01106-19

Lachnit, T., Meske, D., Wahl, M., Harder, T., and Schmitz, R. (2011). Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. *Environ. Microbiol.* 13, 655–665. doi: 10.1111/j.1462-2920.2010.02371.x

Leflaive, J., Danger, M., Lacroix, G., Lyautey, E., Oumarou, C., and Ten-Hage, L. (2008). Nutrient effects on the genetic and functional diversity of aquatic bacterial communities. *FEMS Microbiol. Ecol.* 66, 379–390. doi: 10.1111/j.1574-6941.2008.00593.x

Li, W., Jaroszewski, L., and Godzik, A. (2002). Tolerating some redundancy significantly speeds up clustering of large protein databases. *Bioinformatics* 18, 77–82.

Liu, L., Trevathan-Tackett, S. M., Lewis, C. J. E., Huang, X., and Peter, I. (2020). Beach-cast seagrass wrack contributes substantially to global greenhouse gas emissions. *Macreadie Environ. Sci. Technol.* 54, 14750–14760. doi: 10.1016/j.jenvman.2018.10.047

Liu, Y. X., Qin, Y., Chen, T., Lu, M., Qian, X., Guo, X., et al. (2021). A practical guide to amplicon and metagenomic analysis of microbiome data. *Protein Cell* 12:315. doi: 10.1007/s13238-020-00724-8

McDonough, A. M., and Watmough, S. A. (2015). Impacts of nitrogen deposition on herbaceous ground flora and epiphytic foliose lichen species in southern Ontario hardwood forests. *Environ. Pollut.* 196, 78–88. doi: 10.1016/j.envpol.2014.09.013

Miranda, L. N., Hutchison, K., Grossman, A. R., and Brawley, S. H. (2013). Diversity and abundance of the bacterial community of the red Macroalga *Porphyra umbilicalis*: did bacterial farmers produce macroalgae? *PLoS One* 8:e58269. doi: 10.1371/journal.pone.0058269

Naeem, S. (1998). Species redundancy and ecosystem reliability. *Conserv. Biol.* 12, 39–45. doi: 10.1111/j.1523-1739.1998.96379.x

Nakanishi, K., Nishijima, M., Nishimura, M., Kuwano, K., and Saga, N. (1996). Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *J. Phycol.* 32, 479–482. doi: 10.1111/j.0022-3646.1996.00479.x

Paix, B., Carriot, N., Barry-Martinet, R., Greff, S., Misson, B., Briand, J. F., et al. (2020). A multi-omics analysis suggests links between the differentiated surface metabolome and epiphytic microbiota along the thallus of a Mediterranean seaweed holobiont. *Front. Microbiol.* 11:494. doi: 10.3389/fmicb.2020.00494

Pei, P., Aslam, M., Du, H., Liang, H., Wang, H., Liu, X., et al. (2021). Environmental factors shape the epiphytic bacterial communities of *Gracilariopsis lemaneiformis*. *Sci. Rep.* 11:8671. doi: 10.1038/s41598-021-87977-3

Persiani, A. M., Maggi, O., Montalvo, J., Casado, M. A., and Pineda, F. D. (2008). Mediterranean grassland soil fungi: patterns of biodiversity, functional redundancy and soil carbon storage. *Plant Biosyst.* 142, 111–119. doi: 10.1080/11263500701872713

Pregnall, A. M. (1983). Release of dissolved organic carbon from the estuarine intertidal macroalga *Enteromorpha prolifera*. *Mar. Biol.* 73, 37–42. doi: 10.1007/BF00396283

Provasoli, L., and Pintner, I. J. (1980). Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae) 1. *J. Phycol.* 16, 196–201. doi: 10.1111/j.1529-8817.1980.tb03019.x

Qu, T., Zhao, X., Hao, Y., Zhong, Y., Guan, C., Hou, C., et al. (2020). Ecological effects of *Ulva prolifera* green tide on bacterial community structure in Qingdao offshore environment. *Chemosphere* 244:125477. doi: 10.1016/j.chemosphere.2019.125477

Roth-Schulze, A. J., Pintado, J., Zozaya-Valdés, E., Cremades, J., Ruiz, P., Kjelleberg, S., et al. (2018). Functional biogeography and host specificity of bacterial communities associated with the Marine Green Alga *Ulva* spp. *Mol. Ecol.* 27, 1952–1965. doi: 10.1111/mec.14529

Sale, P. F. (1976). Reef fish lottery. *Nat. History* 85, 60–65.

Sandhya, V., Ali, S. Z., Grover, M., Reddy, G., and Venkateswarlu, B. (2010). Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. *Plant Growth Regul.* 62, 21–30. doi: 10.1007/s10725-010-9479-4

Schwenzfeier, A., Wierenga, P. A., and Gruppen, H. (2011). Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. *Bioresour. Technol.* 102, 9121–9127. doi: 10.1016/j.biortech.2011.07.046

Selvarajan, R., Sibanda, T., Venkatachalam, S., Ogola, H. J. O., Christopher Obieze, C., and Msagati, T. A. (2019). Distribution, interaction and functional profiles of epiphytic bacterial communities from the rocky intertidal seaweeds, South Africa. *Sci. Rep.* 9:19835. doi: 10.1038/s41598-019-56269-2

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Short, M. F., Staniewski, M. A., Short, C. M., Long, A. M., Chaban, Y. V., and Short, S. M. (2015). Isolation and characterization of a virus infecting the freshwater algae *Chrysochromulina parva*. *Virology* 486, 105–115. doi: 10.1016/j.virol.2015.09.005

Spoerner, M., Wichard, T., Bachhuber, T., Stratmann, J., and Oertel, W. (2012). Growth and thallus morphogenesis of *Ulva mutabilis* (Chlorophyta) depends on a combination of two bacterial species excreting regulatory factors. *J. Phycol.* 48, 1433–1447. doi: 10.1111/j.1529-8817.2012.01231.x

Stevens, V., Thijs, S., McAmmond, B., Langill, T., Van Hamme, J., Weyens, N., et al. (2017). Draft genome sequence of *Rhodococcus erythropolis* VSD3, a diesel fuel-degrading and plant growth-promoting bacterium isolated from *Hedera helix* leaves. *Genome Announc.* 5:e01680-16. doi: 10.1128/genomeA.01680-16

Stratil, S. B., Neulinger, S. C., Knecht, H., Friedrichs, A. K., and Wahl, M. (2014). Salinity affects compositional traits of epibacterial communities on the brown macroalga *Fucus vesiculosus*. *FEMS Microbiol. Ecol.* 88, 272–279. doi: 10.1111/1574-6941.12292

Tujula, N., Crocetti, G., Burke, C., Thomas, T., Holmström, C., and Kjelleberg, S. (2010). Variability and abundance of the epiphytic bacterial community associated with a green marine *Ulvacean alga*. *ISME J.* 4, 301–311. doi: 10.1038/ismej.2009.107

Urgun-Demirtas, M., Sattayatewa, C., and Pagilla, K. R. (2008). Bioavailability of dissolved organic nitrogen in treated effluents. *Water Environ. Res.* 80, 397–406. doi: 10.2175/106143007X221454

Vergani, L., Mapelli, F., Suman, J., Cajthaml, T., Uhlik, O., and Borin, S. (2019). Novel PCB-degrading *Rhodococcus* strains able to promote plant growth for assisted rhizoremediation of historically polluted soils. *PLoS One* 14:e0221253. doi: 10.1371/journal.pone.0221253

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840. doi: 10.1038/nrmicro2910

Vukanti, R. V. N. R. (2020). "Structure and function of rhizobiome," in *Plant Microbe Symbiosis*, eds A. Varma, S. Tripathi, and R. Prasad (Cham: Springer). doi: 10.1007/978-3-030-36248-5_13

Wahl, M., Goecke, F., Labes, A., Dobretsov, S., and Weinberger, F. (2012). The second skin: ecological role of epibiotic biofilms on marine organisms. *Front. Microbiol.* 3:292. doi: 10.3389/fmicb.2012.00292

Weiss, A., Costa, R., and Wichard, T. (2017). Morphogenesis of *Ulva mutabilis* (Chlorophyta) induced by *Maribacter* species (Bacteroidetes, Flavobacteriaceae). *Bot. Mar.* 60, 197–206. doi: 10.1515/bot-2016-0083

Wiese, J., Thiel, V., Nagel, K., Staufenberger, T., and Imhoff, J. F. (2009). Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. *Mar. Biotechnol.* 11, 287–300. doi: 10.1007/s10126-008-9143-4

Xu, J., Zhang, Y., Zhang, P., Trivedi, P., Riera, N., Wang, Y., et al. (2018). The structure and function of the global citrus rhizosphere microbiome. *Nat. Commun.* 9:4894. doi: 10.1038/s41467-018-07343-2

Yin, B., Crowley, D., Sparovek, G., De Melo, W. J., and Borneman, J. (2000). Bacterial functional redundancy along a soil reclamation gradient. *Appl. Environ. Microbiol.* 66, 4361–4365. doi: 10.1128/AEM.66.10.4361-4365.2000

Yong, Y. S., Yong, W. T., and Anton, A. (2013). Analysis of formulae for determination of seaweed growth rate. *J. Appl. Phycol.* 25, 1831–1834. doi: 10.1007/s10811-013-0022-7

Zarraonaindia, I., Owens, S. M., Weisenhorn, P. B., West, K., Hampton-Marcell, J. T., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6:e02527-14. doi: 10.1128/mBio.02527-14

Zhang, J., Zhang, N., Liu, Y. X., Zhang, X., Hu, B., Qin, Y., et al. (2018). Root microbiota shift in rice correlates with resident time in the field and developmental stage. *Sci. China Life Sci.* 61, 613–621. doi: 10.1007/s11427-018-9284-4

Zhang, Y., He, P., Li, H., Li, G., Liu, J., Jiao, F., et al. (2019). *Ulva prolifera* green-tide outbreaks and their environmental impact in the Yellow Sea. *China Natl. Sci. Rev.* 6, 825–838. doi: 10.1093/nsr/nwz026

Zhou, J., Bruns, M. A., and Tiedje, J. M. (1996). DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* 62, 316–322. doi: 10.1128/aem.62.2.316-322.1996

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 February 2022
doi: 10.3389/fmicb.2022.775002

Colonization and Interaction of Bacteria Associated With Chinese Chives Affected by Ecological Compartments and Growth Conditions

Nan Sun[1], Yuxin Wang[1], Jianhua Chen[2], Pingzhi Wang[1], Weitang Song[1], Peifang Ma[2], Yabin Duan[2], Ziyuan Jiao[2] and Yixiao Li[2]*

[1] College of Water Resources and Civil Engineering, China Agricultural University, Beijing, China, [2] Pingdingshan Academy of Agricultural Sciences, Pingdingshan, China

OPEN ACCESS

Edited by:
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Diptaraj Sangramsing Chaudhari, Mylab Discovery Solutions Pvt. Ltd., India

***Correspondence:**
Yuxin Wang
wangyuxin@cau.edu.cn

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *13 September 2021*
Accepted: *14 January 2022*
Published: *14 February 2022*

Citation:
Sun N, Wang Y, Chen J, Wang P, Song W, Ma P, Duan Y, Jiao Z and Li Y (2022) Colonization and Interaction of Bacteria Associated With Chinese Chives Affected by Ecological Compartments and Growth Conditions. Front. Microbiol. 13:775002. doi: 10.3389/fmicb.2022.775002

Chinese chive has a long history of planting in China. At present, there are many studies on endophytic bacteria and rhizosphere microorganisms of Chinese chive, but the effects of ecological compartment and growth conditions on bacterial communities in Chinese chives are unclear. Here, we aimed to elucidate the differences in bacterial a-diversity, β-diversity, community structure, core species differences, interaction networks and predicted metabolic functions among bacterial communities in different ecological compartments (the phylloplane, leaf endosphere, stem endosphere, root endosphere, and rhizosphere) in Chinese chives in an open field, a solar greenhouse, an arched shed, and a hydroponic system. Sixty samples were collected from these five ecological compartments under four growth conditions, and we compared the bacterial profiles of these groups using 16S rRNA sequencing. We evaluated the differences in diversity and composition among bacterial communities in these ecological compartments, analyzed the bacterial interaction patterns under the different growth conditions, and predicted the bacterial metabolic pathways in these ecological compartments and growth conditions. The results showed that the effects of ecological compartments on bacterial diversity, community composition, interaction network pattern, and functional expression of Chinese chives were greater than those of growth condition. Ecological compartments ($R^2 = 0.5292$) could better explain bacterial community division than growth conditions ($R^2 = 0.1056$). The microbial interaction networks and indicator bacteria in different ecological compartments showed that most of the bacteria that played the role of key nodes (OTUs) in each ecological compartment were bacteria with high relative abundance in the compartment. However, the bacteria that played the role of key nodes (OTUs) in bulbs were not Proteobacteria with the highest relative abundance in the compartment, but Actinobacteria that were significantly enriched in the root endosphere and rhizosphere ecological compartments. In addition, interactions among bacteria were interrupted in the hydroponic system, and specific bacterial communities and interaction

patterns in Chinese chives varied among growth conditions. Prediction of metabolic functions indicated that plant metabolic activity related to stress responses and induction of system resistance was greater in belowground ecological compartments.

Keywords: Chinese chive, endophyte, ecological compartment, growth condition, colonization, interaction

1. INTRODUCTION

Chinese chives (*Allium tuberosum* Rottl. ex Spreng.) is a perennial plant species in which the leaves are flat and long, succulent and pungent, and the flowers are mostly white. Chinese chives are rich in nutrients, containing iron, zinc, and other trace elements (Wang et al., 2017a), and high in calcium content, which can effectively prevent iron deficiency anaemia. Glutamic acid, proline, and alanine are abundant in Chinese chive leaves (J.Y. et al., 2021), so this plant is a high-quality amino acid provider. The leaves of Chinese chives also contain high levels of total dietary fiber and insoluble dietary fiber, which can effectively promote gastrointestinal peristalsis (Dai and Chau, 2017) and thus reduce the risk of colorectal cancer (Wu et al., 2019). In recent years, the medicinal value of Chinese chives has attracted increased attention from scholars in various countries. The content of unsaturated fatty acids in Chinese chive seeds is approximately 85–90%, mainly in the form of the polyunsaturated fatty acid linoleic acid (Sun et al., 2014a), which can reduce levels of cholesterol and triglycerides in the human body and help regulate the concentration of blood lipids after eating (Wang et al., 2014). Chinese chives have long been cultivated in China and are the most widely distributed among all vegetables, and even on the Qinghai-Tibet Plateau, considerable area is planted in Chinese chives. Therefore, research on the diversity of endophytic bacteria in this plant is highly applicable to agricultural practice.

Endophytes are microorganisms (mainly fungi and bacteria) that live inside healthy plants during almost all life cycle stages without harming the host plants (Yang and Cao, 2016). Endophytes are present in numerous tissues and organs in plants and constitute a natural component of plants. In recent years, endophytes (Jagannath et al., 2019) isolated from bulbs of Liliaceae have been found to synthesize abundant growth promotion hormones (Khan et al., 2020), such as indole acetic acid, that can promote plant growth (Lang, 2018). A highly significant difference in chlorophyll content has been found in maize infected by endophytic bacteria, which indicates that endophytic bacteria can effectively (Emami et al., 2020) stimulate the synthesis of chlorophyll in plants, thereby promoting photosynthesis and growth. Endophytic fungi and bacteria also increase phosphorus efficiency and the N-use efficiency index (Da Silveira et al., 2019) in host plants, and some endophytic bacteria planted on the leaves of host plants can inhibit the infection and growth of pathogens in plants by secreting specific metabolites to form competitive advantages (Moin et al., 2020; Chaouachi et al., 2021). Therefore, microbial endophytes should be further studied for their potential in plant control disease.

Previous studies have found that intercropping cabbage and Chinese chives crops can enhance their quality by increasing the content of vitamin c and soluble sugar in cabbage (Ping, 2020), and reduce the incidence of diseases (Xu et al., 2016) such as cabbage soft rot (Li et al., 2020). At the same time, Chinese chives intercropping with other crops can effectively improve the activity of urease and phosphatase in soil (Meng ZiLi, 2018). Researchers have speculated that Chinese chives can also inhibit the occurrence of soft rot. Further studies have shown that extracts of Chinese chives plants can significantly reduce decay from bacterial soft rot resulting from infection by *Erwinia carotovora* (Ndivo et al., 2018) or *Pectobacterium carotovorum* (Simeon and Abubakar, 2014). Moreover, rotation of Chinese chives with other crops affects crop soil microbial quantity, species, community structure, and diversity (Gu et al., 2020). Therefore, it is speculated that Chinese chives contain endophytic bacteria or associate with rhizospheric microorganisms that can inhibit pathogens. In the microbial analysis of various ecological compartments of other plants, it can be found that ecological compartments have effects on microbial composition and microbial community function (Wei et al., 2021). However, the current studies on plant ecological compartments are mostly between underground compartments (especially in the rhizosphere and root surface), and the composition differences of soil microorganisms, rhizosphere microorganisms, and root endosphere microorganisms and the screening mechanism of roots are studied (Reinhold Hurek et al., 2015). At present, the articles on the effects of ecological compartments on plant microorganisms mainly focus on one of the studies on the differences in microbial community composition, interaction network, and function among ecological compartments. There are few articles that comprehensively analyze the differences among ecological compartments of plants. Similar to the decrease of microbial diversity from rhizosphere to root endosphere (Edwards et al., 2015) and significant differences in microbial composition in underground compartments, we believe that different ecological compartments have effects on bacterial community composition and function of Chinese chives. Previous studies have shown that different facilities will affect the soluble sugar content, vitamin c content and propylene glycol content of plants (Sun et al., 2014b; Chao and Yingcui, 2019), thereby affecting the quality. In our cultivation base, due to the lack of heat preservation wall on the north side, the air temperature and relative humidity in the arched shed are quite different from those in the solar greenhouse (**Supplementary Table S1**). Therefore, we believe that the large-scale growth condition compared with the small-scale ecological compartment also affects the community distribution and interaction of phylloplane, rhizosphere, and endophytic bacteria in Chinese chives.

In this study, the 16S rRNA V5-V6 gene sequences of Chinese chives bacteria were sequenced using Illumina-MiSeq to compare

the differences in bacterial diversity, community composition, interaction network pattern, and functional expression of phylloplane, rhizosphere, and endophytic bacteria of Chinese chives in different ecological compartments and growth conditions. Filling the current research gap on endophytes in Chinese chives is helpful for us to analyze the abundance changes of differential microbiota at the level of bacterial distribution, and provide a theoretical basis for the subsequent isolation of endophytes in Chinese chives.

2. METHODS

2.1. Sample Plots and Methods

Sampling was conducted in an open field, a solar greenhouse, an arched shed (**Supplementary Table S1**) and a hydroponic system under similar production management at Lizhuang Planting Base (33°66′N, 113°26′E) of Pingdingshan Academy of Agricultural Sciences, Pingdingshan, Henan Province, China, and the area planted in Chinese chives in the solar greenhouse, arched shed and field exceeded 600 m^2. The Chinese chives in the field was planted in spring, and the Chinese chives in greenhouse and arch shed was planted in winter. All Chinese chives in the three cultivation environments were cultivated in soil. Fertilization management: a compound water-soluble fertilizer (N: P2O5:K2O=20%: 20%: 20%, 375–450 kg/ha) was used as the base fertilizer, applied once in spring and twice in autumn. Nutrient components in hydroponics: Ca(NO3)2.4(H2O) 425 mg/kg, KNO3 668 mg/kg, KH2PO4 200 mg/kg, (NH4)2SO4 380 mg/kg, K2SO4 116 mg/kg, MgSO4 185 mg/kg. In December 2019, a total of 60 samples (5*4*3) were collected in each growth condition, including 3 duplicate plant samples (complete Chinese chives containing leaves, roots, and stems), 3 rhizosphere soil samples (3 water samples near the roots were collected in a sterile test tube in a hydroponic environment) and 3 phylloplane microbial samples (wiped with sterile cotton swabs). Sampling of plants followed this protocol: healthy Chinese chives about 1 year of age and with growth consistent with surrounding Chinese chives were selected from each growth condition, which were planted homogeneously in Chinese chives, and the sampling distances within each growth condition exceeded 100 m. The rhizospheric soil sampling followed the following (Courchesne and Gobran, 1997) method: healthy roots were excavated along the base of each sampled plant, loose soil attached to the root was gently shaken off, leaving the soil closely bound to the root (about 1 mm attached to the root), then the root samples were sealed in sterile plastic bags, samples were transferred to a car refrigerator and transported to the laboratory, and the rhizospheric samples from the hydroponic environment were obtained by absorbing water samples near the roots (Chi et al., 2012).

2.2. Study Subjects and Biopsy Collection

In this study, rhizospheric soil samples from the same growth condition were mixed evenly, then the animal and plant residues visible in the soil were removed, and finally combined samples were screened with a 0.2-mm sieve. The collected Chinese chive samples were rinsed with tap water and then rinsed in distilled water three times (Zhu et al., 2020). This was done to fully remove materials attached to leaves, bulbs and roots. Then, clean Chinese chive leaves, bulbs, and roots were placed in 50-mL Falcon centrifuge tubes each containing 25 mL PBS buffer, shaken at 180 r/min for 15 min, and cleaned a total of three times. Samples were then dried by sterile filter paper, and impurities in the samples were removed by sterile tweezers. Finally, Chinese chive leaves, bulbs, roots, and corresponding soil were stored at −80°C. These samples from different ecological compartments were used to extract plant and soil microbial genomic DNA, and high-throughput sequencing (HTS) was performed.

2.3. DNA Extraction

First, the frozen samples were homogenized with an MP Fastprep-24 5G (MP Biomedicals, USA) at 7200 r/min 4 times for 30 s each time. Then, according to the manufacturer's instructions, the DNA of bacteria in Chinese chive leaves, bulbs, and roots, soil and water samples was extracted by the FastDNA®SPIN Kit for Soil (MP Biomedicals, USA). Finally, the quality and concentration of DNA were determined by NanoDrop 2000 UV-Vis spectrophotometry (Thermo Scientific, USA), and DNA fragments were separated by 1% agarose gel electrophoresis. High-quality DNA templates were diluted to 10 ng/μl for subsequent PCR of 16S rRNA.

2.4. Bacterial 16s rRNA Amplification and Sequencing

Nested PCR (Xia et al., 2018) was used in this study. Degenerate PCR primers 799F-1392R with a barcode added at the 5′ ends to distinguish different samples were used in the first round. Each PCR amplification system (20 μL) contained 5 × Fast Pfu Buffer (4μL), 2.5 mmol/L dNTPs (2 μL 5 μmol/L forward primer (0.8 μL), 5 μmol/L reverse primer (0.8 μL), FastPfu DNA Polymerase (0.4 μL), BSA (0.2 μL), template DNA (10 ng), and sufficient ddH2O to bring the volume to 20 μL. The PCR amplification procedure was as follows: predenaturation at 95°C for 3 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s); followed by a final extension at 72°C for 10 min in the analyzer (ABI GeneAmp® 9700, USA). For the second round of PCR, primers were 799F-1193R with a barcode added at the 5′ ends to distinguish different samples for amplifying the V5-V7 hypervariable regions of the bacterial 16S rRNA gene (Wang et al., 2020c), and the total volume of each PCR amplification system was also 20 μL. The PCR amplification procedure in the second round was as follows: predenaturation at 95°C for 3 min; 13 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; followed by final extension at 72°C for 10 min.

Three PCR amplifications were performed per sample, and the PCR products were detected by 2% agarose gel electrophoresis. The target length segment (∼400 bp) was cut from each gel with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA), eluted with Tris-HCl, and detected by 2% agarose gel electrophoresis. Then, the PCR products were detected and quantified by a Quantus.Fluorometer (Promega, USA). Finally, a library was constructed with the NEXTflex® Rapid

TABLE 1 | Effective sequence number in different ecological compartments.

	Phylloplane	Leaf endosphere	Stem endosphere	Root endosphere	Rhizosphere
Total sequences	529727	652761	610660	838167	788626
Effective sequences	489118	618959	581622	475384	334513
Effective rate	92.33%	94.82%	95.24%	56.42%	42.42%

DNA-Seq Kit, and the purified PCR products were sequenced (2 × 300 nt) on the Illumina MiSeq platform (Illumina, San Diego, USA).

2.5. Bacterial 16s rRNA Sequence Analysis

After constructing an HTS reference dataset, data were analyzed by Wang et al. (2018) fastp software and FLASH software (http://www.cbcb.umd.edu/software/flash, version 1.2.7). The process is described below. First, the barcode was removed from the sequences, and quality control and splicing of sequencing sequences at both ends were performed. Each sample was distinguished according to the barcode sequence, and then the chimera was identified and removed to obtain the optimized sequence. Chimera recognition was based on the SILVA database, and then with this database as the reference sequence, the nucleotide sequences with lengths greater than 250 bp were classified into operational taxonomic units (OTUs) with 97% similarity by UPARSE software (http://drive5.com/uparse/) and used for analysis of relative abundances of bacterial taxa in different growth condition samples.

Finally, the sequence data were compared with the Silva (Release.138) 16S rRNA gene database. The algorithm applied was Rdp Classifier (http://rdp.cme.msu.edu), and the confidence threshold was set to 70% (Amato et al., 2013) to determine the taxonomic status of microorganisms corresponding to sequences.

2.6. Statistical Analysis

The Kruskal-Wallis H test and Tukeys HSD test were used to evaluate differences in alpha index values (Tan et al., 2021), and bar charts were used to show the relative abundances of phyla and genera in different ecological compartments and different growth conditions. The top 50 bacterial OTUs were selected for each ecological compartment, and IQ-Tree software was utilized to construct phylogenetic trees (Katoh et al., 2002; Price, 2009). Then, the Interactive Tree of Life (ITOL) (Letunic and Bork, 2007) website was used for visualization. Based on the Bray-Curtis method, a principal coordinate analysis (PCOA) (Lozupone and Knight, 2005) map was drawn by using the relative abundances of OTUs in different ecological compartments and growth conditions.

Based on OTUs, a Venn diagram was drawn for analysis of group-specific bacterial microbiota. Bubble diagrams were used to show indicator species in each ecological compartment. The abundance of the bacteria at the genus level was analyzed by using QIIME2 and the vegan package in R software. The Kruskal-Wallis test and Wilcoxon rank sum test (between two groups) were used to evaluate the intergroup significant differences at the phylum and genus levels based on quadrat community abundance data. P values were corrected using the false discovery rate (FDR) (Liu et al., 2007), and Tukey-Kramer was used for multiple comparisons. Average values of samples within groups were calculated, analyzed, and visualized by STAMP and R software (R 4.0.3 statistical package).

To examine the microbial interaction patterns under different growth conditions, the Spearman method was used to analyze correlations between bacterial abundances at the genus level. Coefficients of correlation between bacterial abundances were calculated by the psych package of R. Cytoscape 3.8.2 was released to visualize the patterns of microbial interaction networks, showing only significant correlations ($P < 0.05$). MENA was used to analyze the microbial interaction network between different ecological compartments, and the OTU of more than 6 times in 12 samples was analyzed. Randomize the network structure and then calculate network properties in MENA. Visual analysis was performed by Gephi 0.9.2. Within-module connectivity (Zi) and between-module connectivity (Pi) are used for key nodes (OTUs) analysis.

Tax4Fun software was used to predict gene functions from 16s rRNA data based on the Silva database to obtain the metabolic pathway composition of microorganisms in each ecological compartment and growth condition, and Statistical Analysis of Metagenomic Profiles (STAMP 2.1.3) and SPSS Statistics (SPSS 25) were used for statistical analysis and mapping. STAMP was used for pairwise comparisons of ecological compartments and growth conditions, and Tukey-Kramer was used to adjust for multiple comparisons. The effect size for differences in proportions between groups was greater than 0.05.

3. RESULTS

3.1. Sequencing Results for Microorganisms in Chinese Chives

A total of 3,419,941 reads with an average length of 377 bp were obtained by 16S rRNA high-throughput sequencing analysis of bacterial communities from 60 samples of Chinese chive plants and rhizospheres. Among them, a total of 2,499,596 effective sequences (effective rate 73.09%) were obtained, and the original sequences were then grouped and filtered by barcode tag sequence (**Table 1**). When samples were grouped by growth condition, 607,947 optimal reads (effective rate 68.69%), 639,087 reads (effective rate 71.54 %), 716,750 reads (effective rate 74.77%), and 535,812 reads (effective rate 78.46%) were

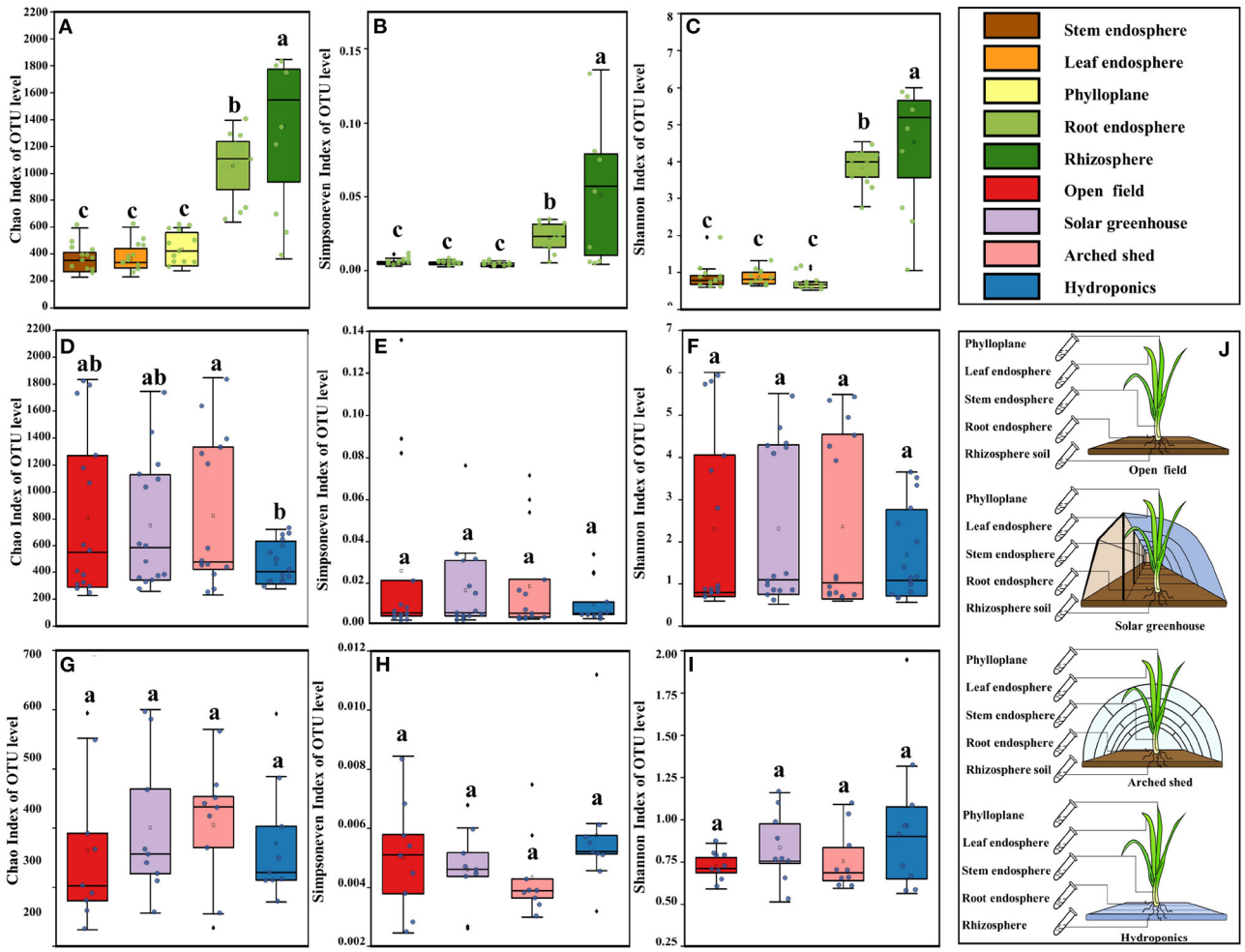


FIGURE 1 | Bacterial diversity in the different ecological compartments and growth conditions. The bacterial alpha diversity in the different ecological compartments: **(A)** Chao index, **(B)** Simpson evenness index, **(C)** Shannon index. Bacterial alpha diversity in the different growth conditions: **(D)** Chao index, **(E)** Simpson evenness index, **(F)** Shannon index. Bacterial alpha diversity in aboveground ecological compartment in different growth conditions: **(G)** Chao index, **(H)** Simpson evenness index, **(I)** Shannon index, **(J)**. Diagram of sampling in the different ecological compartments and growth conditions. The horizontal bars within boxes represent medians. The tops and bottoms of boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to 1.5 × the interquartile range from the upper edge and lower edge of the box, respectively. Different letters indicate differences between groups ($P < 0.05$, ANOVA, Tukey-HSD test). The numbers of replicated samples in this figure are as follows: in different ecological compartments, $n = 12$, in different growth conditions, $n = 15$, root and rhizosphere in different growth conditions, $n = 6$.

found from the open field, solar greenhouse, arched shed, and hydroponic system.

The number of sequences was then standardized to 20,112 (minimum number of sequences among samples) for each sample, and 3,835 OTUs were clustered with 97% similarity, with a coverage index above 98.8%. Rarefaction curves (**Figure 1**) showed that the number of species detected from 10,000 sequences tended to be saturated, indicating that most microbial populations were covered in the sequencing results, which could thus accurately reflect the community structure of endophytic and rhizospheric microorganisms. The data described above showed that this method was suitable for the prediction and analysis of the composition and functions of bacterial populations associated with Chinese chives in different ecological compartments and growth conditions.

3.2. Differences in the Diversity of Bacterial Communities Associated With Chinese Chives Among Ecological Compartments and Growth Conditions

3.2.1. Differences in Alpha Diversity of Bacterial Communities Associated With Chinese Chives in Different Ecological Compartments and Growth Conditions

The alpha diversity of bacteria in the phylloplane, leaf endosphere, stem endosphere, root endosphere, and rhizosphere

was analyzed with the Chao index, Shannon index, and Simpson evenness index (**Figures 1A–C**). Compared with the belowground compartments (root endosphere and rhizosphere), diversity was low in the aboveground compartments (phylloplane, leaf endosphere, and stem endosphere), the richness and evenness were poor, and there was no significant variation among the three compartments, indicating that the diversity of bacterial communities in the aboveground compartments was uniform. Bacterial diversity was significantly higher in root endosphere and rhizosphere than in the aboveground compartments, and the richness and evenness were also significantly different ($P < 0.001$).

In the analysis of alpha diversity of bacteria in the open field, solar greenhouse, arched shed and hydroponic system, Chao, Shannon and Simpson evenness indexes were also included (**Figures 1D–F**). The bacterial diversity index of each growth condition was high, and the evenness was low, but it was not significantly different. The richness index of bacteria in hydroponic environment was significantly lower than that in arched shed ($P < 0.05$). These results showed that ecological compartment and growth condition had significant indigenous effects on the alpha diversity index of microbial groups, and confirmed that the effect of ecological compartment on bacterial community was higher than that of growth condition on bacterial community. Through the analysis of bacterial diversity in different ecological compartments and growth conditions, there are significant differences between underground ecological compartments in different growth conditions due to different cultivation substrates. We are more curious about whether the growth condition has an impact on the aboveground ecological compartment. Therefore, we analyzed the differences in alpha diversity of microbial bacterial communities in the aboveground ecological compartments of Chinese chives among growth conditions (**Figures 1G–I**). Differences in bacterial alpha diversity between aboveground ecological compartments but not significant in different growth conditions ($P > 0.05$). In the hydroponic environment, the bacterial richness and diversity in the aboveground ecological compartments were high but the evenness was low. The bacterial diversity and richness in the aboveground ecological compartments in the solar greenhouse environment were higher than those in the arched shed environment, but the evenness was lower than that in the arched shed environment. Therefore, although the effect of growth condition on the bacterial diversity of aboveground ecological compartments was not significant, there were some effects.

3.2.2. Differences in Beta Diversity of Bacterial Communities in Different Niches and Growth Conditions

Analysis of beta diversity with PCoA showed separate clustering of the root endosphere and rhizosphere groups (Adonis test. $P = 0.001$) (**Figure 2G**). We further analyzed the bacterial content in the root endosphere and rhizosphere. Diversity, richness and evenness of rhizospheric bacteria were significantly higher than for those in roots ($P < 0.05$). Additionally, bacterial beta diversity showed a specific clustering among root endosphere, rhizosphere, and aboveground compartment (stem endosphere, leaf endosphere, phylloplane) samples (Adonis test, $P = 0.001$). This indicates significant differences in bacterial composition among the root endosphere, rhizosphere, and other ecological compartments.

3.3. Composition of Bacterial Communities Associated With Chinese Chives in Different Ecological Compartments and Growth Conditions

3.3.1. Differences in Endophytic Bacterial Components in Different Ecological Compartments of Chinese Chives

To study the effect of ecological compartment on bacterial community structure, we analyzed the group differences among different ecological compartments and compared the bacterial communities of the phylloplane, leaf endosphere, stem endosphere, root endosphere, and rhizosphere, including comparisons at the phylum and genus level. The differences in abundance among Bacteroidetes, Firmicutes, Chloroflexi, Gemmatimonadetes, Proteobacteria, Actinobacteria, and Acidobacteria were extremely significant ($P < 0.001$) at the phylum level (**Figure 2F**), while at the genus level, the abundance differences among *Pseudomonas*, *Ralstonia*, *Lechevalieria*, *Rhodanobacter*, *Rhodococcus*, *Bacillus*, and *Flavobacterium* were extremely significant ($P < 0.001$).

Consistent with findings of previous studies, Proteobacteria was the dominant phylum among the identified OTUs, and among the five ecological compartments, Proteobacteria was most dominant in the leaf endosphere (95.35% relative abundance) and least abundant in the rhizosphere (51.00%) but was still the most dominant phylum in the rhizosphere. Actinobacteria ranked second in relative abundance, with the highest distribution in root endosphere (32.46%) and the lowest distribution in stem endosphere (29.40%). *Pseudomonas* was the most important genus of bacteria among the identified OTUs (**Figures 2A,E**). *Pseudomonas* was most dominant in the phylloplane, accounting for 90.04% of bacterial genera, accounted for over 85% in the leaf endosphere and stem endosphere, and was least abundant in the root endosphere, accounting for 10.47%. Richness values of aboveground ecological compartments were lower than those of belowground ecological compartments: a total of 716 bacterial genera were detected in the rhizosphere, which exceeded by 100 those in the aboveground compartments. At the genus level, the community composition in aboveground compartments (phylloplane, leaf endosphere, and stem endosphere) was significantly different from that of belowground compartments, and the abundances of *Lechevalieria*, *Rhodanobacter*, *Flavobacterium*, and *Burkholderia* − *Caballeronia* − *Paraburkholderia* in belowground compartments were higher than values in aboveground compartments (**Figure 2B**). At the phylum level, the abundances of Proteobacteria (59.26%), Actinobacteria (32.46%), and Bacteroidetes (4.93%) in the root endosphere were higher than those in the rhizosphere group. At the genus level, differences between the rhizosphere and root endosphere were small, but *Lechevalieria* (9.10%), *Rhodanobacter* (5.14 %), and *Flavobacterium* (4.32%) were more abundant in the root endosphere than in the rhizosphere.

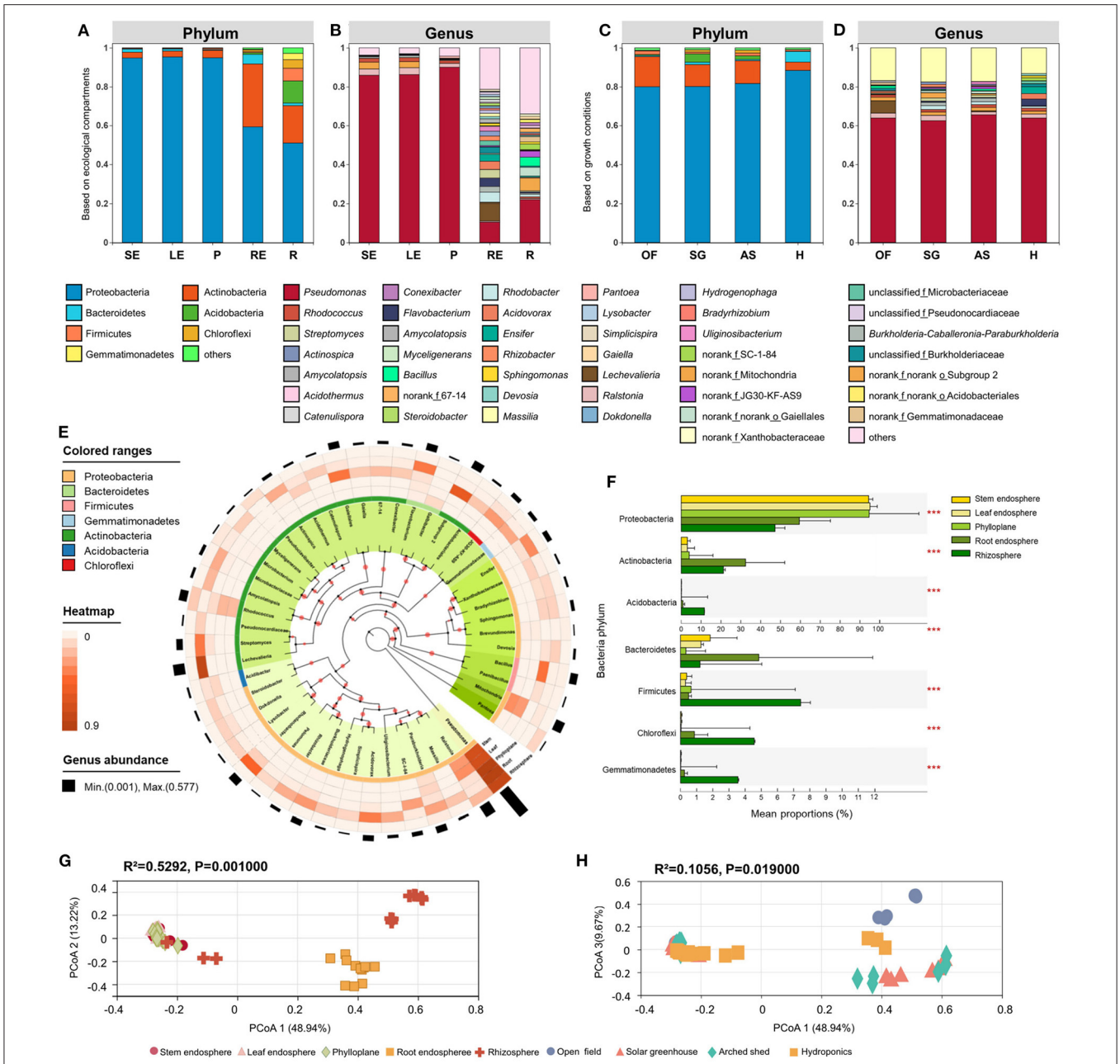


FIGURE 2 | Bacterial community composition in different ecological compartments and growth conditions. The bacterial composition at the phylum **(A)** and genus **(B)** levels in different ecological compartments. SE, stem endosphere; LE, leaf endosphere; P, phylloplane; RE, root endosphere; R, rhizosphere. The bacterial composition at phylum **(C)** and genus **(D)** levels in different growth conditions. OF, open field; SG, solar greenhouse; AS, arched shed; H, hydroponics. **(E)** Bacterial phylogenetic tree. We selected the top 50 bacteria with relative abundance to construct the system tree. Visualization was performed on the iTOL website (https://itol.embl.de/). The colors in the inner circle represents the specific bacterial phyla. Four outer circles represent four different groups, and the height shows the abundance of genera. **(F)** Significant differences in bacterial phyla among different growth conditions. Bacterial beta diversity PCoA based on Bray-Curtis distance in ecological compartments, **(G)** and different growth conditions, **(H)**. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

3.3.2. Differences in Phylloplane, Rhizosphere and Endophytic Bacterial Components in Chinese Chives Under Different Growth Conditions

Proteobacteria was the main phylum under all growth conditions. Relative abundance of Proteobacteria varied little among growth conditions; the highest percentage was 88.42% in the hydroponic environment, and the lowest was 80.2% in the field. Actinobacteria was the second most abundant bacterial phylum, but its distribution pattern was opposite to that of Proteobacteria. Actinobacteria was least abundant in

hydroponics (4.26%) and most abundant in the field (15.48%). At the phylum level, Acidobacteria and Bacteroidetes differed significantly in relative abundance among groups ($P < 0.001$). Compared with the three groups with soil as the cultivation substrate, the relative abundances of Proteobacteria (88.42%) and Bacteroidetes (5.40%) in the hydroponic group were significantly elevated, but the relative abundance of Actinobacteria (4.26%) was significantly lower (**Figure 2C**).

At the genus level, the differences in abundance of *Lechevalieria*, *Burkholderia* − *Caballeronia* − *Paraburkholderia*, *Streptomyces*, and *Ensifer* among groups were extremely significant ($P < 0.001$). *Pseudomonas* was the dominant genus among these, with the greatest abundance in the arched shed (65.45%) and the lowest abundance in the solar greenhouse (62.41%). The relative abundance of *Burkholderia* − *Caballeronia* − *Paraburkholderia* in solar greenhouse and arched shed was significantly higher than that in open field and hydroponic environments. The abundance of *Lechevalieria* (6.37%) in the open field was higher than numbers under the other three conditions, but the abundance of *Rhodanobacter* (0.05%) was significantly reduced. The abundances of *Flavobacterium* (3.61%), *Acidovorax* (2.83%), *Ensifer* (3.48%), and *Rhizobacter* (1.44%) were significantly higher in the hydroponics (**Figure 2D**).

3.4. Specific Bacterial Communities and Differences in Bacterial Communities in Different Ecological Compartments and Growth Conditions

First, specific bacterial communities were analyzed in different ecological compartments. There were 4,012 OTUs in different compartments and 502 OTUs shared by all groups, but different ecological compartments also featured unique bacterial communities. A total of 762 OTUs existed only in the rhizosphere, 209 OTUs existed only in roots, and the number of unique OTUs in the stem was the lowest at 83. There were 848 OTUs in the root endosphere and rhizosphere. In addition, although the numbers of OTUs in the phylloplane, leaf endosphere, and stem endosphere were similar, there were only 43 shared OTUs, so bacterial colonization was not uniform among aboveground ecological compartments. These data indicate that different ecological compartments have a greater impact on OTUs (**Figure 3A**).

We further analyzed the group-specific bacterial genera in each ecological compartment and growth condition and determined the indicator species in each ecological compartment, namely, the bacterial genera that were significantly enriched in one ecological compartment or growth condition and not present in other groups (**Figure 3B**). We found that *Flavobacterium*, *Rhodanobacter*, *Streptomyces*, *Acidovorax*, *Amycolatopsis*, *Lechevalieria*, *Uliginosibacterium*, and *Ensifer* were enriched in the root endosphere, and *Lechevalieria* could be used as a better indicator of root endosphere ecological communities. *Bacillus*, *Gaiella*, *Conexibacter*, *Ellin*6067, and *MND*1 were enriched in the rhizosphere, and *Bacillus* can be used as a better indicator for the identification of rhizospheric bacterial communities. *Conexibacter* and *Cloacibacterium* were abundant in the stem endosphere and could be used as indicator bacteria for stem endosphere ecological communities. *Lysinimicrobium*, *Pantoea*, and *Weissella* were enriched in phylloplane and can be used as indicators to identify bacterial genera in phylloplane ecological communities. *Bacteroides* only enriched in the leaves can be used as indicator bacteria to identify the ecological compartments on the leaf endosphere. Compared with the above ecological compartments, there were fewer bacteria enriched in the leaf endosphere because most of the bacteria enriched in the leaf endosphere were also enriched in the stem endosphere.

In addition, to characterize bacteria in ecological compartments where external bacterial exchange is more frequent, we compared the bacterial genera in the phylloplane, root endosphere, and rhizosphere under different growth conditions. We found that *Ralstonia* and *Rhodococcus* were significantly more numerous in the hydroponic environment (**Figure 3C**). *Pseudomonas* was more likely to colonize the root endosphere in the arched shed environment, while *Lechevalieria* in the root endosphere was significantly more abundant in the open field environment (**Figure 3D**). Because the hydroponic environment is different from the cultivation substrates in the other three environments, the bacterial differences in the rhizosphere are mainly reflected in the hydroponic environment. Pseudomonas in the rhizosphere of hydroponic culture is significantly higher than that in other environments (**Figure 3E**).

Many bacteria were enriched in the belowground compartments. We wanted to further understand which bacterial genera were highly abundant in the root endosphere and rhizosphere under different growth conditions. Therefore, we compared the differences in bacterial genera in the root endosphere and rhizosphere under different growth conditions. We found that *Pseudomonas* was more enriched in the hydroponic root endosphere (**Figure 3F**), while *Rhodanobacter* was more abundant in the solar greenhouse and arched shed but was absent in the open field (**Figure 3G**). In contrast, *Gaiella* was highly enriched in the rhizosphere (**Figure 3H**), and *Acidothermus* was significantly more numerous in the solar greenhouse and arched shed (**Figure 3I**). This indicates that bacteria will exhibit specific enrichment patterns in different ecological compartments and growth conditions. The same ecological compartment in different growth conditions has a great influence on bacterial colonization, which is more obvious in underground compartments.

3.5. Interaction Patterns and Prediction of Metabolic Pathways of Bacterial Communities Associated With Chinese Chives Under Different Ecological Compartments and Growth Conditions

3.5.1. Differences in Interaction Patterns of Bacteria Associated With Chinese Chives Under Different Growth Conditions

Interactions between bacteria are very important for maintaining microbial homeostasis. To explore the interaction networks among bacterial phyla and bacterial genera under different

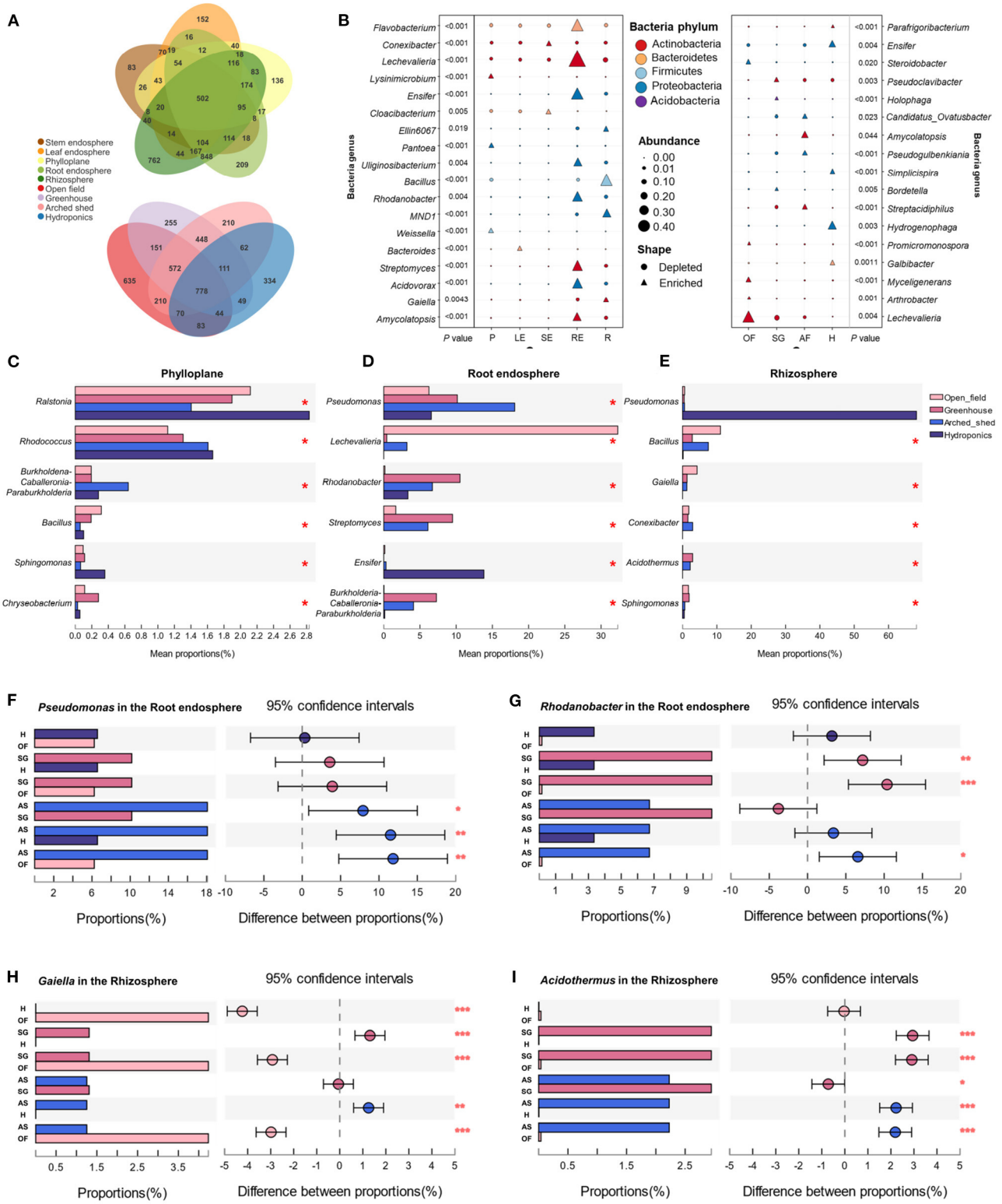


FIGURE 3 | Specific bacterial communities in different ecological compartments. **(A)** UpSet plot shows the OTU count of each group. **(B)** Indicator species in ecological compartments. The bacterial abundance at the genus level was analyzed. The shape of the point represents the enrichment or deletion of OTUs in the population, and the size of the point represents the abundance of OTUs. SE, stem endosphere; LE, leaf endosphere; P, phylloplane; RE, root endosphere; R, rhizosphere. Differences in bacterial genera in the phylloplane, **(C)** root endosphere, **(D)**; and rhizosphere, **(E)** under growth conditions. Comparison and analysis of OTU abundances of *Pseudomonas* **(F)** and *Rhodanobacter* **(G)** in the root endosphere and *Gaiella* **(H)** and *Acidothermus* **(I)** in the rhizosphere among different growth conditions. OF, open field; SG, solar greenhouse; AS, arched shed; H, hydroponics. $^{*}P \leq 0.05$. $^{**}P \leq 0.01$. $^{***}P \leq 0.001$.

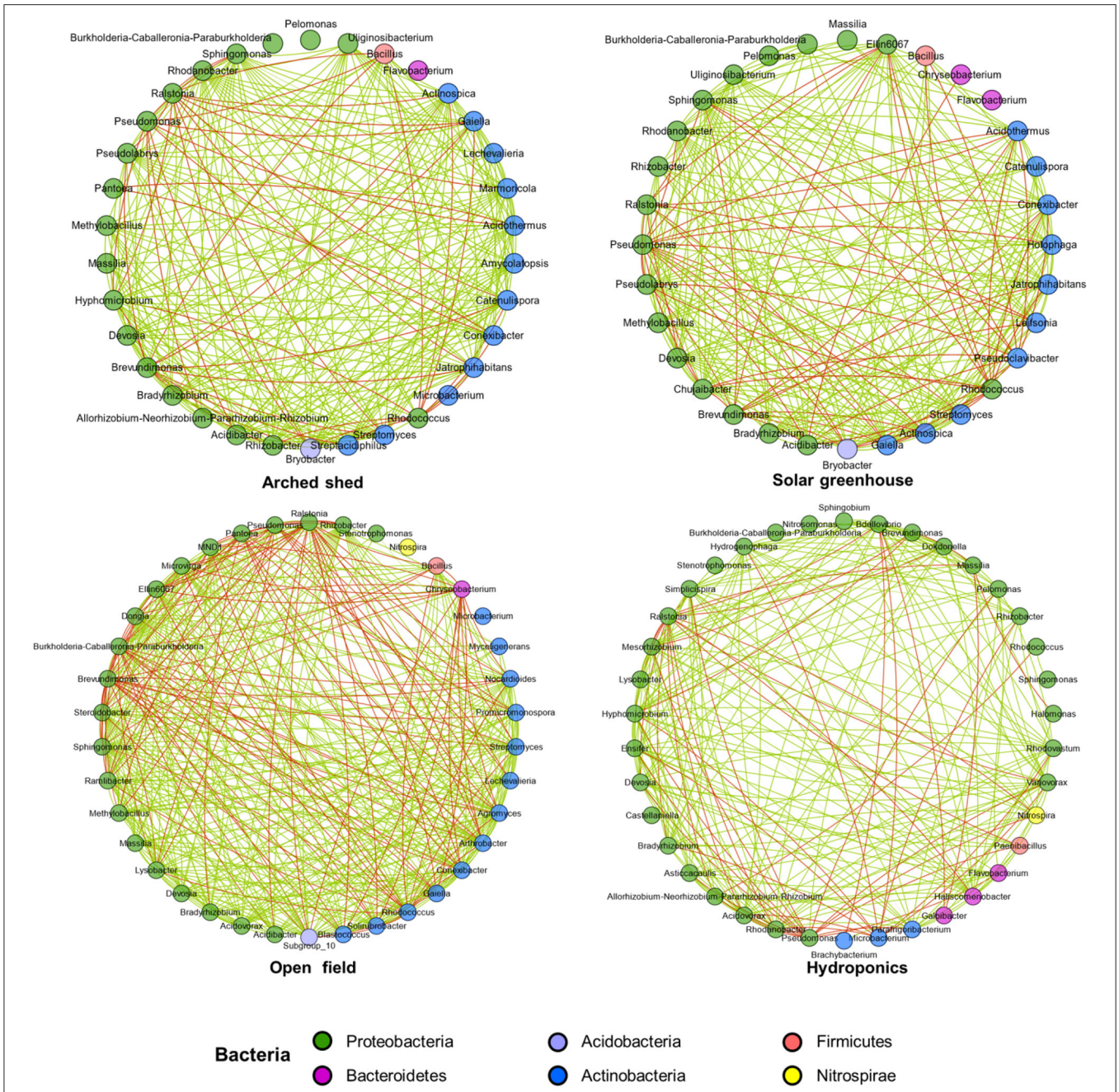


FIGURE 4 | Patterns of interaction of bacteria in different growth conditions. Spearman's test was used to analyze the interactions among the top 50 bacterial OTUs by abundance. Only a significant correlation ($P < 0.05$) is shown as a line. Line color indicates positive (green) or negative (red) correlation depending on the Spearman correlation coefficient. These nodes represent microbial genera, and their colors represent microbial phyla.

growth conditions, we selected the top 50 bacterial genera for correlation analysis. In the hydroponic environment, although interactions between bacteria were weaker than under the other three growth conditions, there were still a greater number of bacterial interactions, and mutual promotion outweighed inhibition, which was also shown in other growth conditions. In the hydroponic environment, a few Actinobacteria (including *Microbacterium* and *Brachybacterium*) had negative interactions with Proteobacteria (**Figure 4**, **Supplementary Figure S2**). The interactions of *Pseudomonas* and *Ralstonia* in Proteobacteria with other bacteria were mostly negative, while the interactions of *Flavobacterium*, *Galbibacter* and *Haliscomenobacter* in Bacteroidetes with other bacteria were mostly positive and frequent. We observed that there were more genera with negative interactions with *Ralatonia* in different growth

TABLE 2 | Topological properties of the empirical ecological networks at different ecological compartments in comparison to the random networks.

Groups	Empirical network indexes					Random networks indexes		
	Average degree (avgK)	Density (D)	Average clustering coefficient (avgCC)	Average path distance (GD)	Modularity	Average clustering coefficient (avgCC)	Average path distance (GD)	Modularity
P	3.087	0.024	0.122	4.58	0.638	0.023 ± 0.011	4.135 ± 0.105	0.563 ± 0.012
LE	2.985	0.047	0.087	4.223	0.626	0.040 ± 0.019	3.697 ± 0.140	0.526 ± 0.017
SE	2.493	0.035	0.131	5.56	0.725	0.027 ± 0.018	4.406 ± 0.219	0.617 ± 0.017
RE	4.078	0.04	0.016	3.137	0.402	0.211 ± 0.029	2.806 ± 0.058	0.395 ± 0.009
R	13.678	0.073	0.155	3.157	0.132	0.335 ± 0.010	2.464 ± 0.017	0.094 ± 0.002

P, phylloplane; LE, leaf endosphere; SE, stem endosphere; RE, root endosphere; R, rhizosphere.

conditions, such as *Lysobacter*, *Variovorax*, *Hyphomicrobium*, and *Mesorhizobium*.

It is worth noting that in the open field environment, negative interactions between bacteria are more frequent, and there are more competitive relationships. Negative correlations of *Chryseobacterium* with Proteobacteria, Actinobacteria, and Acidobacteria were more frequent in the open field. For close interactions in the open field, *Brevundimonas* showed a strong negative interaction with *Bacillus* and *Conexibacter*, and there was also a negative correlation between *Rhodococcus* and *Agromyces* in Actinobacteria. The interactions of *Rhizobacter* and *Stenotrophomonas* in Proteobacteria with other bacteria were mostly positive.

Compared with the open field, the positive and negative interactions between bacteria in the artificial environment were reduced. Therefore, the original relevant models established by some bacteria were interrupted, and new interactions were reconstructed in the artificial environment. Although the arched shed and solar greenhouse were similar sheltered structures, according to the grid of interactions between bacteria, the arched shed seemed to be more conducive to positive interactions between bacteria.

3.5.2. Analysis of the Bacterial Interaction Network in Chinese Chives Between Different Ecological Compartments

To further analyze the effect of ecological compartments on the interaction between bacteria in Chinese chive, we selected OTUs with more than 6 OTUs in each sample to carry out the analysis of the bacterial community cooccurrence network and the calculation of the topological characteristic index in MENA. In this study, the modular index of aboveground compartments was higher than 0.60 (**Table 2**) and much higher than that of underground compartments, indicating that microorganisms in aboveground compartments had modular structure, and ecological compartments had a significant indigenous effect on the modular of the microbial interaction network. The average clustering coefficient and network density reflect the degree of aggregation of nodes and the complexity of the network structure in the co-occurrence network. In this analysis, there were great differences between rhizosphere ecological compartments and other compartments. The average clustering coefficient and average degree of the rhizosphere were high, and the positive interaction relationship was strong, but the network density was low. This indicates that the aggregation of bacterial communities between underground compartments is poor, the positive correlation between microorganisms is strong, and other functions are more complex. This is consistent with the conclusion that underground compartments have higher species richness and community diversity than aboveground compartments. To identify the microbial groups that play key nodes (OTUs) in the interaction network, we calculated the within-module connectivity (Zi) and among-module connectivity (Pi) of each network node (OTUs). All networks have higher modularity characteristics than their random networks (**Table 2**), indicating that it is reasonable to divide these networks into modules. According to the topological characteristics of nodes, node attributes can be divided into the following four types: nodes with high connectivity within the module, Modulehubs ($Zi>2.5$); nodes with high connectivity between the two modules, Connectors ($Pi>0.62$); nodes with high connectivity throughout the network, Networkhubs ($Zi<2.5$ and $Pi>0.62$) and nodes without high connectivity within and between modules, Peripherals ($Zi>2.5$ and $Pi<0.62$). Except for peripherals, all nodes of three types belong to the key nodes (OTUs). It can be found in the network we construct (**Figure 5**) that the vast majority of nodes in each network belong to peripherals, and only a few nodes have a high degree of connection. Compared with the aboveground compartment microbial networks, the rhizosphere and root endosphere microbial networks of Chinese chives have more Modulehubs and Connectors, and Networkhubs were also detected in the rhizosphere and root endosphere microbial networks. These phenomena consistently indicate that there are multiple key microbial groups in the microbial community of Chinese chive underground compartments that dominate the occurrence of more complex interspecific interactions. We show the gate phylum level (**Figure 5**) and its genus classification level (**Table 2**) of all network nodes to determine the inherent attributes of key network nodes. The results showed that more than half of Modulehubs occupied the dominant relative abundance group, while most connectors mainly existed in lower relative abundance, which was more obvious in the underground ecological compartment. Proteobacteria are the most prominent key groups except for the microbial network of the stem endosphere, and the nodes that play a

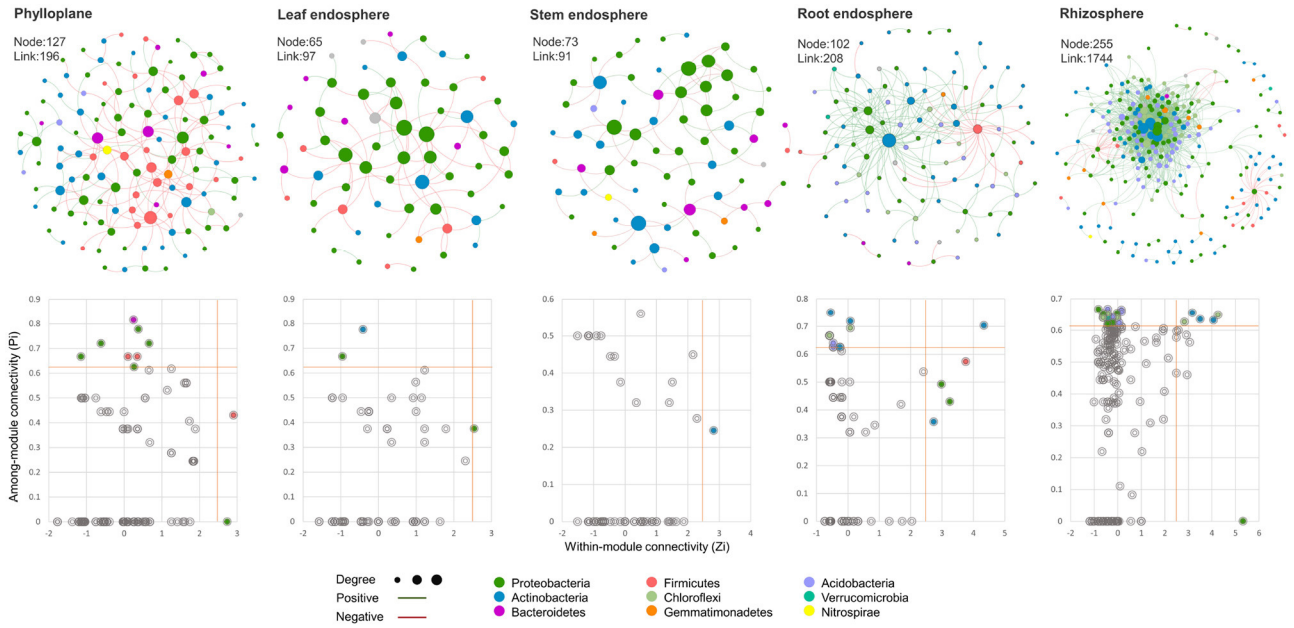


FIGURE 5 | The correlation networks of bacteria in different ecological compartments. One node represents an OTU, and the size of the node is determined by the degree. The larger the degree is, the larger the corresponding node size is, and the color of the node represents the microbial phylum which the OTU belongs. Line color indicates positive (green) or negative (red) correlation.

key role in the microbial network of the stem endosphere are not the highest relative abundance of Proteobacteria in the stem endosphere but the highest relative abundance of Actinobacteria in underground ecological compartments. In addition to Proteobacteria, Acidobacteria, and Actinobacteria are the main key nodes in rhizosphere and root endosphere microbial networks, respectively. Chloroflexi, Firmicutes, Gemmatimonadetes, Verrucomicrobia, and Armatimonadetes also play a key role in underground ecological compartments.

3.5.3. Prediction of the Metabolic Pathways of Endophytic Bacteria Associated With Chinese Chives in Different Ecological Compartments and Growth Conditions

To further predict and analyze the functions of endophytic bacteria in different growth conditions, we used the sequencing results and the SILVA database to annotate the functions of microbial communities in different ecological compartments and growth conditions through the Tax4fun package of R. The metabolic functions of all microorganisms were mainly focused on carbohydrate metabolism, amino acid metabolism, signal transduction, and membrane transport within metabolism, as well as environmental information processing. There were significant group differences among ecological compartments, which were mainly differences between the aboveground and belowground compartments. In most metabolic functions, the differences between the stem endosphere, leaf endosphere, and phylloplane of the aboveground compartment were small, and the differences between the root endosphere and rhizosphere were only significant for carbohydrate metabolism and energy metabolism. Most of the secondary pathways of genetic information processing and metabolism, such as amino acid metabolism, metabolism of cofactors and vitamins, energy metabolism, xenobiotic degradation and metabolism, lipid metabolism, and metabolism of terpenoids and polyketides, were more prevalent in the belowground compartments than in the aboveground compartments, but the secondary pathways of environmental information processing and cellular processes were less prevalent belowground. It is worth noting that cell motility within cellular processes was significantly lower in the root endosphere than in other compartments.

In contrast to the significant differences in the prevalence of group pathways among ecological compartments, differences in growth conditions had little effect on microbial metabolic pathways. Differences observed were mainly present between hydroponic and other growth conditions. Infectious microbial pathogens were more abundant in the hydroponic system than under other growth conditions, while amino acid metabolism was significantly lower in the hydroponic environment than in other growth conditions. The differences between hydroponic and other growth conditions were mainly reflected in organismal systems. The environmental adaptation pathway was significantly more prevalent in the hydroponic system than in the other groups, but endocrine, digestive, and nervous functions were significantly depressed in the hydroponic system ($P < 0.01$).

Among all microbial metabolic pathways, a total of 277 tertiary metabolic pathways were detected, and group difference tests were performed on 15 tertiary metabolic pathways under carbohydrate (**Supplementary Figure S3**) metabolism, the

secondary metabolic pathway with the greatest prevalence. We found that the variation was mainly manifested in differences between the aboveground and belowground compartments, and differences among the aboveground compartments were small. The levels of citrate cycle (TCA cycle) metabolism, butanoate metabolism, inositol phosphate metabolism, propanoate metabolism, and glyoxylate and dicarboxylate metabolism were significantly lower than those in the belowground compartments ($P < 0.05$).

4. DISCUSSION

In this study, the specific differences in bacteria in different ecological compartments and growth conditions were described, and their metabolic pathways were predicted. The results showed that bacterial communities differed significantly among plant ecological compartments, and modes of interaction and function were also significantly different among groups.

4.1. Diversity of Chinese Chive Bacteria Was Affected by Ecological Compartment and Growth Condition

The Chinese chive microbial community consists of multiple ecological compartments (phylloplane, leaf endosphere, stem endosphere, root endosphere, and rhizosphere), each with different microbial compositions. Within the study, we found that the diversity and richness of bacteria in the stem endosphere, leaf endosphere and phylloplane were significantly lower than those in the root endosphere and rhizosphere (**Figure 1**). Plants often distribute many nutrients to roots (Bulgarelli et al., 2012) during their growth and release large amounts of nutrients and energetic compounds, such as monosaccharides, polysaccharides, organic acids, phenolic compounds, amino acids, and proteins (Bais et al., 2006), to the surrounding environment through their roots, and these resources attract large aggregations of microbes. In rhizosphere secretions, compounds belonging to the chemical class of phenolics and terpenoids have strong external antibacterial and antifungal properties. Among them, phenolic metabolites can also effectively attract some soil-borne microorganisms and have beneficial effects on local soil microbial communities. Phenolic metabolites alter internal and external plant environments and various ecological compartments and promote the colonization and growth of specific bacteria. However, this phenomenon was not obvious in the hydroponic environment. The overall bacterial diversity and richness in the hydroponic environment were low, and differences in alpha diversity among the plant ecological compartments were not significant. Diversity was lower in the rhizospheric water, and diversity and evenness in the stem endosphere, leaf endosphere, and phylloplane in the hydroponic system tended to be more consistent. These results are consistent with the research findings of Stouvenakers (Stouvenakers et al., 2020). Microbial diversity and interaction among ecological compartments of plants are related to their ability to resist external disturbance (Kusstatscher et al., 2019; Xie et al., 2021). In this study, the alpha diversity in the Chinese chive rhizosphere was significantly higher than that in other ecological compartments, the rhizosphere microbial network average degree and average clustering coefficient were higher than those in other ecological compartments, and the interaction of the microbial network was more complex. This may be related to the direct contact between the rhizosphere and soil (nutrient solution), the generation of a bacterial screening mechanism and the inhibitory effect of the rhizosphere (Reinhold Hurek et al., 2015; Itumeleng et al., 2020). Inhibition may result from (trace) nutrient competition, antibiotic compounds, production of decomposing enzymes, consumption (Lugtenberg and Kamilova, 2009; Doornbos et al., 2012) of pathogen-stimulating compounds or other pathways. The different growth conditions we sampled had little effect on the diversity of microorganisms, which might be because although the air temperature and humidity were significantly different under different growth conditions, fertilization and management methods were similar. Therefore, the differences in soil physical and chemical properties among open fields, solar greenhouses and arched sheds were small, and the impact on microorganisms was small.

4.2. Ecological Compartments and Growth Conditions Create Different Microbial Community Structures and Succession Patterns

Proteobacteria were dominant in the stem endosphere, leaf endosphere and phylloplane (**Figure 2A**), which was consistent with the findings of previous studies (Wang et al., 2020c). Proteobacteria were significantly negatively correlated with the abundance of Actinobacteria, the second most abundant phylum (**Figures 2A,C**, **Supplementary Figure S2**), which was one of the most important features distinguishing aboveground and belowground ecological compartments. The genus *Pseudomonas* showing the highest abundance in Proteobacteria is consistent with the results obtained by Lugtenberg and Kamilova (2009; Zhuang et al., 2020) in the rhizosphere of *Allium* plants. *Pseudomonas* plays a more important role in the co-occurrence network of rhizospheric bacteria during the late growth stage and under favourable growth conditions for *Allium* plants, and because *Pseudomonas* is present in aboveground ecological compartments, it can be isolated and purified by culturomics for biological control of Chinese chives gray mold. Among species in this genus, *Pseudomonas* aeruginosa (Wang et al., 2020b), *Pseudomonas* chlororaphis (Cw et al., 2021), *Pseudomonas fluorescens* (Zhu et al., 2021), and *Pseudomonas* extremorientalis (Wang et al., 2019) have been successfully isolated from pear rhizosphere soil, tomato and Chinese cherry and purified and show over 85% inhibitory effects on *Botrytiscinerea*. Compared with aboveground ecological compartments, the relative abundance of *Lechevalieria* in the Actinobacteria phylum in roots and rhizosphere was high. For *Lechevalieria*, mangromicins, one of their secondary metabolites, could eliminate free radical activity (as assayed by DPPH, Takuji et al., 2014) and exhibit high inoxidizability. In addition, secondary metabolites have also been found to exert antimicrobial activity and significant inhibitory effects

on *Bacillussubtilis*, *Kocuriarhizophila*, *Xanthomonascampestrispv. oryzae* KB88, and *Candidaalbicans* (Kimura et al., 2018). These results show that the response of microbial communities in the root endosphere to fluctuations in the growth environment was more stable than those in the rhizosphere or soil (Almario et al., 2017; Han et al., 2020; Xie et al., 2021). Bacteroidetes is an important component of the bacterial microbial community (Fernández-Gómez et al., 2013) in a normal aquatic environment (Chen et al., 2011), which is related to the requirement of a strict anaerobic environment as a growth condition for most bacterial strains in this phylum. Bacteria in Bacteroidetes can hydrolyze organic matter such as carbon polymers and proteins in aquatic environments and produce a variety of extracellular enzymes to decompose or utilize complex carbon sources (Kirchman, 2002). Therefore, Bacteroidetes may play an important role in maintaining the aquatic ecosystem balance in hydroponic Chinese chives. Moreover, the bacterial communities classified within Bacteroidetes are closely associated with the decomposition of protein organic substances (Ding et al., 2019), and the conversion of these organic substances is an important aspect of carbon cycling inside plants and in the rhizosphere. Therefore, the metabolic functions of microorganisms, such as signal transduction, membrane transport and metabolism of other amino acids, are more highly expressed in hydroponics (**Figure 6**) than under other growth conditions.

4.3. Different Colonization Patterns of Bacteria in Ecological Compartments

There are vertical changes in the establishment of plant endophytic microorganisms and phyllosphere and rhizosphere microorganisms, and the structure of microbial community changes with plant ecological compartments. In this study, the dynamic patterns of phylloplane, rhizosphere and endophytic microbial communities were also significantly correlated with the ecological compartment of Chinese chive (**Figures 1**, **2**, **6**). The beta diversity analysis based on OTUs revealed that the microbial community had obvious ecologica compartment division characteristics. There are both common bacterial communities and specific bacterial communities in different ecological compartments of Chinese chives. The diversity index of microbial bacteria in the rhizosphere was high, and the number of specific bacteria was large. It can be seen from the relative abundance and interaction network that most of the key nodes in the phylloplane and leaf endosphere of Chinese chives existed in the root endosphere and rhizosphere networks. As key nodes Proteobacteria and Firmicutes in the phylloplane ecological compartment, they are also key nodes in the root endosphere (**Figure 5**). Rhizosphere microorganisms could enter the roots of Chinese chives, and some endophytic bacteria migrated from the root endosphere of Chinese chives to the upper stems and leaves (Edwards et al., 2015; Xie et al., 2021). There were some differences in diversity, bacterial composition, interaction network, and function among the microorganisms in the phylloplane, leaf endosphere, and stem endosphere of Chinese chives (**Figures 1**, **2**, **5**, **6**). This may be because the phylloplane microorganisms of Chinese chives are derived from the diffusion of endophytes in the stem endosphere and leaf endosphere, and on the other hand, the phylloplane is close to the soil (nutrient solution) and directly or indirectly contacts the microorganisms from the soil (nutrient solution) (Melotto et al., 2008), thus forming different bacterial communities from the leaf endosphere and stem endosphere. However, it can be clearly seen in the interaction network that this part of the differential bacterial communities (existing in the leaf endosphere but not in the stem endosphere) has similarities with the rhizosphere bacterial communities. For example, we observed that *Weissella* is an indicator species of Chinese chive phylloplane in our analysis of specific bacterial communities. Because *Weissella* is mostly distributed in soil or river sediments (Fusco et al., 2015), we believe that the original source of *Weissella* in leaves is soil or nutrient solution. According to our analysis of the specific bacterial communities in different ecological compartments, the number of indicator bacteria in the root endosphere was much higher than that in other ecological compartments (**Figure 3B**). The rhizosphere environment exists as a transition boundary between soil (nutrient solution) and plants. Similar to phyllosphere microorganisms, the microorganisms inhabiting the rhizosphere of plants are directly affected by the host plant and the soil (nutrient solution) environment. Therefore, the r root endosphere is more plant-specific (**Figure 3B**), which is quite different from the microorganisms in phylloplane and rhizosphere environments. In the beginning, root endosphere microorganisms are strictly regulated, and then the entry and exit of related groups are more rigorously controlled (Liu et al., 2017). The formation of rhizosphere bacterial communities mainly follows the principle of niche adaptation, while the root endosphere bacterial communities are more significantly regulated by host signals.

4.4. Ecological Compartment and Growth Conditions Have Significant Effects on the Complexity and Functional Expression of the Plant Microbial Network

The results showed that the cooccurrence patterns of microorganisms in each ecological compartment of Chinese chive were nonrandom (**Table 2**), and the nonrandom interaction pattern reflected niche sharing or synergy between microorganisms. The networks between the ecological compartments are dominated by positive correlations (**Figure 5**), indicating that more beneficial interactions exist or occupy similar niche spaces among different types of microorganisms. Due to the characteristics of the microbial community structure in each ecological compartment, the topological characteristics of all networks are significantly related to their corresponding bacterial species diversity. The number of nodes and connections in underground compartments is much higher than that in aboveground compartments (**Figure 5**), indicating that species diversity and interaction patterns in underground compartments are more visible and active. Compared with the aboveground ecological compartment microbial network

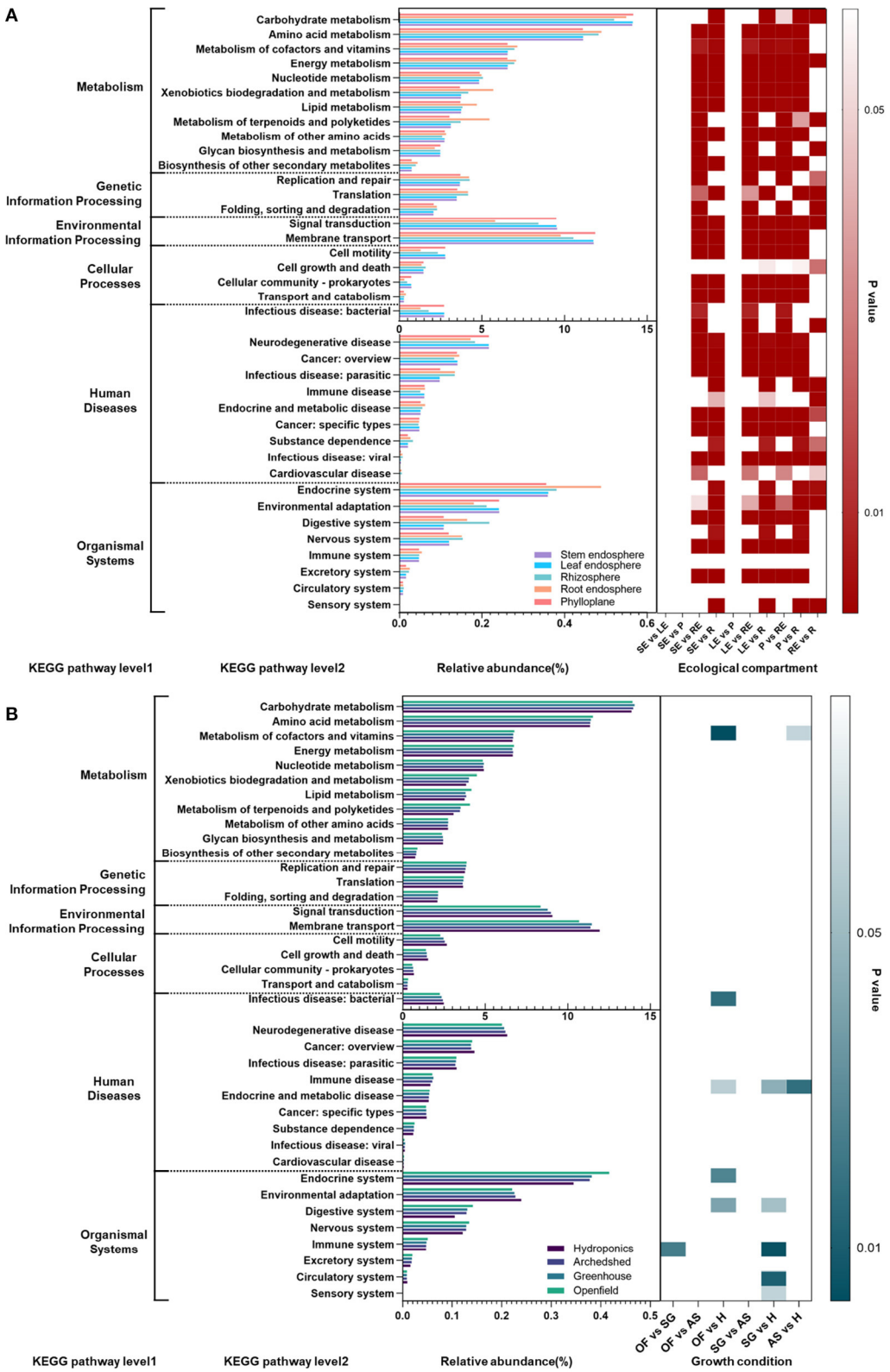


FIGURE 6 | (A) Prediction and analysis of bacterial metabolic pathways in different ecological compartments and different growth conditions. SE, stem endosphere; LE, leaf endosphere; P, phylloplane; RE, root endosphere; R, rhizosphere. **(B)** The bar chart shows the abundance of each group, and the different levels of metabolism are separated by dark gray lines. The heatmaps on the right side indicate significant pairwise differences in abundance and are plotted based on the P values. OF, open field; SG, solar greenhouse; AS, arched shed; H, hydroponics.

of Chinese chives, there are a large number of isolated nodes that do not interact with other nodes in the underground compartment microbial network, resulting in relatively low connectivity of the rhizosphere microbial co-occurrence network (**Figure 5**, **Table 2**). This may be related to the microorganisms in the rhizosphere living in a variety of complex and separated microhabitats (Zhang et al., 2018). which usually do not have a large-scale exchange or niche sharing between different types of microorganisms. Although rhizosphere bacteria have the highest species richness, most species are not involved in the network interaction process because a large number of microbial groups in soil (nutrient solution) exist in an inactive or dormant state (Fierer and Lennon, 2011). Growth conditions have an impact on bacterial growth condition interactions and functional expression. In our study, correlations between bacterial species in different growth conditions were analyzed (**Figure 4**). Mutualistic and inhibitory relationships between bacteria directly affect the bacterial community structure under different growth conditions. For example, we observed genera that were negatively correlated with *Ralstonia* under different growth conditions, such as *Lysobacter*, *Variovorax*, *Hyphomicrobium*, and *Mesorhizobium*, which were effective factors for the extraction of bacteria antagonistic against bacterial wilt in Chinese chives (Wang et al., 2017b). In terms of prediction of metabolic functions, we found that the prevalence in root endosphere and rhizosphere of most metabolic pathways, such as genetic information processing and metabolism (**Figure 6**, **Supplementary Figure S3**), was higher than that in aboveground compartments, which was similar to findings of previous studies (Lian et al., 2016; Wang et al., 2020a; Shehata et al., 2021). We found that inositol phosphate metabolism was significantly more active in the root endosphere than in other ecological compartments in the intergroup difference test for the top 15 tertiary metabolic pathways, with the highest abundance in the secondary metabolic pathway carbohydrate metabolism. Inositol phosphates produced by this metabolic process are closely related to plant stress responses and induced system resistance. In an environment characterized by salt stress and drought stress, the expression of inositol phosphates in plants is upregulated (Tan et al., 2013), which is involved in the response of plants to abiotic stress. In addition, inositol phosphates can stimulate the development of immune cells, thereby stimulating plant defense systems (Sauer and Cooke, 2010).

In conclusion, we observed that the ecological compartments had significant indigenous effects on plant microorganisms, including the diversity of bacteria, the composition of bacterial communities, and the interaction network and functional expression of bacteria in different ecological compartments. Through the analysis of bacterial relative abundance composition, indicator genus, and bacterial interaction network in different ecological compartments, we found that bacteria in different ecological compartments had different assembly methods, but most of the bacteria were still from soil (nutrient solution). The growth conditions had little effect on the bacterial community. Except for the differences in bacterial community composition and functional expression between hydroponic and open fields, arched sheds and solar greenhouses had no significant effect on bacterial community structure.

DATA AVAILABILITY STATEMENT

The (raw sequence reads) data used to support the findings of this study have been deposited in the (NCBI) repository (accession: PRJNA742175).

AUTHOR CONTRIBUTIONS

NS data processing, picture drawing, and paper writing. YW sample collection and theoretical guidance. JC sample planting and theoretical guidance. PW sample collection. WS, PM, YD, ZJ, and YL sample planting and related experimental materials maintenance. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.775002/full#supplementary-material

REFERENCES

Almario, J., Jeena, G., Wunder, J., Langen, G., Zuccaro, A., Coupland, G., et al. (2017). From the cover: pnas plus: root-associated fungal microbiota of nonmycorrhizal arabis alpina and its contribution to plant phosphorus nutrition. *Proc. Natl. Acad. Sci. U.S.A.* 114:E9403. doi: 10.1073/pnas.1710455114

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., et al. (2013). Habitat degradation impacts black howler monkey (alouatta pigra) gastrointestinal microbiomes. *Isme J.* 7, 1344–1353. doi: 10.1038/ismej.2013.16

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Ann. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E. V., and Schulze-Lefert, P. (2012). Structure and functions of the bacterial microbiota of plants. *Ann. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Chao, L. N. W. J. L. Z. H. Y. H. and Yingcui, G. (2019). Quality identification of shawo radish planted in greenhouse. *Bull. Chin. Agron.* 35, 43–46.

Chaouachi, M., Marzouk, T., Jallouli, S., Elkahoui, S., Gentzbittel, L., Ben, C., and Djébali, N. (2021). Activity assessment of tomato endophytic bacteria bioactive compounds for the postharvest biocontrol of botrytis cinerea. *Postharvest Biol. Technol.* 172:111389. doi: 10.1016/j.postharvbio.2020.111389

Chen, W., Zhang, W., Li, S. W., Wang, L., Yun, H. B., Wu, X. K., et al. (2011). Features of soil cultivable microorganism quantity and diversity distribution under different alpine grassland ecosystems in qinghai-tibet plateau. *J. Glaciol. Geocryol.* 33, 1419–1426.

Chi, J., Yang, R., and Wang, A. (2012). Effect of wetland plant species and growth strategy on the distribution of paes and their monoester metabolites in the rhizosphere. *J. Lake Sci.* 24, 416–421. doi: 10.18307/2012.0313

Courchesne, F., and Gobran, G. R. (1997). Mineralogical variations of bulk and rhizosphere soils from a norway spruce stand. *Soil Sci. Soc. America J.* 61, 1245–1249. doi: 10.2136/sssaj1997.03615995006100040034x

Cw, A., Yan, W. A., Lin, W. B., Wf, C., Xz, A., Xc, C., et al. (2021). Biocontrol potential of volatile organic compounds from pseudomonas chlororaphis zl3 against postharvest gray mold caused by botrytis cinerea on chinese cherry - sciencedirect. *Biol. Control.* 159, 104613. doi: 10.1016/J.BIOCONTROL.2021.104613

Da Silveira, A. P. D., Iório, R. d. P. F., Marcos, F. C. C., Fernandes, A. O., de Souza, S. A. C. D., Kuramae, E. E., et al. (2019). Exploitation of new endophytic bacteria and their ability to promote sugarcane growth and nitrogen nutrition. *Antonie van leeuwenhoek* 112, 283–295. doi: 10.1007/s10482-018-1157-y

Dai, F. J., and Chau, C. F. (2017). Classification and regulatory perspectives of dietary fiber. *J. Food Drug Anal.* 25, 37–42. doi: 10.1016/j.jfda.2016.09.006

Ding, S. Y., Wang, L., Han-Chen, X. U., Bao, X. Y., Wang, H., Chang, Y. Q., et al. (2019). Bacterial community structure and function in the intestinal tracts and culture environment of sea cucumber (*apostichopus japonicus*). *Chin. J. Ecol.* 38, 210–220. doi: 10.13292/j.1000-4890.201901.008

Doornbos, R. F., Loon, L., and Bakker, P. (2012). Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. a review. *Agron. Sustain. Develop.* 32, 227–243. doi: 10.1007/s13593-011-0028-y

Edwards, J., Johnson, C., Santos Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Emami, S., Alikhani, H. A., Pourbabaee, A. A., Etesami, H., Motasharezadeh, B., and Sarmadian, F. (2020). Consortium of endophyte and rhizosphere phosphate solubilizing bacteria improves phosphorous use efficiency in wheat cultivars in phosphorus deficient soils. *Rhizosphere* 14:100196. doi: 10.1016/j.rhisph.2020.100196

Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S. G., González, J., et al. (2013). Ecology of marine bacteroidetes: a comparative genomics approach. *Isme J.* 7, 1026–1037. doi: 10.1038/ismej.2012.169

Fierer, N., and Lennon, J. T. (2011). The generation and maintenance of diversity in microbial communities. *Am. J. Botany* 98, 439–448. doi: 10.3732/ajb.1000498

Fusco, V., Quero, G. M., Cho, G. S., Kabisch, J., Meske, D., Neve, H., et al. (2015). The genus weissella: taxonomy, ecology and biotechnological potential. *Front. Microbiol.* 6, 155. doi: 10.3389/fmicb.2015.00155

Gu, Y., Wang, Y., Wang, P., Wang, C., and Li, M. (2020). Study on the diversity of fungal and bacterial communities in continuous cropping fields of chinese chives (allium tuberosum). *BioMed Res. Int.* 2020, 1–14. doi: 10.1155/2020/3589758

Han, Q., Ma, Q., Chen, Y., Tian, B., Xu, L., Bai, Y., et al. (2020). Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *ISME J.* 14, 1915–1928. doi: 10.1038/s41396-020-0648-9

Itumeleng, M., Julien, T., and Tienne, Y. (2020). Temporal and spatial interactions modulate the soybean microbiome. *FEMS Microbiol. Ecol.* 97, 1. doi: 10.1093/femsec/fiaa206

J.Y., H., F.T., M., and L.L., S. (2021). Nutrition and aroma components of hotbed chives and leeks in puding county in summer and winter. *Food Fermentation Ind.* 47, 197–204. doi: 10.13995/j.cnki.11-1802/ts.025522

Jagannath, S., Konappa, N. M., Alurappa, R., and Chowdappa, S. (2019). Production, characterization of indole acetic acid and its bioactive potential from endophytic fungi of cymbidium aloifolium l. *J. Biol. Active Prod. Nat.* 9, 387–409. doi: 10.1080/22311866.2019.1688684

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). Mafft: a novel method for rapid multiple sequence alignment based on fast fourier transform (describes the fft-ns-1, fft-ns-2 and fft-ns-i strategies). *Nucl. Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Khan, M. S., Gao, J., Chen, X., Zhang, M., Yang, F., Du, Y., et al. (2020). Isolation and characterization of plant growth-promoting endophytic bacteria paenibacillus polymyxa sk1 from lilium lancifolium. *BioMed Res. Int.*, 2020:8650957. doi: 10.1155/2020/8650957

Kimura, T., Inahashi, Y., Matsuo, H., Suga, T., Iwatsuki, M., Shiomi K., et al. (2018). Pyrizomicin a and b: structure and bioactivity of new thiazolyl pyridines from lechevalieria aerocolonigenes k10-0216. *J. Antibiot. Int. J.* 71, 606–608. doi: 10.1038/s41429-018-0038-y

Kirchman, D. L. (2002). The ecology of cytophaga–flavobacteria in aquatic environments. *Fems Microbiol. Ecol.* 39, 91–100. doi: 10.1016/S0168-6496(01)00206-9

Kusstatscher, P., Cernava, T., Harms, K., Maier, J., Eigner, H., Berg, G., et al. (2019). Disease incidence in sugar beet fields is correlated with microbial diversity and distinct biological markers. *Phytobiomes*. 3, 22–30. doi: 10.1094/PBIOMES-01-19-0008-R

Lang, C. (2018). Effects of plant endophytic bacteria extracts on growth and yield of corn. *Jiangsu Agricul. Sci.* 46, 70–73. doi: 10.15889/j.issn.1002-1302.2018.20.015

Letunic I., and Bork P. (2007). Interactive tree of life (itol): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127–128. doi: 10.1093/bioinformatics/btl529

Li, W. M., Qin, J., Jiao, J. G., Liu, Q. Y., Huang, Z. Y., and Wu, X. D. (2020). Effects of intercropping garlic combined with nongcanjing on control effect of soft rot, quality and yield of chinese cabbage. *J. Changjiang Vegetables* 14, 71–74.

Lian, Q. G., Gan, L., Qing, M. A., Lan, X. J., Zhang, J., Zong, Z. F., et al. (2016). The disease prevention and growth-promoting effect and mechanism of streptomyces spectabilis sc11 to botrytis cinerea stress. *Acta Phytopathologica Sinica* 3, 8. doi: 10.13926/j.cnki.apps.2016.03.014

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M., et al. (2017). Inner plant values: diversity, colonization and benefits from endophytic bacteria. *Front. Microbiol.* 8, 2552. doi: 10.3389/fmicb.2017.02552

Liu, L., Zhang, L., and Cai, Z. (2007). A multiple hypothesis test and its application in econometrics. *Stat. Res.* 4, 26–30. doi: 10.3969/j.issn.1002-4565.2007.04.007

Lozupone, C., and Knight, R. (2005). Unifrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Ann. Rev. Microbiol.* 2009, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Melotto, M., Underwood, W., and He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* 46, 101–122. doi: 10.1146/annurev.phyto.121107.104959

Moin, S., Ali, S. A., Hasan, K. A., Tariq, A., Sultana, V., Ara, J., et al. (2020). Managing the root rot disease of sunflower with endophytic fluorescent pseudomonas associated with healthy plants. *Crop Protect.* 130:105066. doi: 10.1016/j.cropro.2019.105066

Nakashima, T., Kamiya, Y., Iwatsuki, M., Takahashi, Y., and Omura, S. (2014). Mangromicins, six new anti-oxidative agents isolated from a culture broth of the actinomycete, lechevalieria aerocolonigenes k10-0216. *J. Antibiot.* 67, 533–539. doi: 10.1038/ja.2014.34

Ndivo, F. M., Njeru, E. M., and Birgen, J. (2018). Efficacy of neem, garlic and aloe extracts in the management of postharvest potato soft rot caused by erwinia carotovora. *Asian J. Res. Crop Sci.* 1, 1–7. doi: 10.9734/AJRCS/2018/40255

Ping, L. W. Q. J. J. Q. Z. X. L. R. D. Z. Z. J. (2020). Effects of intercropping garlic combined with nongcanjing on control effect of soft rot, quality and yield of chinese cabbage. *J. Changjiang Vegetables* 14, 71–74. doi: 10.3865/j.issn.1001-3547.2020.14.023

Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). Fasttree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi: 10.1093/molbev/msp077

Reinhold Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Ann. Rev. Phytopathol.* 53, 403–424. doi: 10.1146/annurev-phyto-082712-102342

Sauer, K., and Cooke, M. P. (2010). Regulation of immune cell development through soluble inositol-1,3,4,5-tetrakisphosphate. *Nat. Rev. Immunol.* 10, 257–271. doi: 10.1038/nri2745

Shehata, H. R., Ragupathy, S., Henry, T. A., and Newmaster, S. G. (2021). Niche specificity and functional diversity of the bacterial communities associated with ginkgo biloba and panax quinquefolius. *Sci. Rep.* 11:10803. doi: 10.1038/s41598-021-90309-0

Simeon, A. U., and Abubakar, A. (2014). Evaluation of some plant extracts for the control of bacterial soft rot of tubers. *J. Exp. Agricul. Int.* 4, 1869–1876. doi: 10.9734/AJEA/2014/12309

Stouvenakers, G., Massart, S., Depireux, P., and Hassam, M. (2020). Microbial origin of aquaponic water suppressiveness against pythium aphanidermatum lettuce root rot disease. *Microorganisms* 8, 24. doi: 10.3390/microorganisms8111683

Sun, J., Yin, G. Y., Ding, M. M., and Tao, Z. X. (2014a). Study on extraction and antioxidant activity of protein from chinese chive seed. *Sci. Technol. Food Ind.* 35, 291–294. doi: 10.13386/j.issn1002-0306.2014.12.055

Sun, L., Xie, Y., and Zhang, Y. (2014b). Effect of temperature variation within the facilities on antioxidant properties grape leaves under early and delayed cultivation. *School Agric.* 1, 38–43.

Tan, J., Wang, C., Xiang, B., Han, R., and Guo, Z. (2013). Hydrogen peroxide and nitric oxide mediated cold- and dehydration-induced myo -inositol phosphate synthase that confers multiple resistances to abiotic stresses. *Plant Cell Environ.* 36, 288–299. doi: 10.1111/j.1365-3040.2012.02573.x

Tan, L., Zeng, W.-A., Xiao, Y., Li, P., Gu, S., Wu, S., et al. (2021). Fungi-bacteria associations in wilt diseased rhizosphere and endosphere by interdomain ecological network analysis. *Front. Microbiol.* 12:2536. doi: 10.3389/fmicb.2021.722626

Wang, C., Masoudi, A., Wang, M., Yang, J., Shen, R., Man, M., et al. (2020a). Community structure and diversity of the microbiomes of two microhabitats at the root–soil interface: implications of meta-analysis of the root-zone soil and root endosphere microbial communities in xiong'an new area. *Can. J. Microbiol.* 66, 605–622. doi: 10.1139/cjm-2020-0061

Wang, H. P., Qiu, Y., Fang Wei, L. I., Song, J. P., Zhang, X. H., and Xi Xiang, L. I. (2017a). Investigation and nutrition components analysis of wild chives in hezhang county of guizhou province. *J. Plant Gen. Resour.* 18, 1137–44.

Wang, J., Zeng, X., Zhang, H., Li, Y., and Zhao, S. (2018). Effect of exogenous phosphate on the lability and phytoavailability of arsenic in soils. *Chemosphere Environ. Toxicol. Risk Assess.* 196, 540–547. doi: 10.1016/j.chemosphere.2017.12.191

Wang, R., Zhang, H., Sun, L., Qi, G., Chen, S., and Zhao, X. (2017b). Microbial community composition is related to soil biological and chemical properties and bacterial wilt outbreak. *Sci. Rep.* 7, 343. doi: 10.1038/s41598-017-00472-6

Wang, X., Ye, S., and Li, G. (2014). Effect on the protection of alcoholic liver injury and antihyperlipidemic of extracts from chinese chives(allium tuberosum). *J. Chin. Inst. Food Ence Technol.* 14, 28–32.

Wang, X., Zhou, X., Cai, Z., Guo, L., and Wang, A. (2020b). A biocontrol strain of pseudomonas aeruginosa cq-40 promote growth and control botrytis cinerea in tomato. *Pathogens* 10, 22. doi: 10.3390/pathogens10010022

Wang, Y., Wang, C., Gu, Y., Wang, P., and Yang, X. (2020c). The variability of bacterial communities in both the endosphere and ectosphere of different niches in chinese chives (allium tuberosum). *PLoS ONE* 15:e0227671. doi: 10.1371/journal.pone.0227671

Wang, Y., Wang, C., Wang, L., Liu, Q., Dong, T., Hou, P., et al. (2019). Identification, optimization of fermentation conditions of antagonistic bacterium pseudomonas extremorientalis against botrytis cinerea on pear and evaluation of its biocontrol efficacy. *Chin. J. Biol. Control.* 35, 437–448. doi: 10.16409/j.cnki.2095-039x.2019.03.007

Wei, G., Ning, K., Zhang, G., Yu, H., Yang, S., Dai, F., et al. (2021). Compartment niche shapes the assembly and network of cannabis sativaassociated microbiome. *Front. Microbiol.* 12:714993. doi: 10.3389/fmicb.2021.714993

Wu, X., Shi, J., Fang, W. X., Guo, X. Y., Zhang, L. Y., Liu, Y. P., et al. (2019). Allium vegetables are associated with reduced risk of colorectal cancer: a hospital-based matched case-control study in china. *Asia Pac. J. Clin. Oncol.* 15, e132–e141. doi: 10.1111/ajco.13133

Xia, F., Wang, J. G., Zhu, T., Zou, B., Rhee, S. K., and Quan, Z. X. (2018). Ubiquity and diversity of complete ammonia oxidizers (comammox). *Appl. Environ. Microbiol.* 84:e01390-18. doi: 10.1128/AEM.01390-18

Xie, J., Wang, X., Xu, J., Xie, H., Cai, Y., Liu, Y., et al. (2021). Strategies and structure feature of the aboveground and belowground microbial community respond to drought in wild rice (oryza longistaminata). *Rice* 14, 1–17. doi: 10.1186/s12284-021-00522-8

Xu, L., Yang, F., Wu, X., Ding, R., Lu, Y., and Hou, B. (2016). Effect of intercropping with onion or garlic on yield and quality of chinese cabbage. *Northern Horticul.* 14, 6–10. doi: 10.11937/bfyy.201614002

Yang, Z., and Cao, J. (2016). Research progress of endophytic fungi and their secondary metabolites. *J. Microbiol.* 36, 1–6. doi: 10.3969/j.issn.1005-7021.2016.04.001

Zhang, B., Zhang, J., Liu, Y., Shi, P., and Wei, G. (2018). Co-occurrence patterns of soybean rhizosphere microbiome at a continental scale. *Soil Biol. Biochem.* 118, 178–186. doi: 10.1016/j.soilbio.2017.12.011

Zhu, B., Wu, J., Ji, Q., Wu, W., and Qin, L. (2020). Diversity of rhizosphere and endophytic fungi in atractylodes macrocephala during continuous cropping. *PeerJ* 8:e8905. doi: 10.7717/peerj.8905

Zhu, L., Qian, N., Sun, Y., Lu, X., and Qian, L. (2021). Pseudomonas fluorescens dn16 enhances cucumber defense responses against the necrotrophic pathogen botrytis cinerea by regulating thermospermine catabolism. *Front. Plant Sci.* 12:645338. doi: 10.3389/fpls.2021.645338

Zhuang, L., Li, Y., Wang, Z., Yu, Y., Zhang, N., Yang, C., et al. (2020). Synthetic community with six pseudomonas strains screened from garlic rhizosphere microbiome promotes plant growth. *Microb. Biotechnol.* 14, 488–502. doi: 10.1111/1751-7915.13640

ZiLi, M., Mei-jin, Y., Yan-Mei, Y., Wei, Z., Xiang-Quan, Y., Qian, Z. et al. (2018). Soil microorganism quantity and soil enzyme activity in the wheat-garlic intercropping system. *J. Agricul. Resour. Environ.* 35, 430–438. doi: 10.13254/j.jare.2018.0080

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 February 2022
doi: 10.3389/fmicb.2022.831783

Fungal Diversity in the Soil of the *Oxytropis glacialis* Root System on the Qinghai-Tibet Plateau

Peng-xi Cao[1,2,3], Yixuan Liu[1,2,3], Hong-mei Ma[1,2], Ning Zhao[1,2], Shu-ting Chen[1,2], Guo-qi Xu[1,2] and Xing Liu[1,2,3]**

[1] Ecological Field Station Real-Time Monitoring Center, Research Center for Ecology, Tibet University, Lhasa, China, [2] Laboratory of Adaptation and Evolution of Plateau Biota to Extreme Environments, Research Center for Ecology and Environment of Qinghai-Tibetan Plateau, Tibet University, Lhasa, China, [3] College of Life Science, Wuhan University, Wuhan, China

OPEN ACCESS

Edited by:
Prem Lal Kashyap,
Indian Institute of Wheat and Barley Research (ICAR), India

Reviewed by:
Anukool Vaishnav,
Agroscope, Switzerland
Anna Gałązka,
Institute of Soil Science and Plant Cultivation, Poland
Divjot Kour,
Eternal University, India
Kiran Preet Padda,
University College Dublin, Ireland

***Correspondence:**
Yixuan Liu
yixuan.liu@foxmail.com
Xing Liu
xingliu@whu.edu.cn

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: 08 December 2021
Accepted: 25 January 2022
Published: 24 February 2022

Citation:
Cao P-x, Liu Y, Ma H-m, Zhao N, Chen S-t, Xu G-q and Liu X (2022) Fungal Diversity in the Soil of the Oxytropis glacialis Root System on the Qinghai-Tibet Plateau. Front. Microbiol. 13:831783. doi: 10.3389/fmicb.2022.831783

Because of swainonine-producing endophytic fungal, *Oxytropis glacialis* is one of the main poisonous weeds in the alpine grassland and desert grassland of the Qinghai-Tibet Plateau (QTP). It has a severe impact on grassland degradation on the QTP. In this manuscript, the Internally Transcribed Spacer (ITS) region of fungal communities in the soil of the *O. glacialis* root system was sequenced by high-throughput sequencing and analyzed by bioinformatics methods. The physical and chemical properties of the soil samples were analyzed in combination with the fungal diversity and its relationship with the soil physical and chemical factors. The results showed that the soil fungal community in the *O. glacialis* root system are rich in diversity in different ecological environments and are most affected by the soil pH value and organic matter. The swainonine-producing fungal *Embellisia oxytropis* was first detected in the soil of the *O. glacialis* root system. This finding provides data to support the next step in demonstrating the horizontal spread of swainone-producing fungal from *O. glacialis* to soil. In addition, a stable network of core flora has a facilitating effect on the formation of *O. glacialis* as a dominant species in alpine ecosystems.

Keywords: core microbiota, *Oxytropis glacialis*, QTP, soil fungal of root system, swainonine-producing fungal

INTRODUCTION

The Qinghai-Tibet Plateau (QTP) is commonly called the third pole in the world. Its ecological type is unique. Biodiversity bred in extreme environments is extremely rich and has special biological resources (Xiao et al., 2003). To adapt to this extreme environment, root microorganisms and plants have constructed a complex and stable interaction system, reflecting the adaptation strategies of microorganisms and plants (Hong et al., 2018). According to statistics, the alpine grassland area of the QTP accounts for 37.64% of the national grassland; out of this area, the alpine degraded grassland area accounts for nearly 1/3 of the QTP grassland area, which is as much as 425,000 Km^2 (Zhou et al., 2005; Tian et al., 2009). In recent years, the alpine grasslands on the QTP have been degraded more seriously. Locoweeds are an important part of the alpine grassland ecosystem. They are mainly distributed in alpine degraded grasslands, alpine grasslands, alpine saline deserts, and alpine deserts with high soil sand. The expansion rate of locoweed is consistent with the degradation rate of natural grassland, and some areas or local environments have an irreversible trend

(Hu et al., 2011; Chen et al., 2012). Among more than 20 locoweeds of *Oxytropis* in China, 11 species are distributed in the alpine grasslands of the Tibetan Plateau, including *Oxytropis glacialis*, *Oxytropis ochrocephala*, *Oxytropis sericopetala*, *Oxytropis glabra*, and *Oxytropis falcate* (Zhao et al., 2004). These species can quickly form dominant species or subdominant species in their habitats or local areas, and they pose a serious threat to the stability of the ecological environment. *O. glacialis* is one of the poisonous plants and is the most widely distributed and unique Madagascar genus of alpine grasslands on the Tibetan Plateau (Quan et al., 2009).

Oxytropis glacialis is a perennial herb with extremely shortened stems and well-developed root systems. It grows on hillside gravel, riverbeach gravel, sandy land, and other places at an altitude of 4500–5400 m. These plants are widely distributed in Tibetan Gaize, Bangor, Coqin, Geji, Zhongba, Tingri, Nima, Shenza, Shuanghu, Ritu, Pulan, Jilong, Sakya, and other places. The whole plant of *O. glacialis* is poisonous. It contains a toxic alkaloid swainonine (SW, 1,2,8-trihydroxyoctahydro indolizidine), which is highly toxic to animals and can cause dysfunction of the central nervous system of animals (Guo, 2005). Locoweed species that contain SW, produced by an endophytic fungal called *Embellisia oxytropis* rather than *Oxytropis* itself, have a strong ecotoxicological effect and play a key role in the degradation of alpine grasslands. In addition, SW-producing fungal play a certain role in many hosts and habitats (Yu et al., 2009). *E. oxytropis* can be isolated and purified from plant roots, stems, leaves, petioles, flowers, pods, and seeds (Cook et al., 2011), and its propagation occurs through vertical transmission of seeds (maternal transmission) or horizontal transmission between infected plants (Ralphs et al., 2008; Huang and Huang, 2012). Most of the current research on *O. glacialis* focuses on its ecological toxicology, the chemical properties and biological toxicity of SW, the life history of *E. oxytropis*, and the diversity of fungal in its root soil or whether the root soil contains SW; nevertheless, no research has been conducted on the horizontal transmission and transmission routes of fungal or swainonine (SW) fungal from root soil.

This study takes the soil fungal of *O. glacialis* roots from different ecological environments on the QTP as the research object. This research explores the spatial distribution, community diversity, and structure of the soil fungal of *O. glacialis* roots in different ecological environments, as well as its relationship with the soil physical and chemical factors to explore the possible impact of fungal community on the formation of dominant species of *O. glacialis* in alpine ecosystems. Besides, this study provides a reference for grassland degradation and desertification control in the QTP.

MATERIALS AND METHODS

Sampling Area Summary

Samples were taken from four locations (**Table 1**): the Yangbajing Basin, which is rich in geothermal resources; the core experimental area of the Everest National Nature Reserve; and Zarinanmucuo and Zabuyecuo, which are located in Qiangtang National Nature Reserve in the northern Tibetan Plateau. This scientific investigation has been reported to and approved by the Tibet Autonomous Region Forestry Department and the competent departments of nature reserves.

Plot Setting and Sampling Method

This research started in August 2017, and *O. glacialis* was sampled during the peak period. There were a total of four sampling points (**Table 1**). Each sample point was set with three repeats, and the distance between the three repeats was above 100 m (Dombrowski et al., 2017). A total of 12 samples were collected. In particular, soil of root system, also called root-zone soil, refers to soil that is loosely attached to or close to the roots, while bulk soil is soil outside the root zone, far from the plant's roots (Derr et al., 2016). For the sampling, the whole plant of *O. glacialis* was dug up, the bulk soil was shaken into a sterile bag for the determination of the soil physical and chemical factors, and then the soil of root system of the plants was rinsed into 95% ethanol and loaded into sterile tubes. Then, the samples were stored at −20°C in a car refrigerator (Meigu Mobicool, CF-50), brought back to the laboratory, and placed in a −80°C ultralow temperature refrigerator (Jiangsu Shenglan, DW86L-158) for cryopreservation. Finally, the samples were used for total DNA extraction of soil fungal microbiome.

Analysis of the Soil Physical and Chemical Properties

The environmental factors to be measured included pH value, soil moisture (SM), electrical conductivity (EC), organic matter (OM), available K (AK), available P (AP), and ammonium N (AN), for a total of seven indicators. The pH was measured by the potentiometer method (HANA, HI98103), and the ratio of water and soil was 1:1. The soil water content was measured by the high-temperature drying method. Five grams of fresh soil was weighed and placed in a constant-temperature drying box (Shanghai Jinghong, DK-420S) and measured at 105 ± 2°C for 48 h. The calculation formula was SM (%) = (5-dry weight)/5 × 100%. The conductivity was determined by the conductivity meter method (HANA, HI98304), and the water-soil ratio was 3:1. OM, available potassium, available phosphorus, and ammonium nitrogen were measured by a portable soil composition analyzer (Beijing Youputongyong, UPA–B506); OM measurement was based on the potassium complicate method, available potassium was based on ammonium acetate extraction-atomic absorption spectrophotometry, available phosphorus was based on sodium bicarbonate extraction-molybdenum antimony anti-spectrophotometry, and ammonium nitrogen is based on Nessler reagent colorimetry (Cao et al., 2019).

Extraction and Sequencing of Soil Fungal Total DNA From the Root System

The total DNA of the soil fungal of root system was extracted by the sodium dodecyl sulfate (SDS) plus enzyme method (Liu et al., 2015). The primers were ITS1F

TABLE 1 | Sample site information.

Location	Samples	Longitude (E)	Latitude (N)	Altitude/(m)	Ecotype
Yangbajing Town, Dangxiong County	YBJ	90.48799°	30.04268°	4,287	Alpine depleted range
Everest Base Camp, Dingri County	ZF	86.84309°	28.16793°	5,003	Alpine desert steppe
Zabuyecho, Zhongba County	ZBY	84.02377°	31.39431°	4,453	Alpine salt desert
Zharinanmucuo, Couqin County	CQ	86.04237°	31.03991°	4,710	Alpine steppe

(5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) for PCR amplification of the fungal ITS1-ITS2 region (Adams et al., 2013). PCR used a 20 μl reaction system: 10X buffer, 2 μl; 2.5 mM dNTPs, 2 μl; bidirectional primers (5 μM), 0.8 μl; rTaq polymerase, 0.2 μl; BSA, 0.2 μl; template, DNA 10 ng; and ddH2O supplement to 20 μl. The PCR parameters were 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, then 72°C for 10 min, and 10°C until halted by user. NanoDrop One was used to detect the purity and concentration of PCR products, and the samples were sent to Shanghai Majorbio Biopharm Technology Co., Ltd. for sequencing using the Illumina HiSeq platform.

Data Analysis

Using Illumina HiSeq sequencing to obtain paired-end double-end sequence splicing with FLAS software (FLAS 1.2.11), the raw tags obtained by Trimmomatic software were subjected to sequence defiltering to obtain high-quality clean tag data. Usearch software was used to perform sequence classification and annotation on clean tag data in the Silva database base, and clustering and species classification statistical analysis were performed based on the operational classification unit OTU (operational taxonomic unit) data of 97% similarity pairs (Nouioui et al., 2018). Analysis of similarities (ANOSIM) similarity analysis based on the minimum number of samples was used to determine whether the sample grouping setting is feasible. Mothur software (version v.1.30.1) was used to analyze the alpha diversity, including the Shannon, Sobs, Simpson, Ace, Chao, and coverage indexes, and a *t*-test was used to test the difference between the diversity groups. Beta diversity was analyzed through non-metric multidimensional scaling (NMDS) non-metric multidimensional scaling analysis. The bar graph and Venn diagram reflect the structure and function of fungal community. Different species and functional genes of different fungal community, using One_way ANOVA single factor analysis of variance, Kruskal-Wallis *H* test multiple test correction Fdr, *Post hoc* test Tukey-kramer level value is 0.95, and passed Linear discriminant analysis Effect Size (LEfSe) Discriminant analysis of multilevel species differences determined the species and genes with significantly different enrichment. In terms of fungal structure association and model prediction, random forest analysis was used to determine the ecological evolution process of the fungal community, and the network co-occurrence network and the correlation network were used to determine the core flora and key species. Using MEGA software, the selected SW-producing fungal sequences were combined with known related fungal sequences to construct an NJ phylogenetic tree. Variance expansion factor (VIF) was used to screen environmental factors and select environmental factors that have a greater effect on the flora, analyze the impact of environmental factors on the flora through canonical correspondence analysis (CCA) and use the correlation heatmap based on the Spearman coefficient to analyze the diversity of environmental factors on the flora and the specific impact of sexuality and structural function. The original pictures of related SVGs were grouped and processed by PS (Adobe Photoshop CS6) and AI (Adobe Illustrator CS6).

The raw sequencing read dataset was deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive with accession No. PRJNA630993.

RESULTS

Sequencing Results and Alpha and Beta Diversity Analysis

Sequencing Results

Based on high-throughput sequencing, a total of 711,620 original sequences were obtained, and 388,248 high-quality sequences were obtained after quality control. The average number of reads for each sample was 32,354. The number of OTUs annotated at a similar level of 97% was 1,014, including 5 phyla, 22 classes, 68 orders, 129 families, and 231 genera. The average coverage of the library was 99.91%, and the Shannon dilution curve tended to be flat (**Figure 1**), indicating that the sequencing data of this study are reasonable. More sequencing data would only generate a small number of new species OTUs, and most of the fungal are included in the library, which can reflect the community structure of fungal in the sample. The *R*-value obtained by ANOSIM similarity analysis was 0.9259, indicating that the difference between the groups was greater than the difference within the group, and the grouping was feasible. The Adonis substitution multivariate analysis of variance analysis showed that the *P*-value was 0.001, and the grouping reliability was high.

Alpha Diversity Analysis

Analysis of the species richness and diversity indexes of the four groups of samples included the six indexes of Ace, Chao, Coverage, Shannon, Simpson, and Sobs. **Table 2** shows that the species richness and diversity of the soil fungal in the roots of *O. glacialis* in the four regions were ranked as YBJ > CQ > ZF > ZBY. The richness index of Ace, Chao, and Sobs was the highest for YBJ and the lowest for ZBY. The Shannon diversity index is the highest for YBJ

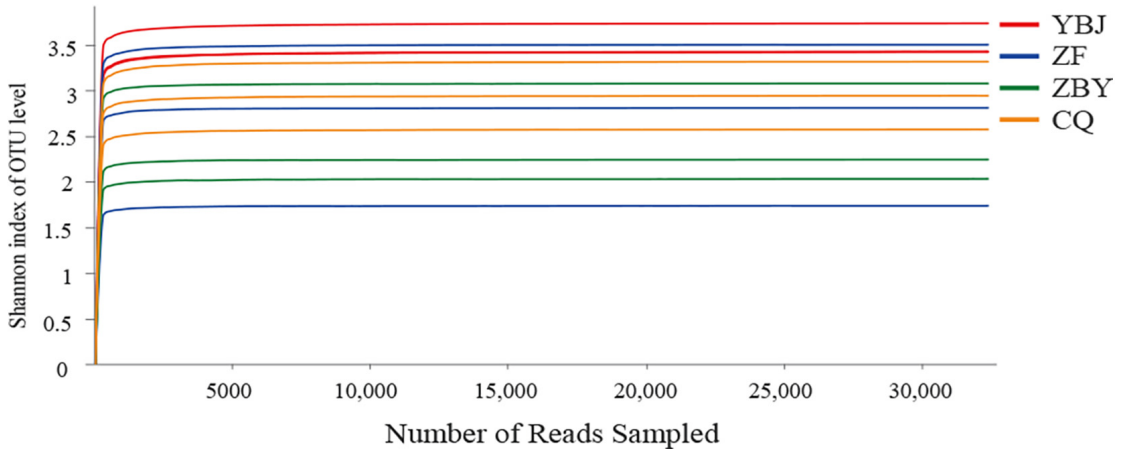


FIGURE 1 | Shannon dilution curve and diversity index difference.

TABLE 2 | The species richness and diversity index of soil fungal in the *Oxytropis glacialis* root system.

Samples	Sequences	OTUs	Alpha diversity					
			Ace	Chao	Coverage	Shannon	Simpson	Sobs
YBJ	53637	312	344	342.69	99.85%	3.53	0.06610	312
ZF	63856	161	178	182.11	99.92%	2.69	0.16196	161
ZBY	60764	123	128	129.94	99.97%	2.45	0.23022	123
CQ	58949	231	260	262.99	99.88%	2.95	0.16423	231

and the lowest for ZBY. The Simpson diversity index is the highest for ZBY and the lowest for YBJ; these findings are consistent with the performance of the Shannon index. The Coverage index table is between 99.85 and 99.97%; the ZBY has the highest coverage, while the YBJ has the lowest coverage.

Analysis of the Species Composition of the Soil Fungal Community in the *Oxytropis glacialis* Root System

Community Structure of Fungal Community at the Phylum and Genus Levels

According to the taxonomic analysis results, samples were annotated to a total of 5 phyla (**Figure 2A**), including Ascomycota (87.24%), Zygomycota (2.65%), Basidiomycota (1.99%), Chytridiomycota (0.83%), and Glomeromycota (0.01%), except for 7.29% that were unclassified. According to the statistics, the four groups of samples all contained more than five annotated fungal. Ascomycota was the main dominant entry for the YBJ (88.37%), ZF (76.32%), ZBY (89.63%), and CQ (94.63%) samples. The samples for YBJ (4.3%), ZF (16.61%), ZBY (4.7%), and CQ (3.54%) all accounted for a large proportion, indicating that there are abundant unknown potential taxa for the root fungal of *O. glacialis*. The average relative abundance of the four groups of samples was in the top 1%. The YBJ samples contained Zygomycota (3.48%) and Basidiomycetes (3.51%), the ZF samples contained Zygomycota (6.01%), the ZBY samples contained Basidiomycetes phyla (2.89%) and Phytophthora (2.54%), and the CQ samples had no average relative abundance in the top 1%.

The results of the genus level analysis (**Figure 2B**) show that all samples were annotated to 232 genera and 7.29% were undetermined taxa. The average relative abundance of the top 1% of the species was distributed in 20 genera of 3 phyla, accounting for 64.62% of all genera. Based on the genus level, a Venn diagram was used to count the number of common and unique genera in the four groups of samples (**Figure 2C**). There were 37 genera (15.95%) in four groups, 39 (16.81%) in three groups, 53 (22.84%) in two groups, and 103 (44.4%) in a single group, including 51 (21.30%) endemic genera in the YBJ sample, 25 (10.78%) endemic genera in the ZF sample, 11 (4.74%) endemic genera in the ZBY sample, and 16 (6.9%) endemic genera in the CQ sample. At the genus level, in at least two samples, the total genera accounted for 55.6%, and the endemic genera accounted for 44.4%. Statistics show that YBJ, ZF, ZBY, and CQ have 165, 104, 83, and 122 genera, respectively. The YBJ samples contain *Endocarpon* (15.72%), *Ascomycota* (11.99%), and *Pleosporales* (10.49%), the ZF samples contain *Gibberella* (14.76%), *Leptosphaeria* (13.63%), and *Cyphellophora* (10.08%), the ZBY samples mainly contain *Sordariomycetes* (33.60%) and *Podospora* (18.01%), and the CQ samples mainly contained *Ascomycota* (19.92%), *Gibberella* (15.70%), and *Coniosporium* (9.52%). The YBJ endemic species

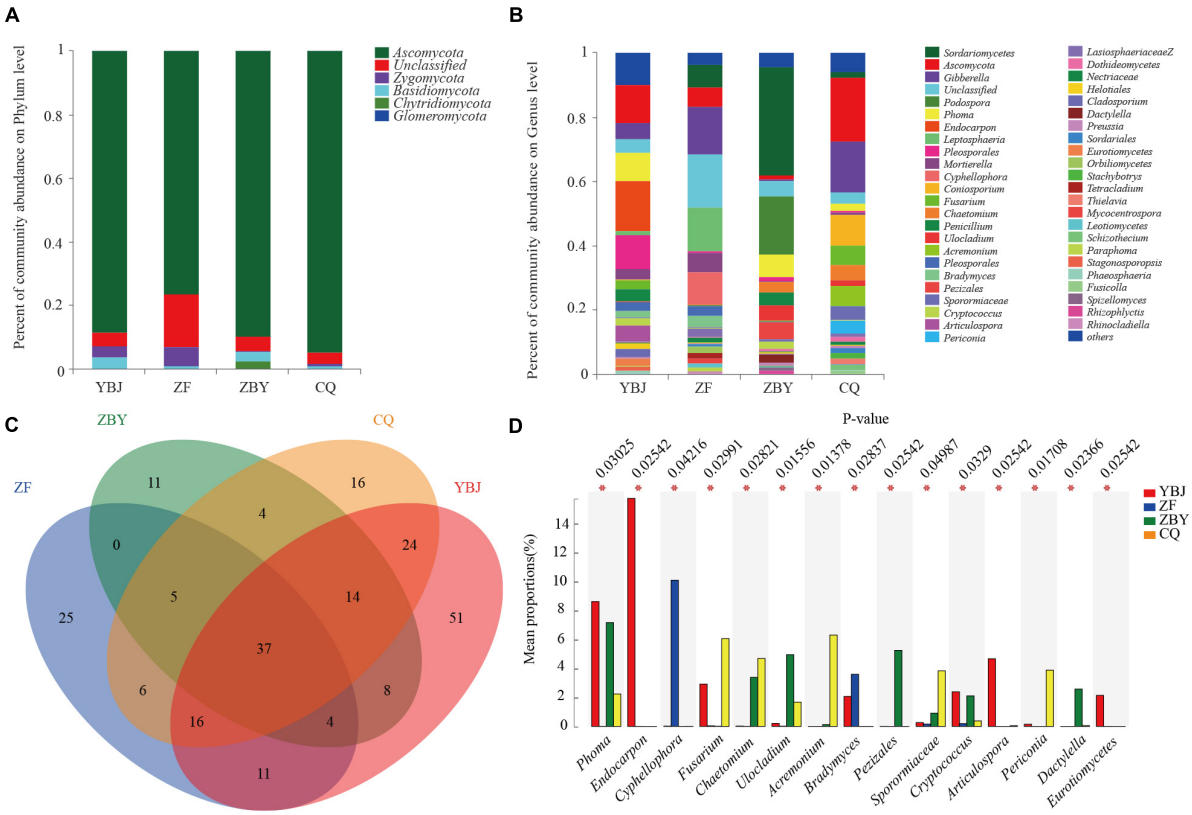


FIGURE 2 | Root system soil fungal community structure at the genus and phylum levels and a Venn diagram based on the genus level. **(A,B)** Fungal community structure based on the phylum and genus levels. **(C)** Venn diagram based on the genus level. **(D)** Analysis of differences between groups of fungal communities (*$0.01 < P \leq 0.05$).

are *Endocarpo*, *Trichoderma*, and *Bovista*, the ZF endemic species are *Orbiliomycetes*, *Rhinocladiella*, and *Spizellomycetales*, the ZBY endemic species are *Rotiferophthora*, and the CQ endemic species are *Amphisphaeriaceae* and *Ascobolaceae*.

Analysis of the Differences in the Soil Fungal of Root System Communities Between Different Groups

At the genus level, one-way analysis of variance (one-way ANOVA) was used to test the significance of the difference between the four groups of differential species. Then, the *post hoc* test was performed for the species with differences, resulting in the top 15 different species based on relative average abundance (**Figure 2D**), and four samples have significant differences ($P < 0.01$) in *Phoma*, *Endocarpon*, *Cyphellophora*, *Fusarium*, *Chaetomium*, *Ulocladium*, *Acremonium*, *Bradymyces*, *Pezizales*, *Sporormiaceae*, *Cryptococcus*, *Articulospora*, *Periconia*, *Dactylella*, and *Eurotiomyce* ($P < 0.01$).

Discriminant Analysis of LEfSe Multilevel Species Differences

To further determine the flora with significant differences in different ecological types (**Figure 3A**), LEfSe multilevel discriminant analysis (LDA $\geq$ 2) was used to count from the phylum to genus levels (**Figure 3B**). For YBJ, approximately eight types of fungal were significantly enriched: *Tremelellas* to *Cryptococcu*, *Coniochaetales*, *Amphisphaeriaceae*, *Phoma*, *Phaeosphaeriaceae*, *Dothideales* to *Selenophoma*, *Cladophialophora*, and *Chaetothyriales*. Nine types of fungal were significantly enriched for ZF, including *Hirsutella*, *Paraphoma*, *Lentitheciaceae* to *Darksidea*, *Leptosphaeriaceae*, *Helotiales* to *Tetracladium*, *Bradymyces*, *Leotiomycetes*, *Myxotrichaceae*, and *Chaetothyriaceae* to *Cyphellophora*. Five types of fungal were significantly enriched for CQ, including *Chaetomiaceae* to *Chaetomium*, *Thielavia*, *Fusarium*, *Hypocreales* to *Stachybotry*, and *Sporomiaceae*, and six types of fungal were significantly enriched for ZBY, including *Spizellomyces*, *Myrothecium*, *Pezizomycetes* to *Pezizales*, *Sordariales*, *Pleosporaceae* to *Curvularia*, and *Ulocladium*. At the same time, the main groups with a large impact on the difference effect were selected from the four ecological types through LDA linear regression analysis, resulting in YBJ, ZF, CQ, and ZBY containing 16, 14, 8, and 8 groups, respectively. CQ includes Hypocreales, *Fusarium*, Chaetomiaceae, *Chaetomium*, Sporormiaceae, Sporormiaceae, *Thielavia*, *Stachybotrys*. YBJ includes *Phoma*, Tremellales, *Cryptococcus*, Tremellales, Dothioraceae, Phaeosphaeriaceae, Dothideales, Coniochaetales, Coniochaetales

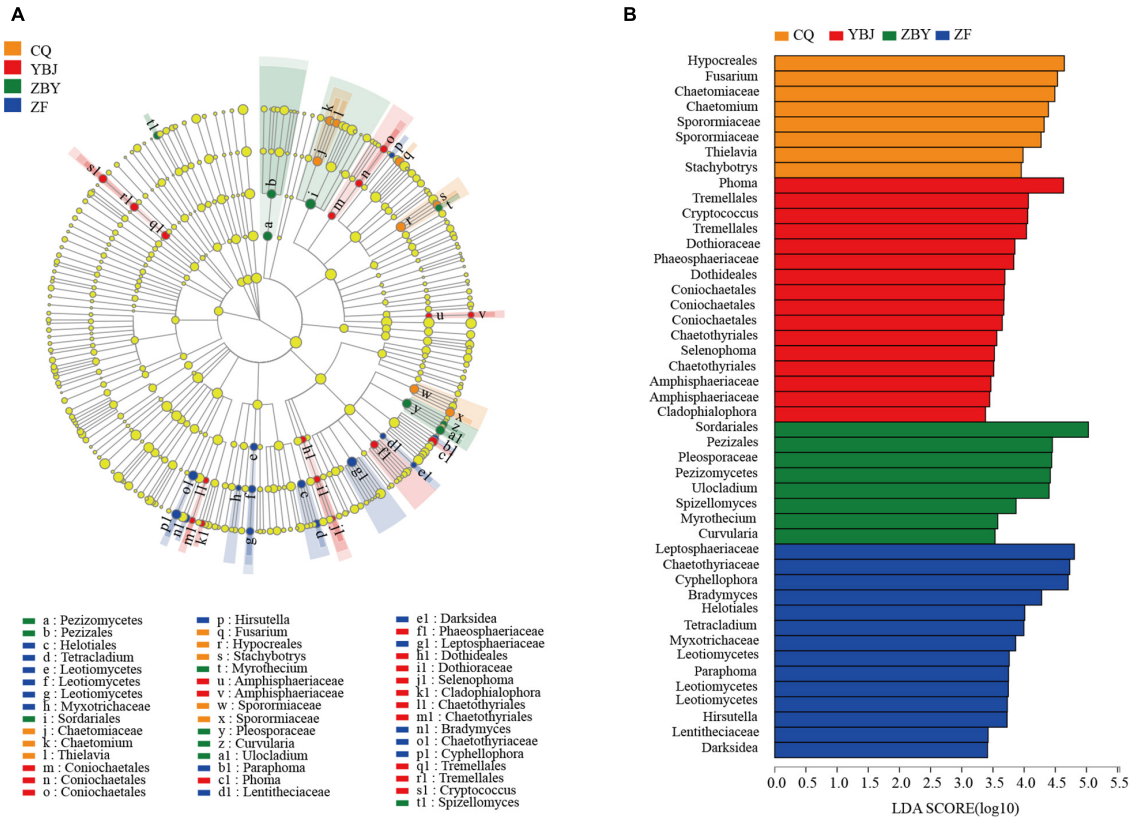


FIGURE 3 | LEfSe multilevel species hierarchy tree diagram and Latent Dirichlet Allocation(LDA) discrimination result diagram. **(A)** LEfSe multilevel species hierarchy tree diagram. **(B)** LDA discriminant result graph. The LDA discriminant column chart counts the microbial groups with significant effects in the four groups. The larger the LDA score is, the greater the impact of species abundance on the difference effect.

Coniochaetales, Chaetothyriales, *Selenophoma*, Chaetothyriales, Amphisphaeriaceae, Amphisphaeriaceae, *Cladophialophora*. ZBY includes Sordariales, Pezizales, Pleosporaceae, Pezizomycetes, *Ulocladium*, Spizellomyces, *Myrothecium*, *Curvularia*. ZF includes Leptosphaeriaceae, Chaetothyriaceae, *Cyphellophora*, Bradymyces, Helotiales, *Tetracladium*, Myxotrichaceae, Leotiomycetes, *Paraphoma*, Leotiomycetes, Leotiomycetes, *Hirsutella*, Lentitheciaceae, Darksidea.

Correlation and Model Prediction Analysis of Soil Fungal Community Structure in the *Oxytropis glacialis* Root System

Co-occurrence Network Analysis

By selecting all the species with the total abundance in the top 50 for co-occurrence network analysis, the coexistence relationship of the four groups of sample flora in environmental samples was obtained (**Figure 4A**). The nodes in the network represent the sample node and the species node, and the connection between the species node and the sample node represents that the sample contains this species. All samples and species have a total of 408 effective nodes (Degree). We found that the YBJ, ZF, ZBY, and CQ samples had 142, 83, 79, and 104 nodes, respectively. Approximately 13.36% of the genera that were collinear with the four groups of samples and at least 45.26% belonged to the same line in more than two groups of samples, indicating that the composition of the four groups of samples was quite different. Thirteen species in the four groups of samples had a collinearity rate of more than 1%, including *Sordariomycetes* (10.95%), *Ascomycota* (10.12%), *Gibberella* (9.30%), unclassified (7.53%), *Phoma* (4.65%), *Pleosporales* (4.85%), *Mortierella* (2.74%), *Fusarium* (2.33%), *Chaetomium* (2.09%), *Penicillium* (2.02%), *Ulocladium* (1.77%), *Sporormiaceae* (1.33%), and *Cryptococcus* (1.31%). We identified these 13 species as the core flora of the soil fungal community in the *O. glacialis* root system.

Correlation Network Analysis

Correlation network analysis (**Figure 4B**) revealed that the network contained a total of 17 nodes and 48 edges at the class level, indicating that the soil fungal community of the *O. glacialis* root system had lower connectivity and that the synergy of the overall community was greater than the antagonistic effect. There is greater competition among the colonies, among which the five classes of Ascomycota, Sordariomycetes, Agaricomycetes, Pezizomycetes, and Cystobasidiomycetes are highly correlated and interact with at least the other four classes, which plays a key role in the entire flora network. Ascomycota is the most critical class for the negative correlation of other species; Sordariomycetes is the most critical class for the

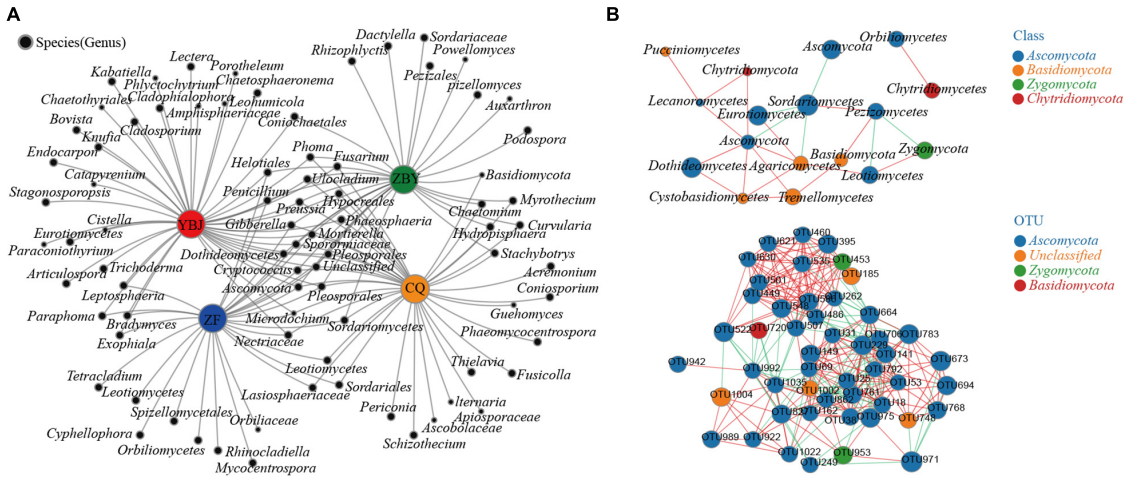


FIGURE 4 | Correlation network of cooccurring and correlation network analysis. **(A)** Collinear network analysis chart based on the genus level. **(B)** Correlation network analysis chart based on 35 phyla, showing species with | SpearmanCoef| > 0.8 and $P < 0.01$.

positive correlation with other species; and Orbiliomycetes and Chytridiomycetes are isolated from the main network and positively related to each other. At the OTU level, the overall positive correlation of the flora with abundance in the top 50 was greater than the negative correlation. The OTU belonging to Ascomycota dominates the interaction of the flora.

Effects of Soil Physical and Chemical Factors on the Fungal Community

The physical and chemical factors of the root soil of different samples are shown in **Table 3**. According to the analysis of VIF, OM, AK, AP, AN, pH, and SM were found to be significantly related to the structure of fungal community after screening the soil physical and chemical factors, which can reflect the impact on fungal community to the greatest extent.

Principal Coordinate Analysis and Canonical Correspondence Analysis

Through principal coordinate analysis (PCoA) (**Figure 5A**), the first sorting axis PC1 explained 20.16%, and the second sorting axis PC2 explained 19.25%; these findings explained 39.41% of the species composition changes. The four samples showed similarities in sex and differences. While the fungal were clustered together, the CQ and ZF samples were relatively similar, and the ZBY sample was discrete from the YBJ, CQ, and ZF samples, which showed a certain degree of heterogeneity.

The CCA analysis results show that the interpretation degree of the first sorting axis (CCA1) is 16.19% (**Figure 5A**), and the correlation coefficients with ammonium nitrogen, available phosphorus, pH and water content are −0.9261, −0.6635, 0.8143, and −0.8943, respectively. The second sorting axis (CCA2) has an explanation degree of 11.92%, and the correlation coefficients with ammonium nitrogen, available phosphorus, pH, and water content are 0.3774, −0.7482, 0.5805, and −0.4474, respectively. In addition, the two axes explain 28.11% of the composition change in species. The fungal community was significantly correlated with pH, water content and ammonium nitrogen ($P < 0.01$) and significantly correlated with available phosphorus ($P < 0.05$). The angle between available phosphorus (**Figure 5B**), ammonium nitrogen and water content was acute and positively correlated, and the angle between pH was obtuse and negatively correlated. Ammonium nitrogen, available phosphorus, pH and water content determine the distribution of community species. The coefficients (R^2 values) were 0.8099, 0.5544, 0.8664, and 0.8292, and the order of influence on the flora was pH > pH > SM > AP > AN.

Heatmap of the Correlation Between the Soil Physical and Chemical Factors and the Microflora

By calculating the Spearman rank correlation coefficient between the soil physical and chemical factors and the flora, the results of the heatmap diagram (**Figure 5C**) show that the soil physical and chemical factors have a significant impact on the fungal community, and different fungal are affected by the soil physical and chemical factors. The study revealed that soil organic matter, available phosphorus, ammonium nitrogen, and water content were positively correlated with the fungal community, while available potassium was mainly negatively correlated with the fungal microflora. We found that 12 genera were significantly positively correlated with the soil physical and chemical factors and that 13 genera were significantly negatively correlated with the soil physical and chemical factors. Additionally, soil available potassium, pH, and conductivity were significantly positively correlated with the genera of fungal belonging to Sordariomycetes and Pezizomycetes and negatively correlated with the genera of fungal belonging to Zygomycota, Chytridiomycota, and Ascomycota. Soil ammonium nitrogen, water content, available phosphorus, and OM were significantly positively correlated with the genera of fungal in Zygomycota, Chytridiomycota, and Ascomycota and negatively correlated with the genera of fungal belonging to

TABLE 3 | Nutrient and chemical properties in the soil of the *Oxytropis glacialis* root system.

Sample	pH	Soil moisture	Electrical conductivity/ (ms/cm)	Organic matte/ (mg/kg)	Available K/ (mg/kg)	Available P/ (mg/kg)	Available N/ (mg/kg)
YBJ	5.80 ± 0.06	0.143 ± 0.023	0.07 ± 0.01	145.9 ± 21.0	164.3 ± 39.3	8.91 ± 2.26	619.7 ± 18.6
ZF	7.45 ± 0.34	0.130 ± 0.007	0.11 ± 0.02	88.3 ± 7.3	134.8 ± 23.8	5.11 ± 1.06	594.9 ± 67.0
ZBY	8.82 ± 0.09	0.054 ± 0.008	0.24 ± 0.00	11.8 ± 0.2	503.9 ± 17.0	4.77 ± 2.58	320.5 ± 4.7
CQ	7.70 ± 0.09	0.091 ± 0.017	0.11 ± 0.00	102.8 ± 11.9	156.8 ± 10.6	4.59 ± 0.28	703.4 ± 96.7
VIF	34.64	8.76	48.19	20.82	35.01	2.53	7.85
Selected VIF	6.31	3.69	/	/	/	2.14	1.69

VIF represents the VIF value of the environmental factors before selection, selected VIF represents the VIF value of the environmental factors after screening, and "-" represents the environmental factors with insignificant related effects.

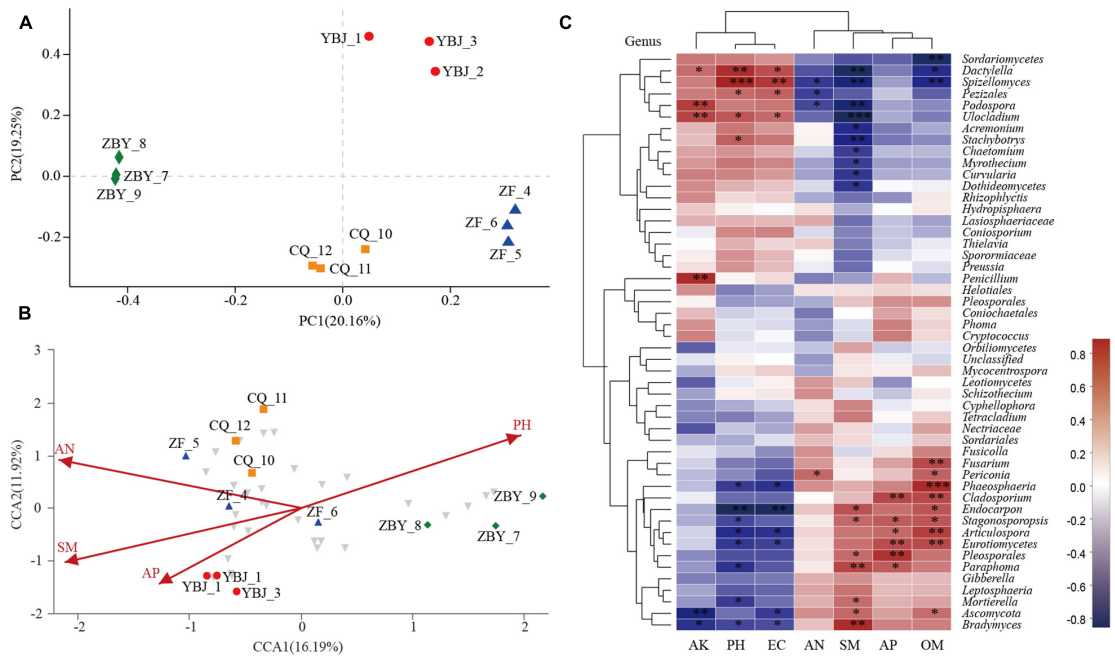


FIGURE 5 | Principal coordinate analysis and Canonical correspondence analysis and correlation heatmap map at the genus level. **(A)** Principal coordinate analysis (PCoA). **(B)** Canonical correspondence analysis (CCA). **(C)** Heatmap diagram of the correlation between soil physical and chemical factors and root-soil flora based on the genus level. *R* values are shown in different colors in the figure. *P*-values less than 0.05 are marked with * (*0.01 < $P \leq$ 0.05, **0.001 < $P \leq$ 0.01, ***$P \leq$ 0.001).

Sordariomycetes and Pezizomycetes. pH and *Spizellomycetaceae* were extremely positively correlated. *Spizellomycetaceae* can promote the growth of plant roots, promote the accumulation and absorption of nutrients through root tissues, and increase the nutrient and organic contents in the root soil. The water content is significantly negatively correlated with *Ulocladium*, which can assist plants in coping with drought resistance stress. OM and *Phaeosphaeria* are highly positively correlated ($P \leq 0.001$), and the intracellular and extracellular metabolites of *Phaeosphaeri* have the function of resisting pathogenic fungal in plants through spectral analysis.

Screening for SW-Producing Fungal

There were 158 unannotated fungal in the Ascomycota, Dothideomycetes, Pleosporales, Pleosporaceae, and *Alternaria* species, where the SW fungal were located. We constructed phylogenetic treesfrom a total of six fungal sequences (**Figure 6**), including SW-producing fungal and known SW-producing fungal, and found that OTU7 (66.61%), OTU592 (13.05%), OTU253 (9.46%), OTU567 (7.78%), OTU557 (2.09%), and OTU271 (1.00%) had the potential to produce swainonine. Among them, OTU576 was similar to *Embellisia grisea*; OTU557, OTU7, OTU592, OTU271, and OTU253 were similar to *E. Oxytropis*; and OTU253 and OTU271 were only distributed in YBJ.

DISCUSSION

The regional climate type is a plateau cold zone and subcold zone monsoon semiarid climate. The population variation

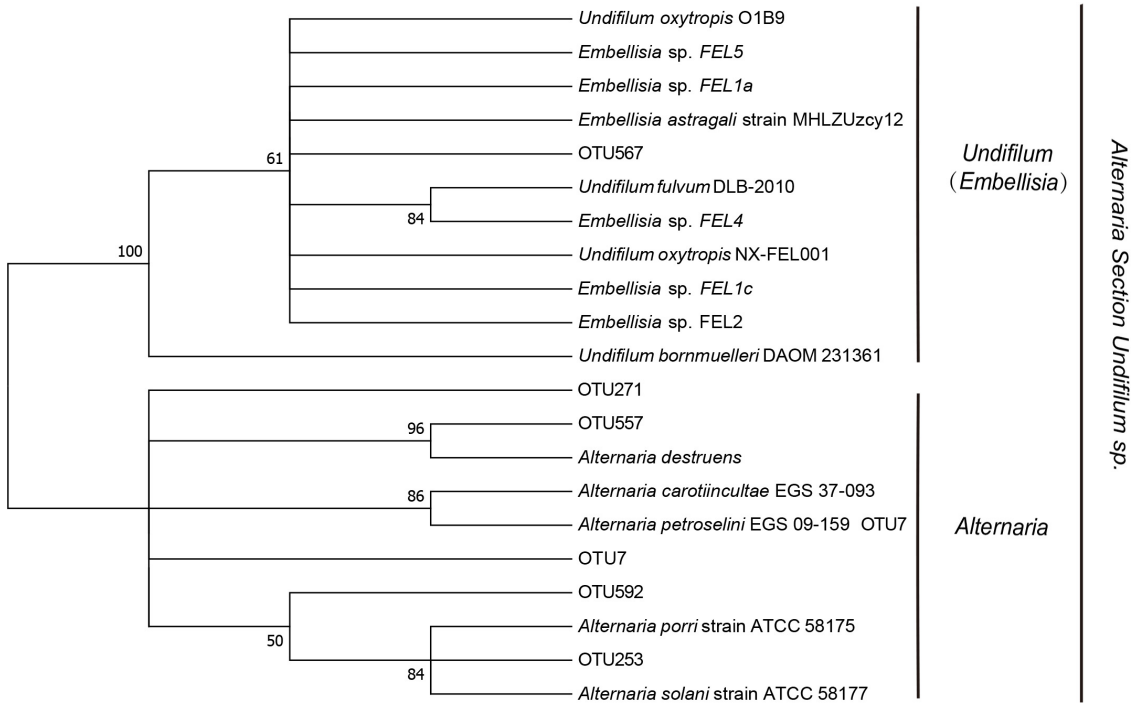


FIGURE 6 | Neighbor-Joining (NJ) phylogenetic tree based on ITS sequence. The number on the evolution tree represents the self-test support rate.

and distribution of soil microbes in plant roots have distinct characteristics, which are of great significance to the climate of the alpine grassland ecosystem on the QTP (Rui et al., 2015; Pérez-Jaramillo et al., 2016; Zhao et al., 2018). This study found significant differences in the soil fungal of the *O. glacialis* root system under different ecological types. Additionally, the diversity of fungal in the alpine grassland and the alpine degraded grassland was significantly higher than that in the alpine desert and alpine saline desert. In addition, locoweeds have better adaptability to this environment under extreme environmental conditions, whereas the growth of locoweed is not enough to make up for the biomass of other plants, which decreases the biomass of grassland and accelerates the degradation of alpine grassland.

The soil fungal microflora of plant roots in the same area is similar at phylum levels, and there are differences as the classification level is refined (Kowalchuk et al., 2002). In this study, as the fungal community structure was classified from the phylum to genus level during the process, the similarity of species gradually decreased, and a certain proportion of endemic genera appeared in various ecological types. Soil microbes respond to active plant selection (Chen et al., 2016). The *O. glacialis* root system is extremely developed, which helps to form a stable fungal community structure (Li, 2005). In recent years, relevant studies have been done about the diversity of soil fungal in the roots of alpine grasslands and alpine meadows and found that the dominant species are mostly distributed in Ascomycetes, Zygomycota, and Basidiomycota (Cui et al., 2019), which is similar to our research results. In addition, we annotated only ascomycetes, zygomycetes, basidiomycetes, chytridiomycetes, and Glomeromycota, while 7.29% of fungal community were unannotated, which is a great potential for the excavation of fungal resources on the QTP. Different ecological types and species that are significantly enriched lead to different fungal diversity and structural function distributions. The nerve co-occurrence network can be used to determine the core species of the flora that can maintain the diversity and structural function of the flora (Cardinale et al., 2015; Aschenbrenner et al., 2017). The 13 core fungal communities we found in this research are of great significance for the study of the adaptive evolution of locoweed such as *O. glacialis* in alpine grasslands. Moreover, it was found that the soil fungal community in the root system of *O. glacialis* increased with the classification from class to genus to OTU, and the connectivity of the microbiome-related network also became closer; thus, the fungal community tended to be more closely synergistic. For example, Ascomycota, Sordariomycetes, and Agaricomycetes have a key role in the construction of the microflora network, which reveals the adaptive evolution mechanism of fungal community on *O. glacialis* and plays an important role in extreme environments.

In previous studies, SW-producing fungal can only be isolated and identified in the roots, stems, petioles, leaves, seeds, fruit pods, and other parts of the locoweeds, and no one has conducted research on the root soil of its habitat. To study the root soil of its habitat, this study was the first to identify six OTUs with potential SW production from the root soil of *O. glacialis* by high-throughput sequencing technology. The SW-producing fungal was a pleasant surprise in our analysis

of the results. OTU clustering analysis and taxonomic analysis of species based on 97% similarity using Usearch 7.0 software in the Silva database. However, SW-producing fungal were not annotated. We speculate that SW-producing fungal have been reported in recent years and are not yet included in the Silva database. We believe that SW-producing fungal are present in the soil of the *O. glacialis* root system. We re-analyzed all OTUs using MEGA7 software. Six highly similar fungal sequences were obtained by comparison with fungal sequences that have been reported to SW-producing fungal and a phylogenetic tree was constructed. With this important finding, we hope to validate it in subsequent experiments and obtain more meaningful results. This research provided evidence for the further study of the horizontal propagation of SW-producing plants. The SW-producing fungal of locoweeds have a greater ecotoxicological effect on alpine grassland ecosystems and exacerbate grassland degradation (Zhao et al., 2010). Research related to grass-produced SW fungal is in its infancy. In previous studies, gramineous plant fungal in grasslands can improve plant stress resistance such as to extreme environments, pests, diseases, cold drought, saline conditions, and alkali conditions (Nan and Li, 2004; Huang and Huang, 2008), and related studies have shown that phosphorus stress and osmotic stress stimulate seedling growth of *O. punctatus* (Cui et al., 2009). Locoweeds can grow better under low pH and drought stress in the field (Barillas et al., 2007). Locoweeds containing SW fungal reduce the number of animals that feeding on them, but the ecological effects of SW fungal on *locoweeds* have not been studied in depth.

The diversity and differential distribution of soil microbes in roots are driven by the soil physical and chemical factors (Schmidt et al., 2011; Yuan et al., 2014). Studies have shown that the soil microbial diversity in roots is mainly affected by soil pH and OM (Nacke et al., 2011). Yang et al. (2014) discovered that soil pH, temperature, ammonium nitrogen, and plant diversity are the most important factors that affect the diversity and structural function of flora. In this study, we also found that pH, water content, and ammonium nitrogen are the main factors that jointly drive the spatial variability of the fungal community in the root system of *O. glacialis*.

CONCLUSION

In conclusion, the soil fungal of the *O. glacialis* root system in the different ecological environments of the QTP are rich in fungal diversity, and its core flora is stable. The spatial variation in fungal diversity is driven by pH, SM, and available N. It was also found for the first time that swainonine-producing fungal coexist in rhizosphere soil microorganisms of *O. glacialis*. This finding provides data to support the next step in demonstrating the horizontal spread of swainone-producing fungal from *O. glacialis* to soil. In addition, a stable network of core flora has a facilitating effect on the formation of *O. glacialis* as a dominant species in alpine ecosystems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

P-XC drafted the manuscript. P-XC, YL, NZ, and G-QX collected the samples. P-XC, YL, and XL conceived and designed the experiments. P-XC, H-MM, NZ, and G-QX performed the experiments and data analysis. P-XC, S-TC, and XL revised the manuscript. All authors have read and approved the submitted version.

FUNDING

The work described in this manuscript was supported by the Tibet Autonomous Region Science and Technology Project [XZ202001ZR0023G, XZ2019ZRG-12(Z)], the Campus Ecological Campus Construction of Tibet University 2020 Central Financial Support Special Funds for local colleges and Universities ([2020] No. 01), 2020 Central Financial Support Special Funds for Local Colleges and Universities (XZ202001YD0028C), and the Growth Plan of University-Level Cultivation Project of Tibet University (ZDCZJH21-04).

ACKNOWLEDGMENTS

We thank Xiao-yan Li (Wuhan University) and Tajie (Tibet University) for their help in the field experiment.

REFERENCES

Adams, R. I., Miletto, M., Taylor, J. W., and Bruns, T. D. (2013). Dispersal in microbes: Fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. *ISME J.* 7, 1262–1273. doi: 10.1038/ismej.2013.28

Aschenbrenner, I. A., Cernava, T., Erlacher, A., Berg, G., and Grube, M. (2017). Differential sharing and distinct co-occurrence networks among spatially close bacterial microbiota of bark, mosses and lichens??. *Mol. Ecol.* 26, 2826–2838. doi: 10.1111/mec.14070

Barillas, J. R. V., Paschke, M. W., Ralphs, M. H., and Child, R. D. (2007). White locoweed toxicity is facilitated by a fungal endophyte and Nitrogen-Fixing bacteria. *Ecology* 88, 1850–1856. doi: 10.1890/06-0728.1

Cao, P. X., Liu, Y. X., Xu, G. Q., Chen, F. F., Zhen, H., and Liu, X. (2019). Study on bacterial diversity rhizosphere soil of *Androsace tapete*. *Environ. Ecol.* 1, 1–9.

Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., and Berg, G. (2015). Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environ. Microbiol.* 17, 239–252. doi: 10.1111/1462-2920.12686

Chen, J. P., Zhao, B. Y., Lu, H., Ding, L. D., Zhang, G. F., Li, X., et al. (2012). Study on Isolation, Identification and the Antibacterial Activity of Endophytic Fungi from the Major Locoweed in China. *Acta Veterinaria Zootechnica Sinica* 43, 1471–1478. doi: 10.1007/s11783-011-0280-z

Chen, W. L., Li, J., Zhu, H. H., Chen, J. Z., and Yao, Q. (2016). A review of the regulation of plant root system architecture by rhizosphere

microorganisms. *Acta Ecol. Sin.* 36, 5285–5297. doi: 10.5846/stxb201502260390

Cook, D., Gardner, D. R., Grum, D., Pfister, J. A., Ralphs, M. H., Welch, K. D., et al. (2011). Swainsonine and Endophyte Relationships in Astragalus mollissimus and Astragalus lentiginosus. *J. Agr. Food Chem.* 59, 1281–1287. doi: 10.1021/jf103551t

Cui, C., Yu-mei, W., Meng-li, Z., Guo-dong, H., Na, L., and Ai-ping, Z. (2009). Effects of Endophyte on Germination, Growth and Resistance to Osmotic Stress of *Oxytropis glabra*. *Chin. J. Grassland* 31, 84–89.

Cui, Y., Bing, H., Fang, L., Wu, Y., Yu, J., Shen, G., et al. (2019). Diversity patterns of the rhizosphere and bulk soil microbial communities along an altitudinal gradient in an alpine ecosystem of the eastern Tibetan Plateau. *Geoderma* 338, 118–127. doi: 10.1016/j.geoderma.2018.11.047

Derr, D. C., Desgarennes, D., Garcia, C. F., Gross, S., Clingenpeel, S., Woyke, T., et al. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. *New Phytol.* 209:13697. doi: 10.1111/nph.13697

Dombrowski, N., Schlaeppi, K., Agler, M. T., Hacquard, S., Kemen, E., Garrido-Oter, R., et al. (2017). Root microbiota dynamics of perennial Arabis alpina are dependent on soil residence time but independent of flowering time. *ISME J.* 11, 43–55. doi: 10.1038/ismej.2016.109

Guo, L. H. (2005). Swainonine research. *Jiangxi Chem. Ind.* 3, 4–8. doi: 10.14127/j.cnki.jiangxihuagong.2005.03.002

Hong, J., Ma, X., Yan, Y., Zhang, X., and Wang, X. (2018). Which root traits determine nitrogen uptake by alpine plant species on the Tibetan Plateau? *Plant Soil* 424, 63–72. doi: 10.1007/s11104-017-3434-3

Huang, D. Y., and Huang, X. L. (2008). Research and application on fungal endophytes on forage grassess. *Acta Pratacult. Sin.* 17, 128–136. doi: 10.3321/j.issn:1004-5759.2008.03.019

Huang, D. Y., and Huang, X. L. (2012). Study on Isolation, Identification and the Antibacterial Activity of Endophytic Fungi from the Major Locoweed in China. *Chin. J. Anim. Vet. Sci.* 43, 1471–1478.

Hu, Y. C., Liu, L., Liu, P., and Wang, J. H. (2011). An overview of ecological research on locoweed. *Prog. Vet. Med.* 32, 108–112. doi: 10.16437/j.cnki.1007-5038.2011.03.023

Kowalchuk, G. A., Buma, D. S., de Boer, W., Klinkhamer, P. G., and Van Veen, J. A. (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek* 81, 509–520. doi: 10.1023/a:1020565523615

Li, Q. F. (2005). *Studies on Toxicity and Bacteria Degradation of Oxytropis Glacialis Toxin*. Xi'an: Northwest A&F University. Available online at: http://cdmd.cnki.com.cn/article/cdmd-10712-2005111762.htm

Liu, L., He, J. S., Xie, Q., and Wang, K. L. (2015). Genetic diversity of rhizobia isolated from common legumes in the Karst area, Northwest. .*Chin J. Appl. Ecol.* 26, 3663–3669. doi: 10.13287/j.1001-9332.20150929.014

Nacke, H., Thürmer, A., Wollherr, A., Will, C., Hodac, L., Herold, N., et al. (2011). Pyrosequencing-Based assessment of bacterial community structure along different management types in german forest and grassland soils. *PLoS One* 6:e17000. doi: 10.1371/journal.pone.0017000

Nan, Z. B., and Li, C. J. (2004). Roles of the grass-Neotyphodium association in pastoral agriculture systems. *Acta Ecol. Sin.* 3, 605–616.

Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J. P., Woyke, T., Kyrpides, N. C., et al. (2018). Genome-Based taxonomic classification of the phylum actinobacteria. *Front. Microbiol.* 9:2007. doi: 10.3389/fmicb.2018.02007

Pérez-Jaramillo, J. E., Mendes, R., and Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol. Biol.* 90, 635–644. doi: 10.1007/s11103-015-0337-7

Quan, L., Jun-nian, L., Shuang-lun, T., Yu-jing, D., Lan, H., and Zheng, W. (2009). Research progress on toxicity, harm and prevention of *Oxytropis*. *Pratacult. Sci.* 26, 99–105.

Ralphs, M. H., Creamer, R., Baucom, D., Gardner, D. R., Welsh, S. L., Graham, J. D., et al. (2008). Relationship between the endophyte embellisia spp. And the toxic alkaloid swainsonine in major locoweed species (*Astragalus* and *oxytropis*). *J. Chem. Ecol.* 34, 32–38. doi: 10.1007/s10886-007-9399-6

Rui, J., Li, J., Wang, S., An, J., Liu, W., and Lin, Q. (2015). Responses of bacterial communities to simulated climate changes in alpine meadow soil of the Qinghai-Tibet plateau. *Appl. Environ. Microb.* 81, 6070–6077. doi: 10.1128/AEM.00557-15

Schmidt, M. W. I., Torn, M. S., Abiven, S., Dittmar, T., Guggenberger, G., and Janssens, I. A. (2011). Persistence of soil organic matter as an ecosystem property. *Nature* 478, 49–56. doi: 10.1038/nature10386

Tian, Y. Q., Gao, Q., Zhang, Z. C., Zhang, Y., and Zhu, K. (2009). The advances in study on plant photosynthesis and soil respiration of alpine grasslands on the Tibetan Plateau. The advances in study on plant photosynthesis and soil respiration of alpine grasslands on the Tibetan Plateau. *Ecol. Environ. Sci.* 18, 711–721. doi: 10.3969/j.issn.1674-5906.2009.02.058

Xiao, Y., Xie, G. D., and An, K. (2003). The function and economic value of soil conservation of ecosystems in Qinghai-Tibet Plateau. *Acta Ecol. Sin.* 23, 2367–2378. doi: 10.3321/j.issn:1000-0933.2003.11.021

Yang, Y., Gao, Y., Wang, S., Xu, D., Yu, H., Wu, L., et al. (2014). The microbial gene diversity along an elevation gradient of the Tibetan grassland. [Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, Non-P.H.S.]. *ISME J.* 8, 430–440. doi: 10.1038/ismej.2013.146

Yu, Y., Wang, J., Wang, Y., Song, Y., Geng, G., and Li, Q. (2009). Identification of swainonine-Producing fungal endophytes from three species of locoweeds in tibet. *Sci. Agricult. Sin.* 43, 1471–1478.

Yuan, Y., Si, G., Wang, J., Luo, T., and Zhang, G. (2014). Bacterial community in alpine grasslands along an altitudinal gradient on the Tibetan Plateau. [Journal Article; Research Support, Non-U.S. Gov't]. *FEMS Microbiol. Ecol.* 87, 121–132. doi: 10.1111/1574-6941.12197

Zhao, B., Liu, Z., Lu, H., Wang, Z., Sun, L., Wan, X., et al. (2010). Damage and control of poisonous weeds in western grassland of china. *Agricult. Sci. China* 9, 1512–1521. doi: 10.1016/S1671-2927(09)60242-X

Zhao, C. Z., Fan, S. Y., Yin, C. Q., and He, X. B. (2004). Study on vegetation community's structure of degraded grassland of noxious and miscellaneous grass type. *J. Desertres.* 4, 507–512. doi: 10.3321/j.issn:1000-694X.2004.04.023

Zhao, S., Zhuang, L., Wang, C., Li, Y., Wang, S., and Zhu, G. (2018). High-throughput analysis of anammox bacteria in wetland and dryland soils along the altitudinal gradient in Qinghai-Tibet Plateau. *MicrobiologyOpen* 7:e556. doi: 10.1002/mbo3.556

Zhou, H., Zhao, X., Tang, Y., Gu, S., and Zhou, L. (2005). Alpine grassland degradation and its control in the source region of the Yangtze and Yellow Rivers, *China*. *Grassl. Sci.* 51, 191–203. doi: 10.1111/j.1744-697X.2005.00028.x

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frontiers in Microbiology

REVIEW
published: 02 March 2022
doi: 10.3389/fmicb.2022.835931

Metabolomic Insights Into Endophyte-Derived Bioactive Compounds

Sushma Mishra[1]†, Priyanka[2]† and Shilpi Sharma[2]**

[1] Plant Biotechnology Laboratory, Dayalbagh Educational Institute, Deemed-to-be-University, Agra, India, [2] Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi, India

Among the various plant-associated microbiota, endophytes (the microbial communities inhabiting plant endosphere without causing disease symptoms) exhibit the most intimate and specific association with host plants. Endophytic microbes influence various aspects of plant responses (such as increasing availability of nutrients, tolerance against biotic and abiotic stresses, etc.) by modulating the primary and secondary metabolism of the host. Besides, endophytic microbes produce a diverse array of bioactive compounds, which have potential applications in the pharmaceutical, food, and cosmetic industries. Further, there is sufficient evidence for endophyte-derived plant metabolites, which could be pursued as alternative sources of commercially important plant metabolites. The field of bioprospecting, the discovery of novel chemistries, and endophyte-mediated production of plant metabolites have witnessed a boom with the advent of *omics* technologies (especially metabolomics) in endophyte research. The high throughput study of small metabolites at a particular timepoint or tissue forms the core of metabolomics. Being downstream to transcriptome and proteome, the metabolome provides the most direct reflection of the phenotype of an organism. The contribution of plant and microbial metabolomics for answering fundamental questions of plant-endophyte interaction, such as the effect of endophyte inoculation on plant metabolome, composition of metabolites on the impact of environmental stressors (biotic and abiotic), etc., have also been discussed.

Keywords: metabolomics, bioprospecting, bioactive compounds, natural products, endophytes

OPEN ACCESS

Edited by:
Prem Lal Kashyap, Indian Institute of Wheat and Barley Research (ICAR), India

Reviewed by:
Vipin Kumar Singh, Banaras Hindu University, India
Ajay Kumar, Agricultural Research Organization (ARO), Israel

***Correspondence:**
Sushma Mishra
sushmamishra87@gmail.com
Shilpi Sharma
shilpi@dbeb.iitd.ac.in

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *15 December 2021*
Accepted: *31 January 2022*
Published: *02 March 2022*

Citation:
Mishra S, Priyanka and Sharma S (2022) Metabolomic Insights Into Endophyte-Derived Bioactive Compounds. Front. Microbiol. 13:835931. doi: 10.3389/fmicb.2022.835931

INTRODUCTION

Bioactive compounds are metabolites that affect one or more metabolic processes, resulting in the improvement of health conditions (Angiolillo et al., 2015). Chemically, bioactive compounds are a heterogeneous group, including peptides, saccharides, phenolics, terpenes, and alkaloids. They may have widespread biological effects such as antimicrobial, antioxidant, anticancerous, etc., and hence, have great applications in the pharmaceutical, cosmetic, and food industries. The increase in population and emergence of new virulent strains and drug-resistant pathogens necessitates both, increasing the production, as well as discovering novel bioactive compounds (Gakuubi et al., 2021). Plant metabolites are a rich source of pharmacologically important compounds that aid in drug discovery and contribute to ∼80% of all synthetic drugs (Bauer et al., 2014). However, the traditional approach of extracting secondary metabolites (SM) from medicinal plants, suffers

from several limitations including seasonal dependence, a gap in demand and supply, loss of biodiversity, the endangered status of many plant species, and increased costs. Here, it needs to be mentioned that plants are not always the sole producers of their SMs; endophytes (the microorganisms inhabiting the endosphere of plants without causing disease symptoms) have been found to produce host (plant) metabolites including taxol, vincristine, vinblastine, camptothecin, hypericin, etc. Endophytes are associated with almost every higher plant studied till date, inhabiting both above ground and below ground plant parts (Rigobelo and Baron, 2021). The significance of endophytes in determining the host plant's overall fitness has been long established (Gouda et al., 2016). Besides their role in nutrient management (Johnston-Monje and Raizada, 2011; Cipriano et al., 2021; García-Latorre et al., 2021), their contribution to alleviation of stresses is significant (Verma et al., 2021; White et al., 2021). This is majorly governed through production of bioactive metabolites. The cross talk between plant and endophyte, which is affected by various abiotic and biotic factors, regulates the production of bioactive metabolites (Rai et al., 2021). This has been extensively reviewed by Mishra et al. (Mishra et al., 2021a,c).

BIOACTIVE COMPOUNDS FROM ENDOPHYTES

The endophytic microbiota inhabit diverse niches within plants, interacting with numerous co-occurring microbes, and displaying different lifestyles (mutualistic, commensal, and parasitic) (Mishra et al., 2021a). The ability of endophytes, especially fungi and actinomycetes, to produce a plethora of bioactive compounds (**Supplementary Table 1**) could be attributed to various reasons: chemical defense of plants due to their long term association (reported from fossil plants as well), interaction with co-occurring microbes, and protection of their niche (within plant endosphere) against attacking pathogen species (Krings et al., 2007; Kusari et al., 2014a; Mishra et al., 2021a). Several endophyte-mediated/assisted plant responses are mediated through the production of a myriad of compounds by these microbial communities (Maciá-Vicente et al., 2018).

The diversity of endophytes, and thereby of the bioactive compounds produced by them, is dependent on a range of factors including plant species and parts (Wang et al., 2019; Guevara-Araya et al., 2020; Harrison and Griffin, 2020), and environmental factors (Giauque and Hawkes, 2013). Amongst environmental factors, cultivation history (Correa-Galeote et al., 2018), and climate and season (Zimmerman and Vitousek, 2012; Hosseyni-Moghaddam and Soltani, 2014; Wang et al., 2019; Oita et al., 2021) have been established as important criteria in shaping the endophytic communities in plants.

Most of the protocols for discovery of bioactive compounds from microbial culture involve axenic cultivation (either submerged or solid-state fermentation) for 2–4 weeks, followed by bioassay-based screening (eg. anticancerous, antimicrobial, etc.) to guide the subsequent purification process. Alternatively, PCR-based screening for biosynthetic gene clusters (BGCs) such as non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS), could provide crucial leads for identifying promising endophytic isolates (Mohamad et al., 2018). Further, approaches such as co-cultivation with host plant extract or co-occurring microbes, the addition of epigenetic modifiers, and heterologous expression have been used to activate the cryptic BGCs and induce the expression of additional metabolites (Toghueo et al., 2020). These procedures involving optimization of fermentation conditions through standardization of physico-chemical parameters and addition of elicitors are time-consuming and cumbersome. The application of metabolomics has facilitated the rapid and (almost) complete screening for metabolites, identification of novel compounds and metabolite markers in plant and endophyte extracts. This review focuses on the application of metabolomic techniques in endophyte research for the identification of economically important compounds (including plant metabolites), the discovery of novel metabolites, and offering insights into the functional role of endophytes in plant holobiont, but before that, a brief account of the technique is presented.

METABOLOMICS APPROACH: A BRIEF OVERVIEW

With the advent of *omics* technologies (mainly metagenomics, metatranscriptomics, metaproteomics, and microbial metabolomics) in plant microbiome research, there has been a leap in our understanding of the composition and abundance of plant-associated microbial diversity, and the role of the plant microbiome in affecting host's responses (Kaul et al., 2016). While metagenomic analyses of plant samples provide a snapshot of the abundance and functional potential of the plant-associated microbiota (Sessitsch et al., 2012; Mishra et al., 2021b), the metatranscriptomic and metaproteomic approaches reveal the "expressed" (RNA and protein components, respectively) fraction of the microbial genome (Liao et al., 2019; Guo et al., 2021). However, since metabolites constitute the last step of gene expression, the high throughput quantitative analysis of metabolites would provide the ultimate and most authentic representation of the physiological state of an organism. Broadly, metabolite analysis can be performed at various levels: (i) target analysis (precise measurement of the concentration of a limited number of known metabolites), (ii) metabolite profiling (non-targeted measurement of metabolite levels), (iii) metabolic fingerprinting (generates total profile or fingerprint, representing a snapshot of the metabolism, without precise quantification of metabolites), and (iv) metabolomics (detection and quantification of thousands of metabolites, defining the phenotype of the biological system) (Shulaev et al., 2008; Porzel et al., 2014).

Metabolomics is the high throughput study of small metabolites (Mr $\leq$ 1 kDa) present in a sample (Nalbantoglu, 2019). Such metabolites, including peptides, sugars, fatty acids, organic acids, vitamins, etc., could represent intermediates or end-products of metabolic pathways, or those that are formed due to the impact of environmental factors or biotic stressors (Castro-Moretti et al., 2020). The analysis of plant and endophyte

metabolomes provide a direct insight into the contribution of microbiome toward host plant phenotype, as well as the impact of environment on plant-microbe interactions, thereby serving as sensitive and accurate markers. Moreover, the ability to scale up and analyze hundreds of samples simultaneously has brought a revolution in the field of natural product discovery and bioprospecting of endophytes to produce diverse compounds. The application of microbial metabolomics enables the complete analysis of crude extracts as well as the identification of marker metabolites (associated with specific bioactivities), before undergoing tedious and laborious downstream processes (Mohamed et al., 2020).

Based on the objective of the study, there could be two basic experimental designs for conducting a metabolomic analysis, viz. untargeted/non-targeted and targeted metabolomics (**Figure 1**). As the name suggests, non-targeted metabolomics involves a comprehensive analysis of numerous small metabolites in the sample. The sample preparation includes the extraction of metabolites using two or more solvents to increase the range of detection of chemically diverse metabolites. This is a hypothesis-generating method and enables the discovery of novel compounds. On the other hand, targeted metabolomics, also known as directed metabolomics, focuses on the quantitative analysis of a specific class of metabolites (∼50 metabolites), usually by tandem MS/MS method against a standard. It is often performed for validating the results of untargeted metabolomics (Tebani et al., 2018). Therefore, the non-targeted and targeted metabolomic approaches could be summarized as "untargeted-discovery-global" and "targeted-validation-tandem, respectively (Nalbantoglu, 2019).

There are two analytical platforms for metabolite detection, viz. nuclear magnetic resonance (NMR) and mass spectrometry (MS)-based; both with their own set of strengths and drawbacks (**Table 1**). Next, separation techniques such as gas chromatography (GC; for volatile molecules such as organic acids, fatty acids, etc.), liquid chromatography (LC; for thermo-labile substances), and capillary electrophoresis (CE; combines the advanced technology of electrophoresis and microcolumn separation) are used in conjunction with MS or NMR equipment. The readers may refer to a recent review on analytical techniques for metabolomics by Segers et al. (2019).

METABOLOMICS FOR INVESTIGATION OF ENDOPHYTE METABOLOME

Bioassay-Based Screening of Crude Extracts of Endophyte

Metabolomic tools have been used to compare the metabolic profile obtained from *Aspergillus aculeatus* (an endophyte isolated from *Terminalia laxiflora*) inoculated in solid and liquid media (Tawfike et al., 2017). The use of rice culture media was reported to produce a higher number of metabolites than liquid culture media. Next, using MS-based metabolomics, the group reported the production of seven metabolites with anticancerous and/or antitrypanosomal properties from the fermentation culture of *Aspergillus flocculus*, an endophyte isolated from the stem of *Markhamia platycalyx* (an Egyptian medicinal plant) (Tawfike et al., 2019). Specifically, five out of the seven metabolites detected in 30-day-old rice culture media demonstrated antagonistic activity against chronic myelogenous leukemia cell line K562, and two exhibited strong activity against the parasite *Trypanosoma brucei brucei* (causal organism of sleeping sickness).

In another report, Sebola et al. (2020) analyzed the antibacterial (against 11 pathogenic bacteria constituting both Gram-positive and Gram-negative bacteria) and anticancer activity (against glioblastoma and lung carcinoma cell lines) of ethyl acetate extracts of bacterial endophytes (*Bacillus safensis, Pseudomonas cichorii,* and *Arthrobacter pascens*) isolated from *Crinum macowanii* (commonly known as Cape coast lily; used to cleanse blood, and treat cough and kidney diseases) leaves. Further, using LC-Q-TOF-MS analysis of crude extracts of the bacterial endophytes revealed the presence of antibacterial and anticancerous compounds such as lycorine, angustine, crinamidine, and powelline. In another similar report, Nischitha and Shivanna (2021) analyzed the antimicrobial and antioxidant activities of crude extract of *Alloteropsis cimicina* (a grass species) and its endophytic fungus *Penicillium pinophilum*; the antioxidant potential of the fungal extract being more than the host extract. Metabolite profiling using Orbitrap High-Resolution (OHR) LC–MS and Fourier-transform infrared spectroscopy (FTIR) analysis identified 21 antimicrobial and 13 antioxidant compounds in the fungal extract. Some of the identified antimicrobial compounds included L-tyrosine, L-isoleucine, acetophenone, phenacetin, dextramycin, and sulfamethazine; and antioxidant compounds include citrinin, zearalenone, and bilirubin ((Nischitha and Shivanna, 2021). Interestingly, there were eight compounds (namely L-tyrosine, acetophenone, 4-methoxycinnamic acid, isophorone, asarone, zearalenone, dioctyl phthalate, and 4-hydroxycoumarin) that were common to both host and the endophyte. These reports provide encouraging evidence for plant-associated endophytes as a natural reservoir of various pharmacologically important and novel bioactive compounds.

Metabolomics-Guided Discovery of Novel Compounds From Endophytes

In an ambitious project, Maciá-Vicente et al. (2018) analyzed the metabolites produced by 822 root-associated fungi (from different plant species collected from various geographical locations) by untargeted ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS)-based approach. The study enabled comprehensive and unbiased coverage of secondary metabolites, as revealed by the detection of >17,000 compounds by UPLC-ESI-MS/MS, of which ∼9000 compounds had unique chemistries. The study was conducted with the objective to discover novel natural products, chemotaxonomic markers for fungal groups, and to facilitate bioprospecting for drug discovery (**Figure 2**).

Xylaria ellisii is a griseofulvin (antifungal activity by inhibiting microtubule assembly) producing novel fungal endophyte

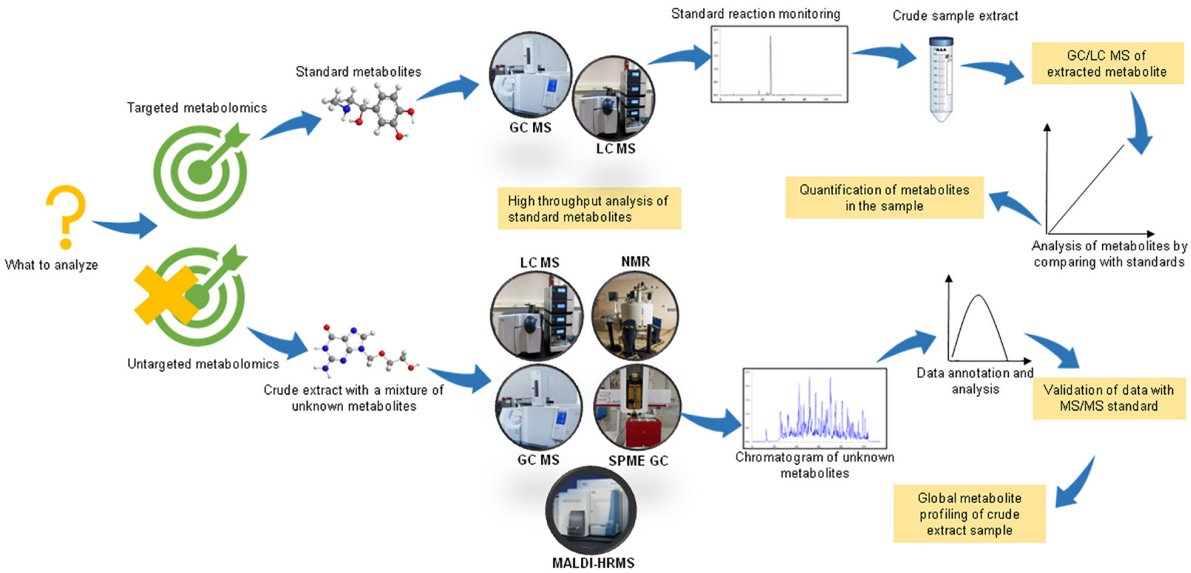


FIGURE 1 | Experimental design for performing untargeted and targeted metabolomic studies. Figure comprised of images taken from google images (source license 'CC0').

TABLE 1 | Advantages and disadvantages of various metabolomic techniques.

Techniques	Advantages	Disadvantages
Nuclear magnetic resonance (NMR)	Non-destructive and rapid, Minimal sample preparation, Highly reproducible	Lack of sensitivity, Expensive instrument
Gas chromatography-mass spectrometry (GC-MS)	High sensitivity and resolution, Easy to use, Cost-effective method, Spectrum library available for unknown materials, Suitable for non–thermosensitive and volatile molecules	Not suitable for less volatile compounds, Derivatisation of samples required
Liquid chromatography-mass spectrometry (LC-MS)	High sensitivity, Suitable for less volatile metabolites, Wide range analysis, Sample derivatisation not required, Both qualitative and quantitative analysis	Lack of reproducibility with fragmentation pattern, Poor separation of different isomers
Capillary electrophoresis-mass spectrometry (CE-MS)	No special treatment required for samples preparation, Low dosage requirement, Cost effective process, Offer shorter test time, Less sample volume required	Low separation reproducibility, Not applicable for high molecular weight proteins
Ultra high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS)	Easy sample preparation, Faster analysis, High mass spectral resolution of metabolites, Interchangeable ion sources, High sensitivity	Formation of dimers belonging to single compound, Expensive instrumentation, Higher maintenance
Solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS)	Simple and versatile, Less time consuming, No harmful solvent required	Loss in analysis and rapidity, Limited availability of commercially available fiber material
Matrix-assisted laser desorption/ionization high-resolution mass spectrometry imaging (MALDI-HRMSI)	Investigate the presence and distribution of the significant secondary metabolites. Requires limited sample preparation provides high spatial resolution	Compounds of low abundance cannot be analyzed, Structurally similar compounds cannot be analyzed

isolated from *Vaccinium angustifolium* (commonly known as wild blueberries). To identify novel natural products, 15 endophytic *Xylaria* strains were subjected to LC-UV/MS-based metabolomic analysis, which led to the discovery of eight new groups of compounds called ellisiiamides (proline-containing cyclic non-ribosomal pentapeptides) from ethyl acetate extract and mycelium (Ibrahim et al., 2020). The structural elucidation of these ellisiiamides was performed using NMR and LC-HRMS/MS analysis. Subsequent analysis demonstrated Ellisiiamide A to have antagonistic activity against *E. coli*, the role of other ellisiiamides is yet to be discovered.

The application of the One Strain Many Compounds (OSMAC) approach enables the researchers to explore the chemodiversity of microorganisms (**Figure 2**). In one such study involving an OSMAC-assisted metabolomics approach, Armin et al. (2021a) investigated the metabolic potential of *Streptomyces pulveraceus* strain ES16 (an apple-associated root endophytic bacteria) by growing it in six different media (ranging from nutrient-rich to minimal media) for a period of 3, 7, and 14 days. Using various state-of-art techniques such as SPME-GC-MS (for expressed volatilome), HPLC-HRMS^n (to target the expressed metabolome), and MALDI-HRMSI

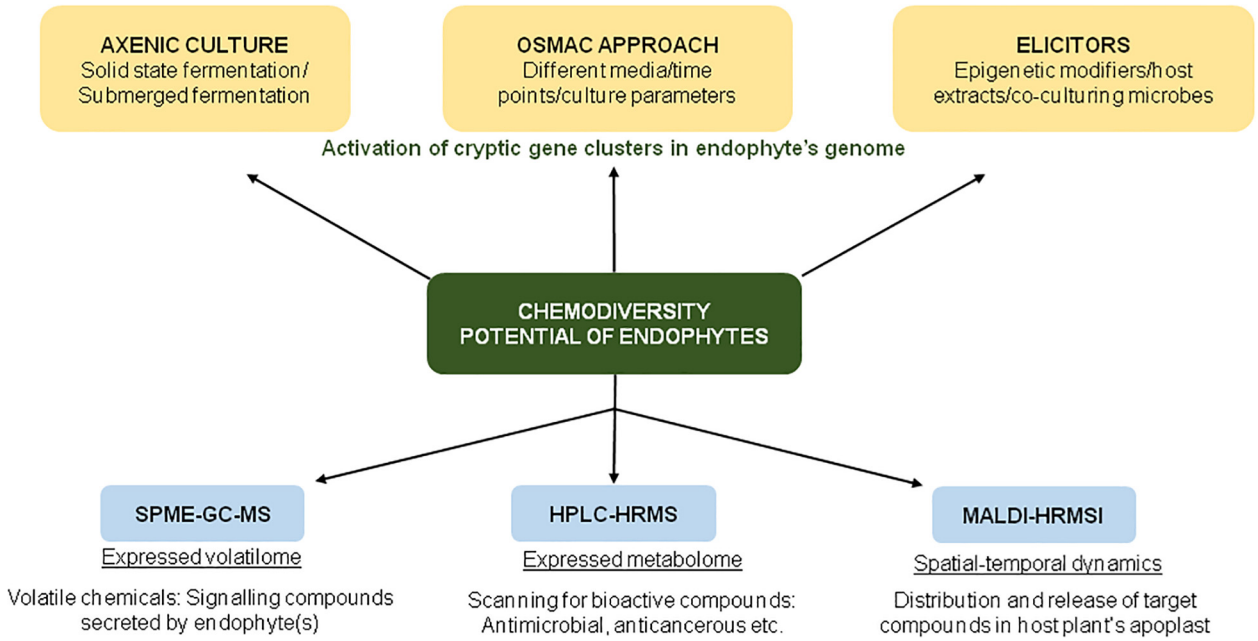


FIGURE 2 | Schematic representation of various approaches for screening of bioactive compounds in endophyte cultures.

TABLE 2 | Studies where endophytic microbes have been reported to produce plant metabolites.

Endophyte	Host plant	Metabolite	Technique(s) used	Pharmacological activity	References
Taxomyces andreanae	*Taxus brevifolia*	Paclitaxel	TLC, HPLC, immunoassay, and radiolabelled precursors	Anticancer	Stierle et al., 1993
Phialocephala fortinii	*Podophyllum peltatum*	Podophyllotoxin	HPLC, ESI-MS	Anticancer	Eyberger et al., 2006
Fusarium solani	*Apodytes dimidiate*	Camptothecin 10-hydroxycamptothecin	LC-MS/MS, LC-HRMS	Anticancer	Shweta et al., 2010
Alternaria alternate	*Capsicum annuum*	Capsaicin	LC-ESI-MS/MS	Antitumor	Devari et al., 2014
Colletotrichum gloeosporioides	*Piper nigrum*	Piperine	HPLC, LC-MS	Antimicrobial, antidepressant, antiinflammatory, and anticancer	Chithra et al., 2014b
Eupenicillium parvum	*Sinopodophyllum hexandrum*	Kaempferol	TLC, HPLC, and NMR	Antioxidant	Huang et al., 2014
Rhizoctonia bataticola	*Coleus forskohlii*	Forskolin	TLC	Reduce blood pressure, antiallergic, antiinflammatory	Mir et al., 2015
Geomyces sp.	*Nerium indicum*	Vincamine	TLC, HPLC, and LC-MS	Cardiovascular and cerebrovascular protective	Na et al., 2016
Fusarium solani	*Camptotheca acuminate*	Camptothecin	TLC, HPLC, and EI-MS	Antineoplastic	Ran et al., 2017
Aspergillus fumigates	*Taxus* sp.	Paclitaxel	TLC, HPLC, FTIR, and NMR	Anticancerous	Kumar et al., 2019

(for spatial-temporal visualization of metabolites), the authors demonstrated the production of geosmin (a volatile chemical that attracts soil arthropods), mirubactin (iron-chelating siderophore) and polycyclic tetramate macrolactams (biocontrol activity against various pathogens) under stress conditions (Armin et al., 2021a). In addition to chemical OSMAC (by alteration/optimization of chemical media), biological OSMAC involving interaction with other microbial species (endophytic or pathogenic) can be used to increase the diversity of endophyte-derived bioactive compounds (Armin et al., 2021b).

Metabolomics for Endophyte-Derived Plant Metabolites

Endophytic microbial communities represent a natural reservoir for obtaining a myriad of bioactive compounds, including host plant metabolites (**Table 2**). It has been proposed that due to long-term evolutionary association with plants, the symbiotic microbiota have acquired some of the host plant genes by horizontal gene transfer (Tiwari and Bae, 2020). Further, genome sequencing of microbes has demonstrated a higher potential to produce metabolites than those observed under standard laboratory conditions. Since the landmark discovery of endophyte-mediated taxol production from an endophytic fungus (*Taxomyces andreanae*) isolated from *Taxus brevifolia* (Stierle et al., 1993), several endophytic microbes have been reported to produce plant metabolites under axenic conditions, some of the reports have been discussed below.

Azadirachtin

Azadirachtin is an oxygenated tetranortriterpenoid found in *Azadirachta indica* (Indian neem; used to cure fever, pain, leprosy, and malaria). It is a broad spectrum insecticide, effective against pests belonging to Heminoptera, Isoptera and Orthoptera (Kilani-Morakchi et al., 2021). The axenic culture of *Eupenicillium parvum* (an endophytic fungus isolated from this plant) was obtained by inoculation in Sabouraud medium for 20 days on a rotary shaker (28°C, 200 rpm). After filtration, both the mycelial pellet and suspension culture were analyzed by LC-HRMS and compared against standard Azadirachtin. The quantitative analysis by LC-MS revealed a yield of ∼0.4 μg per 100 g dry weight of fungal mycelia, and 43 μg per liter in the spent broth for Azadirachtin A, and ∼ 0.05 μg per 100 g dry weight of fungal mycelia and 11 μg per liter in spent broth for Azadirachtin B (Kusari et al., 2012).

Camptothecin

Camptothecin is a pentacyclic quinoline alkaloid with antineoplastic (that prevents or halts tumor growth) properties, obtained from *Camptotheca acuminata* and *Nothapodytes nimmoniana.* Camptothecin causes DNA damage (and hence, apoptosis) by binding to and stabilizing the topoisomerase-DNA complex (Ghanbari-Movahed et al., 2021). Camptothecin and its analogs are synthesized by *Fusarium solani*, an endophytic fungus isolated from *C. acuminata* plant (Kusari et al., 2009). Interestingly, the metabolite could not be detected in the filtrate/culture medium, but accumulated in the fungal biomass, when detected using LC-HRMS and compared with the standard. In a recent report, Mohinudeen et al. (2021) reported the ability of >70% endophytic fungi isolated from *N. nimmoniana* to produce camptothecin in suspension cultures. Interestingly, one of the endophytic isolates, *Alternaria burnsii* NCIM1409, demonstrated high production of camptothecin (∼ 200 μg/g) even after 12 rounds of subculturing (without suffering from attenuation) as detected by LC–MS/MS.

Hypericin

The medicinal plant, *Hypericum perforatum* (commonly known as St. John's Wort), produces hypericin (a naphthodianthrone derivative), which has several medicinal properties such as antimicrobial (bacteria, fungi, and viruses) antioxidant, anticancerous, and antidepressant. Hypericin inhibits virus replication by preventing the shedding of virus particles and its reassembly in infected cells (Dellafiora et al., 2018). The production of hypericin by endophytic fungi (*Thielavia subthermofila*) was first reported by Kusari et al. (2008); the same has been discussed in detail in the later sections of the review. Another endophytic fungi, *Epicoccum nigrum*, was also reported to produce hypericin, as detected by HPLC-UV analysis, and confirmed by LC-HRMS and LC-HRMS/MS analysis (Vigneshwari et al., 2019).

Piperine

Piperine is an amide alkaloid (obtained from various *Piper* species such as *Piper nigrum*, *P. longum*, and *P. chaba*), widely used in Ayurveda, Siddha, and Unani medicine systems to cure respiratory and digestive disorders (Yadav et al., 2020). Besides, piperine is also used as a bioenhancer (to increase the bioavailability of other drugs), by either blocking cytochrome P450 (the drug metabolizing enzyme) or by altering the permeability of intestinal mucosa (Tiwari et al., 2020). There have been a couple of reports on the isolation of endophytes from *P. longum* and *P. nigrum*, and confirmation of piperine production ability by HPLC and LC-MS (Verma et al., 2011; Chithra et al., 2014a,b; Mintoo et al., 2019). The piperine-producing endophytes include endophytic strains of *Colletotrichum gloeosporioides*, *Mycosphaerella* sp., and *Periconia* sp. Interestingly, Chithra et al. (2014a) could detect piperine production by the endophytic fungi *Mycosphaerella* sp. through LC-MS/MS analysis, but it could not be detected using HPLC analysis, thereby highlighting the importance of sensitivity in the screening of bioactive compounds.

Phenylpropanoids

The phenylpropanoid pathway is responsible for the biosynthesis of several classes of SMs such as flavonoids, isoflavonoids, lignans, and stilbenes in plants (Fraser and Chapple, 2011). Phenylpropanoids are important components of cell wall, phytoalexins (against herbivores and pathogens) and floral pigments (mediating pollination) (Deng and Lu, 2017). Interestingly, the phenylpropanoid pathway is not specific to plants; there are a couple of studies reporting the production of phenolics and flavonoids from endophytic strains namely *Aspergillus nidulans* ST22 and *A. oryzae* SX10 from *Ginkgo biloba*

(Qiu et al., 2010). Recently, Lu et al. (2020) reported starvation-induced production of phenylpropanoids in *Alternaria* sp. MG1 (an endophyte isolated from *Vitis vinifera*); the pathway remains repressed under normal growth conditions. Using RT-PCR and GC-TOF-MS-based metabolomics, the authors demonstrated induction in the expression of shikimate and phenylpropanoid biosynthetic genes, as well as accumulation of metabolites such as shikimate, phenylalanine, tyrosine, and 3-(4-hydroxyphenyl)pyruvate under starvation conditions (Lu et al., 2020). The MG1 strain could convert glucose to resveratrol (a polyphenol found in grapes, having high antioxidant and anticancerous activity), thereby acting as a potential alternative source for this compound (Salehi et al., 2018). Apart from resveratrol, the metabolomic analysis also revealed the production of other metabolites such as piceatannol (stilbenoids), taxifolin (flavonoid), lignans (caffeate, sinapate, and sinapoyl aldehyde) and cuminic alcohol (used in food and cosmetic industries), thereby highlighting this endophytic strain to be a treasure house of multiple important bioactive compounds.

Withanolides

Withanolides are secondary metabolites made up of a steroid backbone coupled to a lactone or its derivatives found in *Withania somnifera* (commonly known as Ashwagandha or as Indian ginseng). These metabolites are widely used as memory and stamina enhancer, nerve tonic, and have cardioprotective, neuroprotective, antidiabetic, antioxidant properties, etc. (White et al., 2016). An endophytic fungus, *Taleromyces pinophilus*, isolated from leaves of *W. somnifera*, was found to produce withanolides (C28-steroidal lactones of triterpene ancestry). HPLC and ^{1}H NMR analyses revealed that the endophyte produced a higher yield of withanolides (360 mg/L) than the plant itself (Sathiyabama and Parthasarathy, 2018). Another study reported an increase in withanolide content in leaves upon inoculation of *in vitro* raised *W. somnifera* plants with some endophytic strains, in comparison to non-inoculated control plants (Pandey et al., 2018). The increase in withanolide content was accompanied by modulation of expression of withanolide biosynthetic genes.

METABOLOMICS FOR ANSWERING FUNDAMENTAL QUESTIONS

"Actual" Source of Plant Metabolites

The secondary metabolites produced in plants could be derived either from the host, or endophytes, or a combination of both. There are a couple of examples in literature where endophytes (and not plants) have been reported to be the "actual" source of the metabolite (Mishra et al., 2021c). For example, a HPLC-HRMS-based study revealed that endophytic bacteria associated with the roots of *Putterlickia verrucosa* and *P. retrospinosa* were the original source of maytansine, an anticancerous and cytotoxic compound. The results were further substantiated by MALDI-imaging-HRMS that delineated the localization of maytansine producers in the root cortex of host plants (Kusari et al., 2014b). Another case where the endophytic community was found to be the source of the "plant metabolite" was for astins, a class of macrocyclic peptides with antitumor activity, biosynthesized by the endophytic fungi *Cyanodermella asteris* (earlier known to be produced by the host plant *Aster tataricus*) (Schafhauser et al., 2019). Likewise, the antitumor activity of the bioactive fraction of moss *Claopodium crispifolium* was attributed to *Nostoc microscopium*, an endophytic blue green alga associated with the moss plant (Spjut et al., 1988).

Insights Into Endophytic Hypericin Biosynthesis

Thielavia subthermophila, an endophytic fungus (isolated from the stem of *Hypericum perforatum,* commonly known as St. John's Wort) was found to produce hypericin (a napthodianthrone with antidepressant, antimicrobial, antiinflammatory, and antioxidant properties, as mentioned before) and emodin under submerged fermentation conditions, as detected by HPLC-MS/MS (Kusari et al., 2008). However, in contrast to its host plant, the growth kinetics and hypericin biosynthesis in the endophyte occurs irrespective of light conditions. Further, the *hyp-1* gene encoding for an enzyme that converts emodin to hypericin in *H. perforatum* cell cultures (Bais et al., 2003) could not be amplified from the endophyte genome. Also, the addition of emodin to the endophyte culture did not change the level of hypericin (or emodin), irrespective of illumination conditions. These results obtained for microbial metabolomics suggest a different mechanism of hypericin biosynthesis under axenic conditions. The endophyte has devised a different mode of regulation for hypericin, which exhibits high cytotoxicity when irradiated with visible light. The *H. perforatum* plants store this metabolite in dark glands to prevent autocytotoxicity. In the absence of such dark glands, the endophyte has "evolved" to inhabit the inner stem tissues of the host plant (from where the endophyte was isolated, and where ambient light does not reach). Therefore, microbial metabolomics has unraveled the biosynthetic and regulatory mechanism for a plant metabolite, synthesis by the endophyte, independent of its host.

Deciphering "Taste" Differences in Contrasting Cultivars

In a recent report, a non-targeted LC-MS-based metabolomic analysis of bitter and sweet varieties of sugarcane was carried out (Huang et al., 2021). Around 20% of the total (1245) metabolites (comprising both primary and secondary metabolites) detected in stalk tissues, were differentially abundant between the two varieties. In addition, the microbial communities residing in the sugarcane varieties, as well as the soil, were investigated by 16S rRNA gene sequencing. The results revealed that the flavonoid content in sugarcane stalks and nitrogen content of the soil was correlated with the composition of soil's microbiome. Metabolites such as lamioside, primisulfuron, 3,5-dimethylpyrazin-2-ol, sitaxentan, etc., were >30 to 60-fold higher in the bitter variety. Likewise, some of the metabolites differentially accumulating in the sweet variety included eucoside, isovitexin 2"-O-arabinoside, and vaccarin. Interestingly, the concentrations of many sugarcane metabolites including apigenin, biochanin A, hesperidin, luteolin,

and trigonelline, correlated well ($r > 0.8$) with specific microbial species (Huang et al., 2021).

Investigation of Specific Plant-Endophyte Interaction

Scherling et al. (2009) reported one of the first application of the metabolomic approach for studying specific plant-endophyte interaction using *in vitro* grown (endophyte-free) poplar plants. The inoculation of a specific endophyte, *Paenibacillus* sp. strain P22, to *in vitro* grown endophyte-free plants (the micropropagated plants regenerated from shoot meristems) caused significant changes in the metabolome (affecting levels of 32 metabolites) of inoculated vs. non-inoculated plants. The GC-TOF-MS analyses revealed distinct metabolic signatures for inoculated and non-inoculated (control) plants, indicative of remarkable changes in plants' nitrogen assimilation. The *Paenibacillus*-inoculated poplar plants exhibited elevated asparagine (8-fold) and urea (6-fold) levels (corresponding to nitrogen fixation ability of the endophyte), and reduced levels of sugars and organic acids (intermediates of Krebs' cycle).

METABOLOMICS FOR IMPARTING CLIMATE RESILIENCE TO CROPS

Environmental metabolomics refers to the application of metabolomics for characterizing an organism's response to environmental stresses. Another commonly used term is *eco-metabolomics*; it refers to the application of metabolomic techniques to study ecological principles, including the interaction of species with the environment and with other species (Maciá-Vicente et al., 2018). Such studies can be performed at various levels of ecological organization, namely, individual, population, community, or ecosystem. Though the review mainly focuses on the contribution of metabolomics in the discovery and identification of endophyte-derived bioactive compounds, the contribution of endophyte metabolome toward climate resilience has been briefly discussed.

Genomics-Metabolomics Integrative Approach for Disease Suppression

The bioactive compounds or metabolites produced by endophytic microbiota are responsible for many microbiome-imparted plant phenotypes such as disease suppression. The production of metabolites by the endophytic root microbiome of sugarbeet has been effective in suppressing infection by *Rhizoctonia solani* (a soil-borne fungal root pathogen of rice, wheat, and sugarbeet), as revealed by metagenomics and network analyses (Carrión et al., 2019). The activation of NRPS-PKS biosynthetic gene clusters in bacterial endophytes leads to the synthesis of enzymes that digest fungal cell walls, as well as antifungal compounds including phenazines, polyketides, and siderophores (Carrión et al., 2019). Further, the authors designed a microbial consortium to suppress fungal root disease, using *Chitinophaga* and *Flavobacterium* spp.

In the same year, Hayden et al. (2019) applied environmental metabolomics to facilitate the discrimination of disease suppressive soils for *R. solani*. The researchers analyzed the soil profile of disease suppressive and non-suppressive soils in Australia by ^{1}H NMR and LC-MS techniques, over 2 years of wheat cultivation. The application of metabolomics eliminates the lengthy screening procedures involving pot experiments and quantitative PCR under field conditions. The study revealed that disease suppressive soils had a different ^{1}H NMR and LC-MS profile, mainly involving a higher content of sugar molecules, while the non-suppressive soil had more abundant lipid and terpenes. The study also identified a compound called macrocarpal (an antimicrobial secondary metabolite), as a biomarker for *R. solani* suppressive soil (Hayden et al., 2019).

It needs to be mentioned that rhizospheric microbes have been found to act as the first line of defense against fungal root pathogens in disease suppressive soils (Mendes et al., 2011). The secretion of lipopeptides (encoded by non-ribosomal peptide synthetase gene) by specific strains of *Pseudomonas* are effective in imparting plant resistance to fungal diseases (Mendes et al., 2011). These reports provide encouraging evidence for imparting disease suppression phenotype to disease-conducive soils, similar to fecal transplant in humans. However, the practical feasibility of this concept requires the inoculated microbiota to display active growth rates and tolerance to environmental fluctuations, among others.

In another report, Llorens et al. (2019) performed non-targeted metabolomics using LC-ESI full-scan mass spectrometry to analyze the metabolome of cultivated wheat plants inoculated with two endophytes, *Acremonium sclerotigenum* and *Sarocladium implicatum*, isolated from a wild ancestor *Aegilops sclerotigenum*. The metabolomic analysis revealed that the levels of metabolites involved in 2-oxocarboxilic acid metabolism and amino acid-related metabolism, mainly asparagine and glutamate, were significantly altered between the inoculated and non-inoculated plants. These amino acids are abiotic stress-responsive and involved in the production of osmolytes such as proline and polyamines (Yamamoto et al., 2015). The results indicate that endophytes improve plant performance under stress conditions by improving the physiological status of plants (Llorens et al., 2019).

CHALLENGES IN METABOLOME MINING

In metabolomics, the detection and identification of metabolites involve the comparison of experimental MS/MS spectra to library spectra (a process known as dereplication); therefore, the raw data quality and robustness of the database dictates the authenticity/success of the experiment. Further, owing to the chemical diversity of metabolites, there is no universal protocol for the detection and quantification of all the classes of metabolites. It is a costly method requiring sophisticated instrumentation and facilities, and hence, not feasible for small laboratories. The lack of reference libraries for metabolites and the availability of standards limit the applications of metabolomics. Another challenge is that the synthesis of metabolites in a natural community cannot be specifically ascribed to a specific microbial species (biological source and biosynthetic pathway). Finally, the

results are sensitive to contaminants and experimental artifacts. Nevertheless, metabolomics has added another dimension to our understanding of plant-microbe interactions, and with further technological advancements, these challenges can be addressed.

AUTHOR CONTRIBUTIONS

SM and SS conceived the idea of the manuscript. SM wrote the manuscript. Priyanka prepared the figures and tables. All authors have read and approved the final version of the manuscript.

FUNDING

SM and SS thankfully acknowledge the Science and Engineering Research Board-Teachers Associateship for Research Excellence (SERB-TARE) grant (TAR/2021/000309). Priyanka acknowledges the fellowship received from CSIR and IIT Delhi.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.835931/full#supplementary-material

REFERENCES

Angiolillo, L., del Nobile, M. A., and Conte, A. (2015). The extraction of bioactive compounds from food residues using microwaves. *Curr. Opin. Food Sci.* 5, 93–98. doi: 10.1016/J.COFS.2015.10.001

Armin, R., Zühlke, S., Mahnkopp-Dirks, F., Winkelmann, T., and Kusari, S. (2021a). Evaluation of apple root-associated endophytic *Streptomyces pulveraceus* strain ES16 by an OSMAC-assisted metabolomics approach. *Front. Sustain. Food Syst.* 5:643225. doi: 10.3389/fsufs.2021.643225

Armin, R., Zühlke, S., Grunewaldt-Stöcker, G., Mahnkopp-Dirks, F., and Kusari, S. (2021b). Production of siderophores by an apple root-associated *Streptomyces ciscaucasicus* strain GS2 using chemical and biological OSMAC approaches. *Molecules* 26:3517. doi: 10.3390/molecules26123517

Bais, H. P., Vepachedu, R., Lawrence, C. B., Stermitz, F. R., and Vivanco, J. M. (2003). Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (*Hypericum perforatum* L.). *J. Biol. Chem.* 278, 32413–32422. doi: 10.1074/jbc.M301681200

Bauer, A., Brönstrup, M., and Brönstrup, B. (2014). Industrial natural product chemistry for drug discovery and development. *R. Soc. Chem.* 31:35. doi: 10.1039/c3np70058e

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., et al. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* 366, 606–612. doi: 10.1126/science.aaw9285

Castro-Moretti, F. R., Gentzel, I. N., Mackey, D. A., and Alonso, A. P. (2020). Metabolomics as an emerging tool for the study of plant-pathogen interactions. *Metabolites* 10:52. doi: 10.3390/metabo10020052

Chithra, S., Jasim, B., Anisha, C., Mathew, J., and Radhakrishnan, E. K. (2014a). LC-MS/MS based identification of piperine production by endophytic *Mycosphaerella* sp. PF13 from *Piper nigrum*. *Appl. Biochem. Biotechnol.* 173, 30–35. doi: 10.1007/s12010-014-0832-3

Chithra, S., Jasim, B., Sachidanandan, P., Jyothis, M., and Radhakrishnan, E. K. (2014b). Piperine production by endophytic fungus *Colletotrichum gloeosporioides* isolated from *Piper nigrum*. *Phytomedicine* 21, 534–540. doi: 10.1016/j.phymed.2013.10.020

Cipriano, M. A. P., Freitas-Iório, R. d. P., Dimitrov, M. R., de Andrade, S. A. L., Kuramae, E. E., and Silveira, A. P. D. (2021). Plant-growth endophytic bacteria improve nutrient use efficiency and modulate Foliar N-Metabolites in sugarcane seedling. *Microorganisms* 9:479. doi: 10.3390/microorganisms9030479

Correa-Galeote, D., Bedmar, E. J., and Arone, G. J. (2018). Maize endophytic bacterial diversity as affected by soil cultivation history. *Front. Microbiol.* 9:484. doi: 10.3389/fmicb.2018.00484

Dellafiora, L., Galaverna, G., Cruciani, G., Dall'Asta, C., and Bruni, R. (2018). On the mechanism of action of anti-inflammatory activity of hypericin: an in silico study pointing to the relevance of Janus kinases inhibition. *Molecules* 23:3058. doi: 10.3390/molecules23123058

Deng, Y., and Lu, S. (2017). Biosynthesis and regulation of phenylpropanoids in plants. *Crit. Rev. Plant Sci.* 36, 257–290. doi: 10.1080/07352689.2017.1402852

Devari, S., Jaglan, S., Kumar, M., Deshidi, R., Guru, S., Bhushan, S., et al. (2014). Capsaicin production by *Alternaria alternata*, an endophytic fungus from *Capsicum annum*; LC-ESI-MS/MS analysis. *Phytochemistry* 98, 183–189. doi: 10.1016/j.phytochem.2013.12.001

Eyberger, A. L., Dondapati, R., and Porter, J. R. (2006). Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *J. Nat. Prod.* 69, 1121–1124. doi: 10.1021/np060174f

Fraser, C. M., and Chapple, C. (2011). The phenylpropanoid pathway in *Arabidopsis*. *Arabidopsis Book* 9:152. doi: 10.1199/tab.0152

Gakuubi, M. M., Munusamy, M., Liang, Z.-X., and Ng, S. B. (2021). Fungal endophytes: a promising frontier for discovery of novel bioactive compounds. *J. Fungi* 7:786. doi: 10.3390/jof7100786

García-Latorre, C., Rodrigo, S., and Santamaría, O. (2021). "Endophytes as plant nutrient uptake-promoter in plants," in *Endophytes: Mineral Nutrient Management, Sustainable Development and Biodiversity*, Vol. 3, eds D. K. Maheshwari and S. Dheeman (Cham: Springer). doi: 10.1007/978-3-030-65447-4_11

Ghanbari-Movahed, M., Kaceli, T., Mondal, A., Farzaei, M. H., and Bishayee, A. (2021). Recent advances in improved anticancer efficacies of camptothecin nano-formulations: a Systematic Review. *Biomedicines* 9:480. doi: 10.3390/biomedicines9050480

Giauque, H., and Hawkes, C. V. (2013). Climate affects symbiotic fungal endophyte diversity and performance. *Am. J. Bot.* 100, 1435–1444. doi: 10.3732/ajb.1200568

Gouda, S., Das, G., Sen, S. K., Shin, H. S., and Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. *Front. Microbiol.* 7:1538. doi: 10.3389/fmicb.2016.01538

Guevara-Araya, M. J., Vilo, C., Urzúa, A., and Gonzalez-Teuber, M. (2020). Differences in community composition of endophytic fungi between above- and below-ground tissues of *Aristolochia chilensis* in an arid ecosystem. *Rev. Chil. Hist. Nat.* 93:3. doi: 10.1186/s40693-020-00091-y

Guo, L., Yu, H., Wang, B., Vescio, K., Deiulio, G. A., Yang, H., et al. (2021). Metatranscriptomic comparison of endophytic and pathogenic fusarium-*Arabidopsis* interactions reveals plant transcriptional plasticity. *Mol. Plant Microbe Interact.* 34, 1071–1083. doi: 10.1094/MPMI-03-21-0063-R

Harrison, J. G., and Griffin, E. A. (2020). The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: how far have we come and where do we go from here? *Environ. Microbiol.* 22, 2107–2123. doi: 10.1111/1462-2920.14968

Hayden, H. L., Rochfort, S. J., Ezernieks, V., Savin, K. W., and Mele, P. M. (2019). Metabolomics approaches for the discrimination of disease suppressive soils for *Rhizoctonia solani* AG8 in cereal crops using 1H NMR and LC-MS. *Sci. Total Environ.* 651, 1627–1638. doi: 10.1016/j.scitotenv.2018.09.249

Hosseyni-Moghaddam, M. S., and Soltani, J. (2014). Bioactivity of endophytic Trichoderma fungal species from the plant family Cupressaceae. *Ann. Microbiol.* 64, 753–761. doi: 10.1007/s13213-013-0710-1

Huang, J. X., Zhang, J., Zhang, X. R., Zhang, K., Zhang, X., and He, X. R. (2014). *Mucor fragilis* as a novel source of the key pharmaceutical agents

podophyllotoxin and kaempferol. *Pharm Biol.* 52, 1237–1243. doi: 10.3109/13880209.2014.885061

Huang, W., Sun, D., Chen, L., and An, Y. (2021). Integrative analysis of the microbiome and metabolome in understanding the causes of sugarcane bitterness. *Sci. Rep*. 11:6024. doi: 10.1038/s41598-021-85433-w

Ibrahim, A., Tanney, J. B., Fei, F., Seifert, K. A., Christopher Cutler, G., Capretta, A., et al. (2020). Metabolomic-guided discovery of cyclic nonribosomal peptides from *Xylaria ellisii* sp. nov., a leaf and stem endophyte of *Vaccinium angustifolium*. *Sci. Rep.* 10:4599. doi: 10.1038/s41598-020-61088-x

Johnston-Monje, D., and Raizada, M. N. (2011). "Integration of biotechnologies | plant and endophyte relationships: nutrient management," in *Comprehensive Biotechnology*, 2nd Edn, Vol. 4, ed. M. Moo-Young (Amsterdam: Elsevier), 713–727. doi: 10.1016/B978-0-08-088504-9.00264-6

Kaul, S., Sharma, T., and Dhar, M. K. (2016). "Omics" tools for better understanding the plant–endophyte interactions. *Front. Plant Sci.* 7:955. doi: 10.3389/fpls.2016.00955

Kilani-Morakchi, S., Morakchi-Goudjil, H., and Sifi, K. (2021). Azadirachtin-based insecticide: Overview, risk assessments, and future directions. *Front. Agron.* 3:32. doi: 10.3389/fagro.2021.676208

Krings, M., Taylor, T. N., Hass, H., Kerp, H., Dotzler, N., and Hermsen, E. J. (2007). Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *N. Phytol.* 174, 648–657. doi: 10.1111/j.1469-8137.2007.02008.x

Kumar, P., Singh, B., Thakur, V., Thakur, A., Thakur, N., Pandey, D., et al. (2019). Hyper-production of taxol from *Aspergillus fumigatus*, an endophytic fungus isolated from Taxus sp. of the Northern Himalayan region. *Biotechnol. Rep.* 24:e00395. doi: 10.1016/j.btre.2019.e00395

Kusari, S., Lamshoö, M., Kusari, P., Gottfried, S., Zuü, S., Louven, K., et al. (2014a). Endophytes are hidden producers of maytansine in *Putterlickia* roots. *J. Nat. Prod.* 77, 2577–2584. doi: 10.1021/np500219a

Kusari, S., Singh, S., and Jayabaskaran, C. (2014b). Rethinking production of Taxol§(paclitaxel) using endophyte biotechnology. *Trends Biotechnol.* 32, 304–311. doi: 10.1016/j.tibtech.2014.03.011

Kusari, S., Lamshöft, M., Zühlke, S., and Spiteller, M. (2008). An Endophytic fungus from *Hypericum perforatum* that produces hypericin. *J. Nat. Prod.* 71, 159–162. doi: 10.1021/np070669k

Kusari, S., Verma, V. C., Lamshoeft, M., and Spiteller, M. (2012). An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. *World J. Microb. Biotechnol.* 28, 1287–1294. doi: 10.1007/s11274-011-0876-2

Kusari, S., Zühlke, S., and Spiteller, M. (2009). An endophytic Fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *J. Nat. Prod.* 72, 2–7. doi: 10.1021/np800455b

Liao, H.-L., Bonito, G., Rojas, J. A., Hameed, K., Wu, S., Schadt, C. W., et al. (2019). Fungal endophytes of *Populus trichocarpa* alter host phenotype, gene expression, and rhizobiome composition. *Mol. Plant Microbe Interact.* 32, 853–864. doi: 10.1094/MPMI-05-18-0133-R

Llorens, E., Sharon, O., Camañes, G., García-Agustín, P., and Sharon, A. (2019). Endophytes from wild cereals protect wheat plants from drought by alteration of physiological responses of the plants to water stress. *Environ. Microbiol.* 21, 3299–3312. doi: 10.1111/1462-2920.14530

Lu, Y., Che, J., Xu, X., Pang, B., Zhao, X., Liu, Y., et al. (2020). Metabolomics reveals the response of the phenylpropanoid biosynthesis pathway to starvation treatment in the grape endophyte *Alternaria* sp. MG1. *J. Agricult. Food Chem.* 68, 1126–1135. doi: 10.1021/acs.jafc.9b05302

Maciá-Vicente, J. G., Shi, Y. N., Cheikh-Ali, Z., Grün, P., Glynou, K., Kia, S. H., et al. (2018). Metabolomics-based chemotaxonomy of root endophytic fungi for natural products discovery. *Environ. Microbiol.* 20, 1253–1270. doi: 10.1111/1462-2920.14072

Mendes, R., Kruijt, M., Bruijn, I., de, Dekkers, E., Voort, M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive Bacteria. *Science* 332, 1093–1097. doi: 10.1126/science.1202007

Mintoo, M. N., Mishra, S., and Prem, D. K. (2019). Isolation and characterization of endophytic bacteria from *Piper longum*. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 89, 1447–1454. doi: 10.1007/s40011-018-01064-8

Mir, R. A., Kaushik, P. S., Chowdery, R. A., and Anuradha, M. (2015). Elicitation of forskolin in cultures of rhizactonia bataticola-a phytochemical synthesizing endophytic fungi. *Int. J. Pharm. Pharmaceut. Sci.* 7, 185–189.

Mishra, S., Bhattacharjee, A., and Sharma, S. (2021a). An ecological insight into the multifaceted world of plant-endophyte Association. *Crit. Rev. Plant Sci.* 40, 127–146. doi: 10.1080/07352689.2021.1901044

Mishra, S., Sahu, P. K., Agarwal, V., and Singh, N. (2021c). Exploiting endophytic microbes as micro-factories for plant secondary metabolite production. *Appl. Microbiol. Biotechnol.* 105, 6579–6596. doi: 10.1007/s00253-021-11527-0

Mishra, S., Goyal, D., and Phurailatpam, L. (2021b). Targeted 16S rRNA gene and ITS2 amplicon sequencing of leaf and spike tissues of *Piper longum* identifies new candidates for bioprospecting of bioactive compounds. *Arch. Microbiol.* 203, 3851–3867. doi: 10.1007/s00203-021-02356-w

Mohamed, E. M., Hetta, M. H., Rateb, M. E., Selim, M. A., Aboulmagd, A. M., Badria, F. A., et al. (2020). Bioassay-guided isolation, metabolic profiling, and docking studies of hyaluronidase inhibitors from *Ravenala madagascariensis*. *Molecules* 25:1714. doi: 10.3390/molecules25071714

Mohamad, O. A. A., Li, L., Ma, J. B., Hatab, S., Xu, L., Gua, J. W., et al. (2018). Evaluation of the antimicrobial activity of endophytic bacterial populations from Chinese traditional medicinal plant licorice and characterization of the bioactive secondary metabolites produced by Bacillus atrophaeus against *Verticillium dahlia*. *Front. Microbiol.* 9:924. doi: 10.3389/fmicb.2018.00924

Mohinudeen, K. I. A. H., Pandey, S., Kanniyappan, H., Muthuvijayan, V., and Srivastava, S. (2021). Screening and selection of camptothecin producing endophytes from *Nothapodytes nimmoniana*. *Sci. Rep.* 11:11205. doi: 10.1038/s41598-021-90778-3

Na, R., Jiajia, L., Dongliang, Y., Yingzi, P., Juan, H., Xiong, L., et al. (2016). Indentification of vincamine indole alkaloids producing endophytic fungi isolated from *Nerium indicum*, Apocynaceae. *Microbiol. Res.* 192, 114–121. doi: 10.1016/j.micres.2016.06.008

Nalbantoglu, S. (2019). "Metabolomics: basic principles and strategies," in *Molecular Medicine*, eds S. Nalbantoglu and H. Amri (London: IntechOpen), 137–150. doi: 10.5772/intechopen.88563

Nischitha, R., and Shivanna, M. B. (2021). Metabolite fingerprinting, in vitro antimicrobial and antioxidant activities and in-silico docking in *Alloteropsis cimicina* and its endophytic fungus *Penicillium pinophilum*. *Mol. Biol. Rep.* 48, 4021–4037. doi: 10.1007/s11033-021-06410-0

Oita, S., Ibáñez, A., Lutzoni, F., Miadlikowska, J., Geml, J., Lewis, L. A., et al. (2021). Climate and seasonality drive the richness and composition of tropical fungal endophytes at a landscape scale. *Commun. Biol.* 4:313. doi: 10.1038/s42003-021-01826-7

Pandey, S. S., Singh, S., Pandey, H., Srivastava, M., Ray, T., Soni, S., et al. (2018). Endophytes of *Withania somnifera* modulate in planta content and the site of withanolide biosynthesis. *Sci. Rep.* 8:5450. doi: 10.1038/s41598-018-23716-5

Porzel, A., Farag, M. A., Mülbradt, J., and Wessjohann, L. A. (2014). Metabolite profiling and fingerprinting of *Hypericum* species: a comparison of MS and NMR metabolomics. *Metabolomics* 10, 574–588. doi: 10.1007/s11306-013-0609-7

Qiu, M., Xie, R. S., Shi, Y., Zhang, H., and Chen, H. M. (2010). Isolation and identification of two flavonoid-producing endophytic fungi from *Ginkgo biloba* L. *Ann. Microbiol.* 60, 143–150. doi: 10.1007/s13213-010-0016-5

Rai, N., Keshri, P. K., Verma, A., Kamble, S. C., Mishra, P., Barik, S., et al. (2021). Plant associated fungal endophytes as a source of natural bioactive compounds. *Mycology* 12, 139–159. doi: 10.1080/21501203.2020.1870579

Ran, X., Zhang, G., Li, S., and Wang, J. (2017). Characterization and antitumor activity of camptothecin from endophytic fungus Fusarium solani isolated from *Camptotheca acuminate*. *Afr. Health Sci.* 17, 566–574. doi: 10.4314/ahs.v17i2.34

Rigobelo, E. C., and Baron, N. C. (2021). Endophytic fungi: a tool for plant growth promotion and sustainable agriculture. *Mycology* doi: 10.1080/21501203.2021.1945699

Salehi, B., Mishra, A. P., Nigam, M., Sener, B., Kilic, M., Sharifi-Rad, M., et al. (2018). Resveratrol: A double-edged sword in health benefits. *Biomedicines* 6:91. doi: 10.3390/biomedicines6030091

Sathiyabama, M., and Parthasarathy, R. (2018). Withanolide production by fungal endophyte isolated from *Withania somnifera*. *Nat. Prod. Res.* 32, 1573–1577. doi: 10.1080/14786419.2017.1389934

Schafhauser, T., Jahn, L., Kirchner, N., Kulik, A., Flor, L., Lang, A., et al. (2019). Antitumor astins originate from the fungal endophyte *Cyanodermella asteris* living within the medicinal plant *Aster tataricus*. *Proc. Natl. Acad. Sci. U.S.A.* 116, 26909–26917. doi: 10.1073/pnas.1910527116/-/DCSupplemental

Scherling, C., Ulrich, K., Ewald, D., and Weckwerth, W. (2009). A metabolic signature of the beneficial interaction of the endophyte *Paenibacillus* sp. isolate and in vitro-grown poplar plants revealed by metabolomics. *Mol. Plant Microbe Interact. MPMI* 22, 1032–1037.

Sebola, T. E., Uche-Okereafor, N. C., Mekuto, L., Makatini, M. M., Green, E., and Mavumengwana, V. (2020). Antibacterial and anticancer activity and untargeted secondary metabolite profiling of crude bacterial endophyte extracts from *Crinum macowanii* Baker leaves. *Int. J. Microbiol.* 2020:8839490. doi: 10.1155/2020/8839490

Segers, K., Declerck, S., Mangelings, D., Heyden, Y., and van der Eeckhaut, A. (2019). Analytical techniques for metabolomic studies: a review. *Bioanalysis* 11, 2297–2318. doi: 10.4155/bio-2019-0014

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant Microbe Interact. MPMI* 25, 28–36.

Shulaev, V., Cortes, D., Miller, G., and Mittler, R. (2008). Metabolomics for plant stress response. *Physiol. Plant.* 132, 199–208. doi: 10.1111/j.1399-3054.2007.01025.x

Shweta, S., Zuehlke, S., Ramesha, B. T., Priti, V., Mohana Kumar, P., Ravikanth, G., et al. (2010). Endophytic fungal strains of *Fusarium solani,* from *Apodytes dimidiata* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. *Phytochemistry* 71, 117–122. doi: 10.1016/j.phytochem.2009.09.030

Spjut, R. W., Cassady, J. M., Mccloud, T., Suffness, M., Norris, D. H., Cragg, G. M., et al. (1988). Variation in cytotoxicity and antitumor activity among samples of the moss *Claopodium crispifolium* (Thuidiaceae). *Econ. Bot.* 42, 62–72.

Stierle, A., Strobel, G., and Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* 260, 214–216. doi: 10.1126/science.8097061

Tawfike, A. F., Romli, M., Clements, C., Abbott, G., Young, L., Schumacher, M., et al. (2019). Isolation of anticancer and anti-trypanosome secondary metabolites from the endophytic fungus *Aspergillus flocculus* via bioactivity guided isolation and MS based metabolomics. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1106–1107, 71–83. doi: 10.1016/j.jchromb.2018.12.032

Tawfike, A. F., Tate, R., Abbott, G., Young, L., Viegelmann, C., Schumacher, M., et al. (2017). Metabolomic tools to assess the chemistry and bioactivity of endophytic *Aspergillus* strain. *Chem. Biodiver.* 14:40. doi: 10.1002/cbdv.201700040

Tebani, A., Afonso, C., and Bekri, S. (2018). Advances in metabolome information retrieval: turning chemistry into biology. Part I: analytical chemistry of the metabolome. *J. Inherit. Metab. Dis.* 41, 379–391. doi: 10.1007/s10545-017-0074-y

Tiwari, A., Mahadik, K. R., and Gabhe, S. Y. (2020). Piperine: acomprehensive review of method of isolation, purification, and biological properties. *Med. Drug Discov.* 7:100027. doi: 10.1016/j.medidd.2020.100027

Tiwari, P., and Bae, H. (2020). Horizontal gene transfer and endophytes: An Implication for the acquisition of novel traits. *Plants* 9:305. doi: 10.3390/plants9030305

Toghueo, R. M. K., Sahal, D., and Boyom, F. F. (2020). Recent advances in inducing endophytic fungal specialized metabolites using small molecule elicitors including epigenetic modifiers. *Phytochemistry* 174:112338. doi: 10.1016/j.phytochem.2020.112338

Verma, S. K., Sahu, P. K., Kumar, K., Pal, G., Gond, S. K., Kharwar, R. N., et al. (2021). Endophyte roles in nutrient acquisition, root system architecture development and oxidative stress tolerance. *J. Appl. Microbiol.* 131, 2161–2177. doi: 10.1111/jam.15111

Verma, V. C., Lobkovsky, E., Gange, A. C., Singh, S. K., and Prakash, S. (2011). Piperine production by endophytic fungus *Periconia* sp. isolated from *Piper longum* L. *J. Antib.* 64, 427–431. doi: 10.1038/ja.2011.27

Vigneshwari, A., Vid Rakk, D., Németh, A., Ndor Kocsubé, S., Mi Kiss, N., Csupor, D., et al. (2019). Host metabolite producing endophytic fungi isolated from *Hypericum perforatum*. *PLoS One* 14:60. doi: 10.1371/journal.pone.0217060

Wang, L., Ren, L., Li, C., Gao, C., Liu, X., Wang, M., et al. (2019). Effects of endophytic fungi diversity in different coniferous species on the colonization of *Sirex noctilio* (Hymenoptera: Siricidae). *Sci. Rep.* 9:5077. doi: 10.1038/s41598-019-41419-3

White, J. F., Kingsley, K. L., Zhang, Q., Verma, R., Obi, N., Dvinskikh, S., et al. (2021). Review: Endophytic microbes and their potential applications in crop management. *Pest Manag. Sci.* 75, 2558–2565. doi: 10.1002/ps.5527

White, P. T., Subramanian, C., Motiwala, H. F., and Cohen, M. S. (2016). Natural withanolides in the treatment of chronic diseases. *Adv. Exp. Med. Biol.* 928, 329–373. doi: 10.1007/978-3-319-41334-1_14

Yamamoto, N., Takano, T., Tanaka, K., Ishige, T., Terashima, S., Endo, C., et al. (2015). Comprehensive analysis of transcriptome response to salinity stress in the halophytic turf grass *Sporobolus virginicus*. *Front. Plant Sci.* 6:241. doi: 10.3389/fpls.2015.00241

Yadav, V., Krishnan, A., and Vohora, D. (2020). A systematic review on *Piper longum* L.: bridging traditional knowledge and pharmacological evidence for future translational research. *J. Ethnopharmacol.* 247:112255.

Zimmerman, N. B., and Vitousek, P. M. (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13022–13027. doi: 10.1073/pnas.1209872109

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frontiers in Microbiology

REVIEW
published: 11 March 2022
doi: 10.3389/fmicb.2021.805498

Microbial Community and Function-Based Synthetic Bioinoculants: A Perspective for Sustainable Agriculture

Archna Suman, Venkadasamy Govindasamy, Balasubramanian Ramakrishnan, K. Aswini, J. SaiPrasad, Pushpendra Sharma, Devashish Pathak and Kannepalli Annapurna*

Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Interactions among the plant microbiome and its host are dynamic, both spatially and temporally, leading to beneficial or pathogenic relationships in the rhizosphere, phyllosphere, and endosphere. These interactions range from cellular to molecular and genomic levels, exemplified by many complementing and coevolutionary relationships. The host plants acquire many metabolic and developmental traits such as alteration in their exudation pattern, acquisition of systemic tolerance, and coordination of signaling metabolites to interact with the microbial partners including bacteria, fungi, archaea, protists, and viruses. The microbiome responds by gaining or losing its traits to various molecular signals from the host plants and the environment. Such adaptive traits in the host and microbial partners make way for their coexistence, living together on, around, or inside the plants. The beneficial plant microbiome interactions have been exploited using traditional culturable approaches by isolating microbes with target functions, clearly contributing toward the host plants' growth, fitness, and stress resilience. The new knowledge gained on the unculturable members of the plant microbiome using metagenome research has clearly indicated the predominance of particular phyla/genera with presumptive functions. Practically, the culturable approach gives beneficial microbes in hand for direct use, whereas the unculturable approach gives the perfect theoretical information about the taxonomy and metabolic potential of well-colonized major microbial groups associated with the plants. To capitalize on such beneficial, endemic, and functionally diverse microbiome, the strategic approach of concomitant use of culture-dependent and culture-independent techniques would help in designing novel "biologicals" for various crops. The designed biologicals (or bioinoculants) should ensure the community's persistence due to their genomic and functional abilities. Here, we discuss the current paradigm on plant-microbiome-induced adaptive functions for the host and the strategies for synthesizing novel bioinoculants based on functions or phylum predominance of microbial communities using culturable and unculturable approaches. The effective crop-specific inclusive microbial community bioinoculants may lead to reduction in the cost of cultivation and improvement in soil and plant health for sustainable agriculture.

Keywords: bioinoculants, PGPRs, plant microbial communities, novel biologicals, microbiome

OPEN ACCESS

Edited by:
Prem Lal Kashyap, Indian Institute of Wheat and Barley Research (ICAR), India

Reviewed by:
Ahmed Elhady, Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institute, Germany
Pratiksha Singh, Guangxi University for Nationalities, China

****Correspondence:***
Archna Suman
archsuman@yahoo.com

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *30 October 2021*
Accepted: *29 December 2021*
Published: *11 March 2022*

Citation:
Suman A, Govindasamy V, Ramakrishnan B, Aswini K, SaiPrasad J, Sharma P, Pathak D and Annapurna K (2022) Microbial Community and Function-Based Synthetic Bioinoculants: A Perspective for Sustainable Agriculture. Front. Microbiol. 12:805498. doi: 10.3389/fmicb.2021.805498

INTRODUCTION

Cultivated soils are one of the most diverse microbial ecosystems, harboring bacteria, fungi, archaea, viruses, protists, and many others and supporting various biogeochemical cycles and plant growth. Soil microbial communities are critical to plant health and adapt rapidly to different abiotic and biotic stresses (Abdul Rahman et al., 2021). The soils and their microbial members provide humans with 98.8% of the plant foods we eat (FAO, 2018; Kopittke et al., 2019; Soto-Giron et al., 2021). The Food and Agriculture Organization (FAO) predicts that soil erosion could result in between 20 and 80% losses in agricultural yields due to human activities and climate change events. This erosion of topsoil could result in variable agricultural yields, depending on the soil type and the resource use pattern (Kopittke et al., 2019; Christy, 2021). The agrarian management of soils depends on many synthetic chemical inputs for increasing profitability and productivity. Unfortunately, intensive use of these chemical inputs has led to adverse environmental consequences from regional to global scales. To reduce chemical inputs and their associated undesirable effects in the soil and environment, microbial interventions as biological products are becoming an integral part of plant nutrient management programs and pest and disease management practices.

Microbial communities associated with plants, presently referred to as the plant microbiome, extend the host plant genome and their functions (**Figure 1**). Many studies demonstrate that these microbiomes are the key determinants of plant development, health, and productivity (Conrad et al., 2006; Bulgarelli et al., 2012; Lundberg et al., 2012; Turner et al., 2013; Williams, 2013). The recent investigations have unraveled the complex network of genetic, biochemical, physical, and metabolic interactions among the plant host, the associated microbial communities, and the environment. These interactions shape the microbiome assembly and modulate beneficial traits such as nutrient acquisition and plant health (Trivedi et al., 2021). Nutrient acquisition by plants is mediated by diverse mechanisms that include (i) augmenting the surface area accessed by plant roots for uptake of water and nutrients, (ii) through nitrogen fixation, (iii) P-solubilization, (iv) the production of siderophore and HCN production, and other unknowns. Furthermore, their contributions in protection against biotic (pests and diseases) and abiotic stresses directly or through modulating intrinsic resistance/tolerance have been reported (Pii et al., 2015; Govindasamy et al., 2020; Abiraami et al., 2021). The basis of this review is to highlight strategic approaches for designing novel bioinoculants based on the plant microbiome data generated from both culturable and unculturable approaches. Such plant microbiome-based specific bioinoculants may function in a better way as compared to the conventional bioinoculants with non-specific microbial isolates. The agricultural bioinoculant market is a fast-growing sector with a compound annual growth rate (CAGR) of 6.9% with a predicted value of over 12 billion US dollars by 2025. The growth of the market is driven by increasing health concerns and awareness among consumers, resulting in the inclination toward organic farming practices or low-chemical-input agriculture.

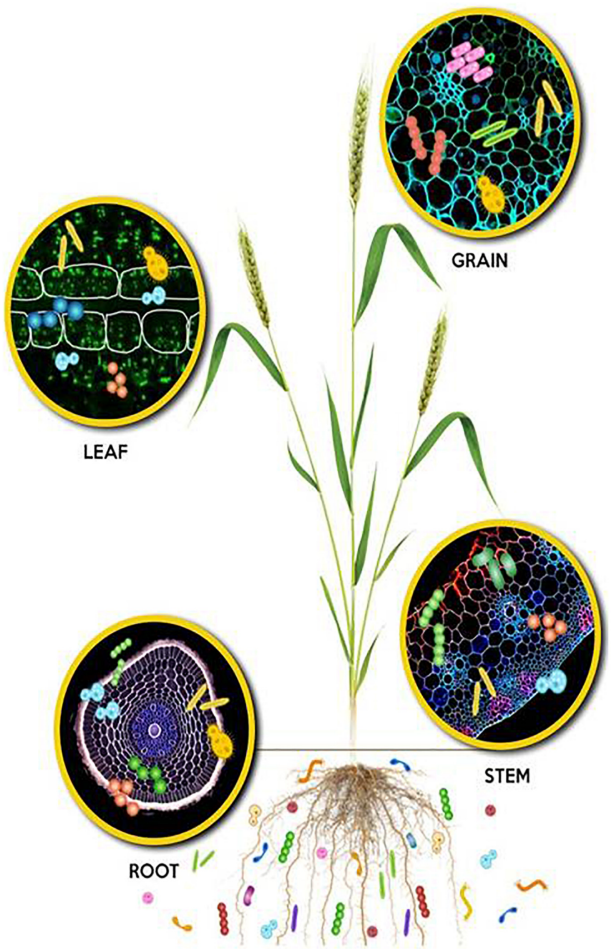


FIGURE 1 | Microbial colonization depicted in different plant niches: Rhizosphere, phyllosphere and endosphere of root, stem, leaf, and grain.

Hence, the bioinoculant technology will move forward toward reducing the cost of cultivation while improving soil and plant health for sustainable agriculture.

PLANT-MICROBIOME-MEDIATED ADAPTIVE FUNCTIONS

The microbiome is playing a significant role, throughout the plant life cycle, in altering the physiologies, and development through phytohormones, metabolites, signals, responses, nutrients, and induction of systemic resistance against pathogens as well as tolerance mechanisms against abiotic stresses such as drought, salinity, or contaminated soils (Mendes et al., 2013; Marag and Suman, 2018; Compant et al., 2019). At the community level, the microbiome functional capability is more than the sum of its individual microbial components as individual microbial species in the microbiome may interact to form a complex network, which interrelates with the host plant(s) in a mutualistic, synergistic, commensalistic, amensalistic, or

parasitic mode of relationship. These interactions influence each member of the complex network for their survival, fitness, and propagation. The sum of all these interactions influences plant health *vis-a-vis* soil fertility (Berg et al., 2020). The advancement in the molecular methods and affordable sequencing has led to a greater understanding of the microbiome composition; however, translating species or gene composition into microbiome functionality still remains a challenge. Using community ecology concepts, Saleem et al. (2019) have indicated that more than individual functions, the overall microbiome biodiversity is critical as the driver of plant growth, soil health, and ecosystem functioning. By meta-analysis of numerous publications on microbial biodiversity and ecosystem functioning (BEF), they indicated that the impacts can be classified into (i) biodiversity effects (negative, no (or unknown), and positive effects of biodiversity on microbial derived services), (ii) assessed functions (nutrient cycling, protection from different stresses, etc.), and (iii) underlying mechanisms (cooperation, mutualism, etc.). Higher diversity can increase the number and resilience of plant-beneficial functions that can be co-expressed and can unlock the expression of plant-beneficial traits that are hard to obtain from any species in isolation. Therefore, the maintenance and modulation of desired microbial activities (functional pools) in the vicinity of the plant system may have more significant potential to provide crops with required nutrition and other protection systems (**Figure 2**).

With increasing knowledge of plant microbiome *vis-à-vis* plant performance, approaches are being devised for tapping the potential of plant-growth-promoting (PGP) isolates, by employing both culturable and unculturable approaches. The advent of "omics" technologies understandably provides the tools for a broader understanding of microbial ecosystems and their dynamic interaction with their hosts. These techniques and methods enable the screening of large microbial populations and easily identify the individual or groups of taxa with functional capabilities. Large-scale genomic analyses of plant-associated bacteria have indicated that the bacteria from phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are dominant in different plant niches (Levy et al., 2018a,b). The exhaustive investigations on wheat seeds followed by rhizospheric, epiphytic, and endophytic bacterial diversity, growing in six diverse agro-climatic zones in India, led to more than 200 diverse bacterial isolates with PGP traits (Suman et al., 2016; Verma et al., 2016, 2019; Verma and Suman, 2018; Sai Prasad et al., 2021). The PGP rhizobacteria (PGPR) can adapt easily to adverse conditions and protect the host plants from the deleterious effects of specific environmental stresses (Glick et al., 1997). Several bacteria like *Bacillus* sp., *Azospirillum*, *Herbaspirillum*, and pink-pigmented methylotrophic bacteria have been shown to mitigate stress conditions in maize, wheat, and other crops (Chakraborty et al., 2013; Vurukonda et al., 2016; Curá et al., 2017; Ahlawat et al., 2018). Various factors related to host, microbes, and the environment influence the community composition and diversity of plant microbiome (Dastogeer et al., 2020). Our knowledge on the underlying mechanism(s) of microbiome assemblages and how they influence the host plants is still lacking. How the entire assembly of microbial communities interfere with the host fitness and health remains largely unknown. Connecting the microbiome composition comprising PGP as well as plant-growth-compromising activities and diversity to their function is a great challenge for future research.

These fundamental, microbial-mediated adaptive functions can help address the significant challenges in sustainable food production under the changing climatic conditions. Likewise, the strategic application of microbial communities rather than as individual isolates to improve plant production offers enormous potential, particularly under adverse environmental conditions. Their applications can serve multiple purposes, such as reducing climate change impact and avoiding excessive reliance on chemical fertilizers and pesticides. Earlier studies solely based on culture-dependent techniques have overlooked the benefits of collective microbial functional and genetic diversity and the advantages of the culture-independent methods (Banik and Brady, 2010; Stewart, 2012; Turner et al., 2013; De Souza et al., 2016; Waigi et al., 2017; Armanhi et al., 2018; Mourad et al., 2018).

The cultivable isolates of the microbial community members such as plant probiotics, biofertilizers, or agricultural bioinoculants have shown their distinct influences on plant growth, fitness, and stress resilience but with certain limitations. The developed formulations containing one or more beneficial microorganism strains (or species) can mediate the cycling of several elements from the soil and transform them into the more readily available form of nutrients for plant uptake. Not only do the probiotic action of these formulations increase the growth, yield, and quality of plants, but they are also a tool to produce high-quality functional foods. The use of microbial-based agricultural inputs has a long history, beginning with broad-scale rhizobial inoculation of legumes in the early twentieth century (Desbrosses and Stougaard, 2011). The "Fresh" Green Revolution, perhaps the Bio-Revolution, needs to be based on fewer intensive inputs with reduced environmental impact. It would be based on biological inputs through utilization of the phytomicrobiome (with inoculants, microbially produced compounds, etc.) and improved crops (by manipulation of the phytomicrobiome community structure) (Timmusk et al., 2017; Backer et al., 2018). With increasing data availability on plant microbiome from different ecological niches, strategic approaches based on the concomitant use of culture-dependent and culture-independent techniques, targeting all the plant-beneficial microbial groups, are necessitated to develop novel biological products in all categories like biofertilizers, biopesticides, bioagents, or bioinoculants and biostimulants.

POTENTIAL OF BIOINOCULANTS FOR FIELD APPLICATION

The current knowledge on functions, ecological adaptations, host interactions, and putative beneficial traits of microorganisms associated with the host plants mainly revolves around a handful of cultivable rhizospheric and endophytic bacteria or fungi. Many microbial formulations having individual or mixture of strains are developed and used at present. These

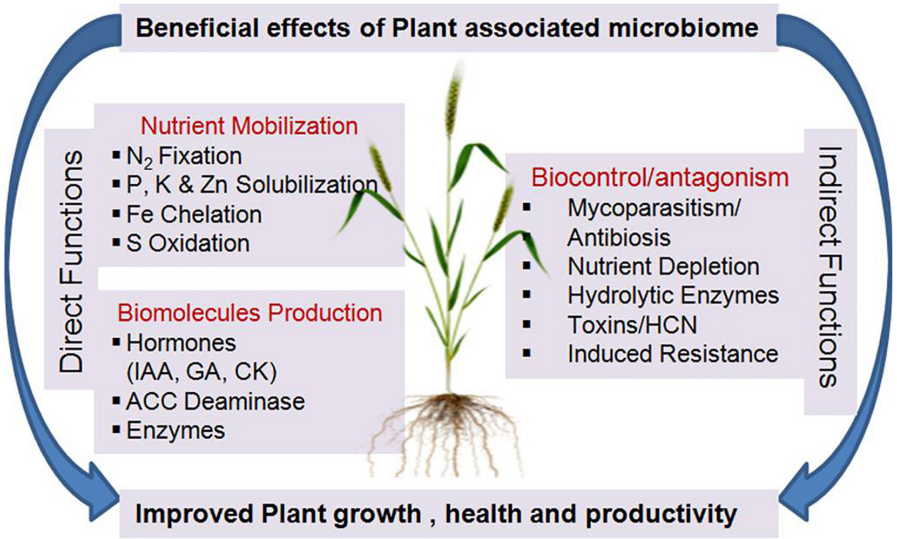


FIGURE 2 | Beneficial functions of Plant associated microbiome. N, Nitrogen; P, Phosphorous; K, Potassium; Zn, Zinc; Fe, Iron; S, Sulfur; IAA, Indole Acetic Acid; GA, Giberrelic Acid; CK, Cytokinin; ACC, 1-AminoCyclopropane Carboxylate; HCN, Hydrocyanic Acid.

biological or bioinoculants are nitrogen fixers, phosphate solubilizers, siderophore producers, photohormone producers, and exopolysaccharide producers. Some of them are involved in lytic enzyme production against pests and pathogens, antibiosis, and induced systemic resistance (Gupta et al., 2015; Sruthilaxmi and Babu, 2017).

The bioinoculants are grouped as either biofertilizers or bioagents depending on the intended purpose of plant growth promotion or protection, respectively. The biofertilizers include the individual species of *Azotobacter*, *Azospirillum*, and *Rhizobium*; phosphate-, potassium-, and zinc-solubilizing bacteria; vesicular–arbuscular mycorrhiza (VAM), and *Acetobacter*. Crop-specific biofertilizers like *Gluconacetobacter diazotrophicus* for sugarcane or generic biofertilizers like *Pantoea* isolates showing multi-PGP activities in several crops have demonstrated benefits in improving crop yield and productivity (Suman et al., 2005, 2008). Not only the rhizosphere-colonizing but also several endosphere-colonizing bacteria have been exploited for their beneficial contributions in sustainable agriculture (White et al., 2019). Presently, bioinoculants are available mostly as single entities (Bashan et al., 2014) but are also being formulated as consortia with multiple bacteria and fungi, which have synergistic PGP traits for improving plant production and productivity. **Tables 1**, **2** summarize the current status of various microbial formulations developed using single, dual, or multiple isolates as bioinoculants to improve nutrient uptake or protect against various biotic and abiotic stresses.

Although the biofertilizer/bioinoculant technology has grown into a proven biological or biotechnological innovation, it is still struggling to get acceptability and popularity with farmers, the end-users. The availability and quality of bioinoculants and their inconsistent performances under field conditions have been identified as significant issues in their adoption by the farming community (Martínez-Hidalgo et al., 2019), which requires the attention of the policymakers in different countries. Along with the development, large-scale production, and assured quality of bioinoculants, one of the most promising ways to increase their efficacy is by introducing effective delivery systems. The farmers may repose the faith, buy these products confidently, and compare their usefulness and cost–benefit ratios with conventional fertilizer inputs. Many studies on bioinoculant development and laboratory-based and field studies proving their worth indicate that these microbial resources must be considered a partial replacement as the application of chemicals may not be wholly replaceable or transferable into biologicals or microbials (Sessitsch et al., 2019).

DESIGNING TARGETED SYNTHETIC BIOINOCULANTS

The natural microbial communities are composed of a mix of microbes with often unknown functions. A promising way to overcome the difficulties associated with studying natural communities is to create artificial synthetic communities that retain the key features of their natural counterparts. With reduced complexity, synthetic microbial communities behave like a defined system and can act as a model system to assess the role of key ecological, structural, and functional features of communities in a controlled way (Großkopf and Soyer, 2014).

The existing thought process of top-down and bottom-up approaches for synthesizing microbial communities is based on the functional character of the individual microbial isolate and metabolic interactions among isolates, respectively. Basic motifs of commensalism, competition, predation, cooperation, and amensalism are the key metabolic interactions for the common substrate or metabolites leading to the community formations (Großkopf and Soyer, 2014). Several reviews have summarized

TABLE 1 | Status of various microbial inoculants developed as synthesized microbial communities in use for improving nutrient uptake and protections against plant pathogens.

S. No.	Microorganism (Bacteria)	Host/Plant associated	PGP Activity	References
	Single culture inoculation			
1.	*Bacillus megaterium* TRS-4	Tea	Biofertilization and biocontrol activity to reduce brown root rot disease (*Fomes lamaoensis*)	Chakraborty et al. (2006)
2.	*Pseudomonas putida* B0	Sub-alpine	Phosphate solubilisation and antagonistic activity	Pandey et al. (2006)
3.	*Pseudomonas fluorescens* GRS1	Pea	Phosphorus solubilisation and increased biomass production	Katiyar and Goel (2003)
4.	*Bacillus pumilus* ES4	Soil	Nitrogen fixation	Hernandez et al. (2009)
5.	*Azospirillum* sp. P1AR6-2	Black pepper	Phosphorus solubilisation along with improved root and shoot growth	Ramachandran et al. (2007)
6.	*Paenibacillus polymyxa* P2b-2R	Canola	Nitrogen fixation, phosphate solubilisation, antibiotic production, and other plant growth regulators for increased plant biomass	Padda et al. (2016)
7.	*Pseudomonas fluorescens* PGPR1	Peanut	Siderophore production, phosphate solubilization, increased yield and biomass production	Dey et al. (2004)
8.	*Bacillus* sp. EUCB 10	Gum trees	IAA production, phosphate solubilization, nitrogen fixation and increased biomass production	Paz et al. (2012)
9.	*Herbaspirillum seropedicae* ZAE94	Rice	Nitrogen fixation and increased biomass production	Alves et al. (2015)
10.	*Bacillus megaterium* B388	Pine	IAA production, phosphate solubilization, antagonistic activity and increased biomass production	Trivedi and Pandey (2008)
11.	*Pseudomonas fluorescens* L321	Pea	Phosphate solubilisation and increased biomass production	Otieno et al. (2015)
12.	*Bacillus aryabhattai* MDSR7	Soybean	Zinc solubilisation, decreased rhizosphere soil pH, increased dehydrogenase, glucosidase, auxin production, microbial biomass	Ramesh et al. (2014)
13.	*Acinetobacter* sp. AGM3	Rice	Zinc solubilisation and IAA production	Gandhi and Muralidharan (2016)
14.	*Bacillus megaterium* CDK25	Cow dung	Phosphate solubilization, IAA production, phytase production, siderophore production and increased plant growth	Bhatt and Maheshwari (2020)
15.	*Enterobacter cloacae* ZSB14	Rice	Zinc solubilization and increased plant growth	Krithika and Balachandar (2016)
16.	*Enterobacter* sp. MN17	Chickpea	Improved productivity, profitability, Zinc use efficiency and quality	Ullah et al. (2020)
17.	*Bacillus* sp. BPR7	Common bean	Production of plant growth regulators and antagonistic activity	Kumar et al. (2012)
18.	*Bacillus* sp. SC2b	Applegate stonecrop	ACC deaminase activity, IAA production, siderophore production, increased chlorophyll content and plant growth	Ma et al. (2015)
19.	*Burkholderia ambifaria* MCI 7	Maize	Siderophore production and antifungal activity	Ciccillo et al. (2002)
20.	*A. brasilense* Ab-V5	Maize	Nitrogen fixation and IAA production	Ferreira et al. (2013)
21.	*Rhizobium leguminosarum* bv. *viciae*	Pea	Increase in nodule number, N accumulation and nitrogen fixation	Clayton et al. (2004)
22.	*P. fluorescens* (PGPR1, PGPR2, and PGPR4)	Peanut	ACC-deaminase activity, IAA production, siderophore production, antifungal activity	Dey et al. (2004)
23.	*Azospirillum* sp. B510	Rice	Nitrogen fixation, IAA production, increase in tiller number and seed yield	Isawa et al. (2009), Bao et al. (2013)
24.	*Bacillus amyloliquefaciens* sks_bnj_1	Soybean	Siderophore production, IAA production, ACC-deaminase activity and antifungal activity, phytases production	Sharma et al. (2013)
25.	*Gluconacetobacter diazotrophicus* Vl27	Sugarcane	Nitrogen fixation, siderophore production, IAA production, phosphorus solubilisation and increase in germination	Beneduzi et al. (2013)
26.	*Azospirillum brasilense* INTA Az-39	Wheat	Nitrogen fixation, IAA production and increased dry matter accumulation	Díaz-Zorita and Fernández-Canigia (2009)
27.	*A. brasilense* (Ab-V5 and Ab-V6)	Wheat and maize	Nitrogen fixation, IAA production and increased yield	Hungria et al. (2010)
28.	*Pseudomonas* sp. PS1	Mung bean	Increase plant dry weight, root nodule, total chlorophyll content, seed yield and seed protein	Ahemad and Khan (2011a, 2012a)
29.	*Bradyrhizobium* sp. MRM6	Mung bean	Increased plant growth parameters	Ahemad and Khan (2011b, 2012b)
30.	*Pseudomonas* sp. A3R3	Cabbage	Increased biomass production	Ma et al. (2011)
31.	*Rhizobium* sp. MRP1	Pea	Nitrogen fixation, increased nodulation, increase in N, P uptake, increase seed yield and seed protein	Ahemad and Khan (2009, 2010)
32.	*Bacillus Weihenstephanensis* SM3	Sunflower	Increased plant biomass and accumulation of trace elements like Cu, Ni and Zn	Rajkumar and Freitas (2008)
33.	Single inoculation of *Brayrhizobium diazoefficiens* USDA 110, B. Elekani USDA 61 and USDA 94	Soybean	Rhizobitoxine production, improved symbiotic effectiveness through high nodulation and nitrogen fixation under drought stress	Govindasamy et al., 2017
34.	Single inoculation of *Ochrobactrum* sp. EB-165*, Microbacterium* sp. EB-65*, Enterobacter sp.* EB-14 *and Enterobacter cloacae strain* EB-48	Sorghum	Multi-PGP traits on molecular regulation of stress responsive genes and improved physiological stress tolerance under drought	Govindasamy et al., 2020

(Continued)

TABLE 1 | Continued

S. No.	Microorganism (Bacteria)	Host/Plant associated	PGP Activity	References
35.	*Gluconacetobacter diazotrophicus* –IS100	Sugarcane	Efficient in promoting plant growth and N recovery more at low nitrogen input	Suman et al. (2005)
36.	*Pantoea* sp *(8) as single inoculant*	Wheat, Maize and Rice	Multi PGP generic bioinoculant for cereals	Suman et al. (2020)
	Dual culture inoculation			
37.	*Azospirillum brasilense* Az39 *Brayrhizobium japonicum* E109	Maize	Promote seed germination, nodule formation, and early development of corn and soybean seedlings	Cassan et al. (2009)
38.	*Pseudomonas fluorescens* Aur6 *Chryseobacterium balustinum* Aur9	Rice	Most effective control against rice blast pathogen	Lucas et al. (2009)
39.	*Bacillus subtilis* SU47 *Arthrobacter* sp. SU18	Wheat	Salinity tolerance and increased dry weight	Upadhyay et al. (2012)
40.	*Pseudomonas jessenii* R62 *Pseudomonas synxantha* R81	Wheat	Increased grain yield	Mäder et al. (2011)
41.	*Azotobacter chroococcum* A-41 *Bacillus mucilaginosus*	Rice	Potassium solubilization, Nitrogen fixation and Mobilization of potassium-bearing minerals.	Basak and Biswas (2010)
42.	*Bacillus subtilis* OSU-142 *Bacillus megaterium* M-3	Chickpea	Nitrogen fixation, Phosphorus solubilisation, increased seed and total biomass yields	Elkoca et al. (2007)
43.	*Gluconacetobacter diazotrophicus* *Trichoderma viride*	Sugarcane	Improves nutrient uptake (N, P and K) on inoculation with FYM	Shukla et al. (2008)
44.	*Chryseobacterium* sp. PSR10 *Escherichia coli* RGR13	Soil	Phosphorus solubilization, enhanced plant growth and yield	Singh et al. (2013)
45.	*Bacillus* sp. ZM20 *Bacillus aryabhattai* ZM31	Bhendi	Zinc solubilisation, improved relative water content and biomass production	Fatima et al. (2018)
46.	*Pantoea dispersa* MPJ9 *Pseudomonas putida* MPJ6	Mungbean	Iron chelation and increased plant growth	Patel et al. (2018)
47.	*Pseudomonas aeruginosa* LSE-2 *Bradyrhizobium* sp. LSBR-3	Soybean	IAA production, phosphorus and zinc solubilization, siderophore production and increased plant growth	Kumawat et al. (2019)
48.	*Pseudomonas jessenii* PS06 *Mesorhizobium ciceri* C-2/2	Chickpea	Higher nodule fresh weight, nodule number and shoot N content, highest in seed yield and nodule fresh weight	Valverde et al. (2007)
49.	*Bacillus cereus* UW85 *B. japonicum*	Soybean	Stimulations in shoot dry weight, increased seed yield and seed N content	John Bullied et al. (2002)
50.	*B. japonicum (*SEMIA 5079 and SEMIA 5080) *A. brasilense* (Ab-V5 and Ab-V6)	Soybean	Nitrogen fixation, IAA production and increased yield	Hungria et al. (2013)
51.	*Azospirillum* sp. *Azotobacter* sp.	Artichoke	Increased radical, shoot length, shoot weight and increased germination	Jahanian et al. (2012)
52.	*Rhizobium leguminosarum* *Pseudomonas. fluorescens*	Lentil	Improved leghemoglobin content, growth and grain yield	Singh et al. (2018)
53.	*Azospirillum sp. AZ204* *Pseudomonas fluorescens Pf1*	Cotton	Nitrogen fixation, Phosphorus solubilisation and biocontrol activity	Marimuthu et al. (2013)
54.	*Enterobacter cloacae* *Bacillus drentensis*	Mung bean	Increase salt tolerance, seed yield, dry biomass, plant height, leaf area, relative water content and chlorophyll	Mahmood et al. (2016)
55.	*Gluconacetobacter* sp. *Burkholderia* sp.	Rice	Higher phosphatase activity, increased P uptake, increased biomass, yield, number of panicles and seeds/panicles.	Stephen et al. (2015)
56.	*Pantoea cypripedii* *Pseudomonas plecoglossicida*	Maize, Wheat	Increased grain yield, P uptake, shoot and root biomass	Gurdeep and Reddy (2015)
57.	*Ochrobactrum ciceri* *Mesorhizobium ciceri*	Kabuli and Desi chickpea	Increased nodulation, biomass and grain yield	Imran et al. (2015)

(Continued)

TABLE 1 | Continued

S. No.	Microorganism (Bacteria)	Host/Plant associated	PGP Activity	References
	Triple culture inoculation			
58.	*Gluconacetobacter azotocaptans* DS1	Maize	Alcohol production, IAA production, phosphorus solubilization, nitrogen fixation and increased biomass production	Mehnaz and Lazarovits (2006)
	Pseudomonas putida CQ179			
	Azospirillum lipoferum N7			
59.	*Bacillus thuringiensis* KR-1	Kudzu	HCN production, IAA production and increased biomass production	Selvakumar et al. (2008)
	Enterobacter asburiae KR-3			
	Serratia marcescens KR4			
60.	*Bacillus cereus* PK6-15	Guinea grass	Zinc solubilization, ammonia production, nitrogen fixation, phosphorus solubilisation and increased plant growth	Bokhari et al. (2019)
	Bacillus subtilis PK5-26			
	Bacillus circulans PK3-109			
61.	*Pseudomonas fluorescens* A506	Pear	Biological control against Fire blight pathogen	Stockwell et al. (2011)
	Pantoea vagans C9-1			
	Pantoea agglomerans			
62.	*Rhizobium* spp.	Chickpea	Nitrogen fixation, biocontrol activity and Phosphorus solubilisation	Elkoca et al. (2007)
	B. subtilis OSU- 142			
	Bacillus megaterium M-3			
63.	*Pseudomonas alcaligenes* PsA15	Maize	Nitrogen fixation and antifungal activity	Egamberdiyeva (2007)
	Bacillus polymyxa BcP26			
	Mycobacterium phlei MbP18			
64.	*P. fluorescens* ACC-5 (biotype G)	Pea	ACC-deaminase activity	Zahir et al. (2008)
	P. fluorescens ACC-14			
	P. putida Q-7 (biotype A)			
65.	*B. vietnamiensis* MG43	Sugarcane	Nitrogen fixation and increased biomass production	Govindarajan et al. (2008)
	G. diazotrophicus LMG7603			
	H. seropedicae LMG6513			
66.	*Bradyrhizobium japonicum*	Soybean and common bean	Nitrogen fixation and increased grain yield	Hungria et al. (2013)
	Rhizobium tropici			
	Azospirillum brasilense			
67.	*Rhizobium leguminosarum*	Common bean	Increased grain yield	Kumar et al. (2016)
	Bacillus sp.			
	Pseudomonas sp.			
68.	*Pseudomonas aeruginosa*	Tomato	Increased root and shoot length, ACC deaminase activity, IAA production, phosphate solubilization and siderophore production	Tank and Saraf (2010)
	Pseudomonas uorescens			
	Pseudomonas stutzeri			
69.	*Xanthomonas sp. WCS2014-23*	Arabidopsis	Less fungal spores and higher plant fresh weight	Berendsen et al. (2018)
	Stenotrophomonas sp. WCS2014-113			
	Microbacterium sp. WCS2014-259			
	Multiple culture inoculations			
70.	*Exiguobacteriumaurantiacum* MS-ZT10, *Trabusiella* sp. MS-ZT1, *Aeromonas* sp. MS-ZT4, *Arthrobacter* sp. MS-ZT5	Wheat	Zinc solubilisation, enhanced N, P, and K concentration	Shaikh and Saraf (2017)
71.	1:1:1:1 ratio of Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes	Arabidopsis	Reciprocal relocation between root and leaf microbiota members and functional overlap in the communities with improved plant growth	Bai et al. (2015)

(Continued)

TABLE 1 | Continued

S. No.	Microorganism (Bacteria)	Host/Plant associated	PGP Activity	References
	Triple culture inoculation			
72.	*Bacillus amylolquifaciens, Bacillus simplex,* MCP of 12 isolates *Azotobacter vinlandii*, *Clostridium* sp., *Lactobacillus* sp., *Bacillus velezensis*, *Bacillus subtilis* (SILo Sil® BS), *Bacillus thuringiensis*, *Pseudomonas fluorescens*, *Acetobacter*, *Enterococcus*, *Rhizobium japonicum*, *Nitrosomonas*, and *Nitrobacter*, as well as fungi: *Saccharomyces*, *Penicillium roqueforti*, *Monascus*, *Aspergillus oryzae*, *Trichoderma harzianum* (TRICHOSIL®), and algae extracts from *Arthrospira platensis* (Spirulina) and *Ascophyllum nodosum*	Tomato	Improved phosphate (P) acquisition, increased biomass production and fruit yield	Bradáčová et al. (2019)
73.	*Arthrobacter nitroguajacolicus E46, Bacillus mojavensis K1, Pseudomonas frederiksbergensis A176, Arthrobacter nitroguajacolicus E46, Bacillus cereus CN2, Bacillus megaterium B55, Bacillus mojavensis K1, Pseudomonas azotoformans A70, Pseudomonas frederiksbergensis A176, Pseudomonas azotoformans A70*	Tobacco	Increased fitness and survival of tobacco plants	Santhanam et al. (2015)
74.	*Bacillus megaterium SOGA_2, Curtobacterium ceanosedimentum SOGA3, SOGA6, Massilia aurea SOGA7, Pseudomonas coleopterorum SOGA5, 11, 12, Pseudomonas psychrotolerans SOGA13, Pseudomonas rhizosphaerae SOGA14 and 19, Frigoribacterium faeni SOGA17, Xanthomonas campestris OGA20*	Tomato	Fewer pathogen (*Pseudomonas syringae* pv. tomato) DNA copies in the phyllosphere of field-grown tomato plants	Berg and Koskella (2018)
75.	*8 Pseudomonas spp.*	Pea, wheat, etc.	Reduced disease severity and pathogen (*Ralstonia solanacearum*) abundance in pea, wheat, cotton, tomato, sugar beet and tobacco	Hu et al. (2016)
76.	*Pseudomonas* spp., *Bacillus amyloliquefaciens*, *Bacillus subtilis*, soil yeast	Rice	Increased grain and straw yields, total N uptake, as well as grain quality in terms of N percentage	Cong et al. (2009)
77.	*Rhizobium*, *Sinorhizobium*, *Bacillus*, *Burkholderia*	Pigeon pea	Increased plant biomass and nodule mass per plant	Pandey and Maheshwari (2007)
78.	*Arthrobacter nitroguajacolicus*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus mojavensis*, *P. azotoformans*, *P. frederiksbergensis*	Tobacco	Reduced disease incidence and mortality without influencing growth or herbivore resistance	Verma et al. (2013)
79.	Mixes of various *Pseudomonas*, *Enterobacter* and *Serratia* strains	Rapeseed	Increased rapeseed oil and grain yields	Lally et al. (2017)
80.	Various consortia involving *Enterobacter*, *Serratia*, *Pseudomonas*, *Microbacterium* and *Achromobacter*	Avocado	Mitigate water shortage and salt stress	Barra et al. (2016)
81.	*Bacillus amyloliquefaciens* strains	Tomato	Decreased disease incidence	Wei et al. (2011)
82.	*Pseudomonas spp. CHA0, PF5, Q2-87, Q8R1-96, 1M1-96, MVP1-4, F113, Phl1C2*	Pea	Reduced disease severity and pathogen abundance in pea, wheat, cotton, tomato, sugar beet and tobacco	Hu et al. (2016)
83.	4 Small communities each of endophytes from sugarcane, maize, brassica and wheat	Wheat-maize cropping system	Improves system productivity at low input of nitrogen and irrigation managing abiotic stress	Suman et al. (unpublished)

the study of ecological interactions among microbes in synthetic as well as in natural microbial communities (Faust and Raes, 2012; Mitri and Richard Foster, 2013). Linking the composition of microbial communities with the functions is a central challenge in microbial ecology. It may be linked in some systems, but not in others, as some functions are restricted to certain taxa (e.g., sulfate reduction), but other functions are widespread across diverse groups (e.g., photosynthesis). A microbiome may

TABLE 2 | Fungal inoculants developed as synthesized microbial communities used for improving nutrient uptake and protections against plant pathogens.

Sr No.	Microorganism (fungal)	Host/plant associated	PGP activity	References
	Single-culture inoculation			
1.	*Glomus* sp. 88	Wheat	Phosphorus solubilization	Singh and Kapoor, 1999
2.	*Penicillium rugulosum* IR-94MF1	Maize	Phosphorus solubilization	Reyes et al., 2002
3.	*Eupenicillium parvum* NRRL 2095	Tea	Phosphorus solubilization and high stress levels of aluminum and iron desiccation	Vyas et al., 2007
4.	*Trichoderma harzianum*	Soil	*Trichoderma*-enriched compost extracts, symbiotic association, and suppression of fungal infections	Siddiqui et al., 2008
5.	*Trichoderma asperellum* Q1	Cucumber	Siderophore production and inducement of plant systemic resistance (broad spectrum), resistance to plant pathogens, and plant growth promotion	Qi and Zhao, 2013
	Dual-culture inoculation (mostly with bacteria)			
6.	*Gluconacetobacter diazotrophicus* IS100	Sugarcane	Improved nutrient uptake (N, P, and K) on inoculation with FYM	Shukla et al., 2008
7.	*Trichoderma viride* *Gluconacetobacter diazotrophicus* IS100	Sugarcane	Consortium brought economy in the use of fertilizer N by 45.2 kg ha^{-1} and also increased the yield by 6.1 t ha^{-1} compared to the control treatment	Yadav et al., 2009
8.	*Trichoderma viride* *Bacillus/Pseudomonas* *Aspergillus/Penicillium*	Soil/rhizosphere	P solubilization and symbiotic association	Sharma et al., 2013
9.	*Pseudomonas aeruginosa*	Soil and rhizosphere	Biocontrol agent against pathogen, pest, symbiotic association	Afzal et al., 2013
10.	*Trichoderma viride* *Microbispora* sp.	Soil	ACC deaminase (stressbuster) and IAA production, N2 fixation, P solubilization, siderophore production, and symbiotic association	Glick, 2014; Souza et al., 2015
11.	*Streptomyces* sp. *Trichoderma harzianum*	Tobacco	Effective *Ralstonia solanacearum* suppression at 68.2% disease incidence	Yuan et al., 2016
12.	*Glomus mosseae* *Aspergillus* sp.	Common bean	Increased P uptake and N content, increased biomass, and increased nodule number	Elias et al., 2016
13.	*Penicillium* sp. *Funneliformis mosseae* *Bacillus sonorensis*	Chili	Increased plant growth, dry weight, fruit yield, and nutrient concentration	Thilagar et al., 2016
14.	*Pseudomonas* AM fungi	Tomato	Sugar and vitamin production and increased sweetness	Bona et al., 2017
	Triple-culture inoculation (mostly with bacteria)			
15.	*Pseudomonas reactans* *Chryseobacterium humi* *Rhizophagus irregularis*	Soil	N fixation and symbiotic association	Moreira et al., 2016
16.	*Pseudomonas putida* *Bacillus megaterium* AM fungi (*Glomus coronatum*, *Glomus constrictum*, or *Glomus claroideum*)	Abiotic (water) stress condition	Stimulation of plant growth, drought tolerance, IAA production, and symbiotic association	Marulanda-Aguirre et al., 2008
17.	Two *Pseudomonas* Mixed mycorrhiza	Tomato	Increased flowering, dimensions, and weight of tomato fruits and improved industrial and nutritional features of fruits	Bona et al., 2017
18.	*Pseudomonas aeruginosa* (PHU094)	Chick pea	Suppression of *Sclerotium rolfsii*	Singh et al., 2013

(Continued)

TABLE 2 | (Continued)

Sr No.	Microorganism (fungal)	Host/plant associated	PGP activity	References
	Trichoderma harzianum (THU0816)			
	Mesorhizobium sp. (RL091)			
19.	*P. aeruginosa* PJHU15	Peas	Suppression of *Sclerotinia sclerotiorum*	Jain et al., 2015
	T. harzianum TNHU27			
	Bacillus subtilis BHHU100			
	Multiple-culture inoculations (with bacteria)			
20.	*Azospirillum*, *Rhizobium*, *Bacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, *Coniothyrium*, *Ampelomyces*, *Trichoderma*	Soil	Soil conditioner, plant pathogen suppressor, biofertilizer, plant straightener, phytostimulator, biopesticide, and symbiotic association	Berg, 2009

contain both phylogenetic and functional redundancy. Many novel insights on the microbial community composition and organization of plant microbiomes of several crops have come from metagenomic studies using high-throughput sequencing (Edwards et al., 2015; Beckers et al., 2016; Wagner et al., 2016). Metagenomics enables the study of all microorganisms, cultured or not, through the analysis of genomic data obtained directly from an environmental sample, providing knowledge of the species present and information regarding the functionality of microbial communities in their natural habitat. Functional metagenomics has been utilized, with much success, to identify many novel genes, proteins, and secondary metabolites such as antibiotics with industrial, biotechnological, pharmaceutical, and medical relevance (Culligan and Sleator, 2016).

A microbiome may contain both phylogenetic and functional redundancy. Phylogenetic redundancy occurs when multiple OTUs from the same lineage are present in a microbiome, while functional redundancy occurs when multiple OTUs perform the same action (e.g., nitrogen fixation) within a microbiome (Shade and Handelsman, 2012). Phylogenetic redundancy is important for defining the core microbiome, which may buffer the ecological disturbances and enable the recovery of community functions. Several reports on human microbiome indicate that gut microbiome disturbances due to heavy antibiotics are restored due to the redundancy of the core group only (Antonopoulos et al., 2009). It carries relevance in agriculture as different agri-management systems lead to the disturbances in soil microbiome *vis-a-vis* plant microbiome. Recently, Berg et al. (2021) summarized the effects of microbial inoculants on the indigenous plant microbiome and termed this unexplored mode of action as "microbiome modulation."

Synthetic microbial community analysis in gnotobiotic systems is a valuable approach to create reproducible conditions to experimentally test microbial interactions *in situ*. Such systems have been developed for animal and plant models including the well-studied plant *Arabidopsis thaliana*. With established huge volume of data on the metagenome of different crops, there is a need for its translation to certain tailored microbiome-based solutions for promoting plant growth under a range of environmental conditions and increasing resilience to biotic and abiotic stresses. The genomic data with taxonomic status, habitat compatibility, and functional trait knowledge including metabolic potential of plant microbiome communities can be followed as the approach for designing effective microbial inoculants. Here, based on phylogenetic or functional redundancy, two approaches for synthesizing microbial-communities-based bioinoculants are discussed.

Community-Based SB

Microbial colonization in the plant root rhizosphere is the outcome of the interplay between roots exuding chemical compounds that microbes capture as signals and on which their survival and perpetuance depend. The differential abundance of colonizing microbes and the establishment of core-microbiome-based microbial communities forms the basis for plant–microbe interactions. The core members remain present throughout the development of the crop, which may be joined by other taxa during the crop growth. The metagenome data about the relative abundance of colonizing phyla/taxa and core microbiome in the plant rhizosphere and endosphere form the basis for developing Community-Based SB (CSB). Microbial isolates representing the abundant phyla can be sourced either from the crop associated culture bank or with targeted culturomics, for developing the synthetic community. The isolates are expected to be rich in community-forming characteristics like motility, chemotaxis ability, quorum sensing, metabolic diversity, and others. This approach is a direct microbiome manipulation where inoculated CSB may serve to reduce the time required for the rhizosphere microbiome to achieve niche saturation and competitive exclusion of pathogens (Bakker et al., 2012).

Taye et al. (2020) reported that in field-grown *Brassica napus*, rhizosphere core genera found at each growth stage were generally part of the overall core taxa at the 75% prevalence threshold. *Arthrobacter*, *Bradyrhizobium*, and an unclassified *Acidobacteria* in the class Ellin6075 were present in all growth stages, while other genera joined at the flowering or harvesting stage, as the recruitment of the microbiome is governed majorly

by the host plant. Metagenome analysis of more than 600 *Arabidopsis thaliana* plants from eight diverse, inbred accessions growing at different locations indicated that the core endophytic microbiome is less diverse than their corresponding rhizosphere soil microbiomes. The soil types influenced the microbial communities in the *A. thaliana* rhizosphere, but the endophytic communities were overlapping and less complex with maximum of actinobacteria and selected proteobacteria. Lundberg et al. (2012) concluded that the host plants influenced the bacterial colonization in the rhizosphere which varied between inbred lines of *Arabidopsis*, but in the endophytic compartment, it remained consistent across different soil types. An extensive bacterial culture collection that captures a large part of the natural microbial diversity of healthy *A. thaliana* plants was established (Bai et al., 2015). Carlström et al. (2019) conducted dropout and late introduction experiments by inoculating *A. thaliana* with synthetic communities from a resource of 62 native bacterial strains to test how arrival order shapes community structure and indicated that individual Proteobacteria (*Sphingomonas* and *Rhizobium*) and Actinobacteria (*Microbacterium* and *Rhodococcus*) strains have the greatest potential to affect community structure as keystone species.

Similar influences of maize inbred lines growing in different soils and agri-management systems suggested the substantial variation in α- or β-bacterial diversity and relative abundances of taxa with a small proportion of heritable variation across fields. Despite significant differences between the microbial community profiles of maize inbreeds, the estimated α- and β-diversity could not define the kinship of the 27 maize inbreeds to supplement the diversification history of maize (Peiffer et al., 2013). Edwards et al. (2015) resolved the distinct nature in the microbiomes associated with rhizosphere, rhizoplane, and endosphere of rice roots, influenced by the growing conditions and genotypes.

The functional diversity within microbial communities enables metabolic cooperation toward accomplishing more complex functions than those possibly exhibited by a single organism. The consortium members or communities can communicate by exchanging metabolites or molecular signals to coordinate their activity through temporal and spatial expression and further execution of required functions. In contrast with monocultures, microbial members at the community level can self-organize to form spatial patterns, as observed in biofilms or soil aggregates. This self-organization enables them to adapt to the gradient changes, improve resource interception, and exchange metabolites more effectively (Zhang and Wang, 2016; Ben Said and Or, 2017). Hence, the selection and sourcing of microbial members are very important for the construction of CSBs, and they can be from the microbial communities specific to plant niches like rhizosphere (Huang et al., 2018), endosphere, and phyllosphere (Kong and Glick, 2017). Kong et al. (2018) reviewed the strategies for developing synthetic microbial consortium (SMC) and suggested that the crops with good quality can be a good origin of SMC. Based on next-generation sequencing and network analysis, the core microbes can be isolated from the rhizospheric soils or the plant roots using the web-based platform KOMODO (Known Media Database). Herrera Paredes et al. (2018) designed synthetic bacterial communities based on predominant phyla and demonstrated their effect on developing specific and predictable phenotypes in *A. thaliana*. Using the plant–bacterium binary-association assays, the effect of bacterial community manipulation was observed on the plant response to phosphate (Pi) starvation. This approach might contribute to microbial communities' rational design and deployment to improve the host response to biotic and nutritional stresses.

In vitro techniques have demonstrated that the host genotypes and abiotic factors influence the composition of plant microbiomes. At the *in vivo* level, it is a challenge to define the mechanisms controlling the community dynamicity, its assembly, and the beneficial effects on the plant hosts. In an earlier study, the host-mediated natural selection of bacteria by maize roots was employed to select a simplified synthetic bacterial community consisting of seven strains (*Enterobacter cloacae*, *Stenotrophomonas maltophilia*, *Ochrobactrum pituitosum*, *Herbaspirillum frisingense*, *Pseudomonas putida*, *Curtobacterium pusillum*, and *Chryseobacterium indologenes*) representing the dominant phyla such as Proteobacteria and Actinobacteria (Niu et al., 2017). By assessing the functional role of these bacterial community combinations using axenic maize seedlings, *E. cloacae* was identified as the keystone member in this model ecosystem. This model community inhibited the phytopathogenic fungus *Fusarium verticillioides*, both *in vitro* and *in planta*, indicating a stronger benefit to the host plant. The reductionist approaches to disentangle the inherent complexity of microbial communities' interactions have also been suggested for SynComs to be used as inoculants for a given host to decipher their key functions under the gnotobiotic system (Vorholt et al., 2017). Thus, these recent reports support the strategy of combining unculturable and culturable methods, giving the possibility of assembling a representative, yet simplified, bacterial synthetic communities from the pool of dominant genera present in the system. **Figure 3** represents an outline for developing CSB based on the metagenome data and bioinformatic applications for predominant taxa and core microbiome. The key functions for developing such communities are collection of available individual isolates representing predominant taxa or isolating them using culturomic tools. Furthermore, such communities can be strengthened by their ecological interactions and probable functional annotations under gnotobiotic conditions.

Function-Based SB

Due to high organic matter, soils with dynamic microbial ecologies typically have lower fertilizer requirements than conventionally managed soils (Bender et al., 2016). Focusing on the functional groups of microorganisms rather than on taxonomic relatedness and manipulating their activities (functional pools) in the vicinity of the plant ecosystem have more significant potential for providing nutrients and stress protection requirements of crops. Further exploration into the mechanisms and specificity of plant growth promotion from these key microorganisms will refine their specific use and maximize the potential inherently possessed by the microbiomes of plants or soils (Parnell et al., 2016). As only a limited proportion of microbial diversity is cultured, there is much

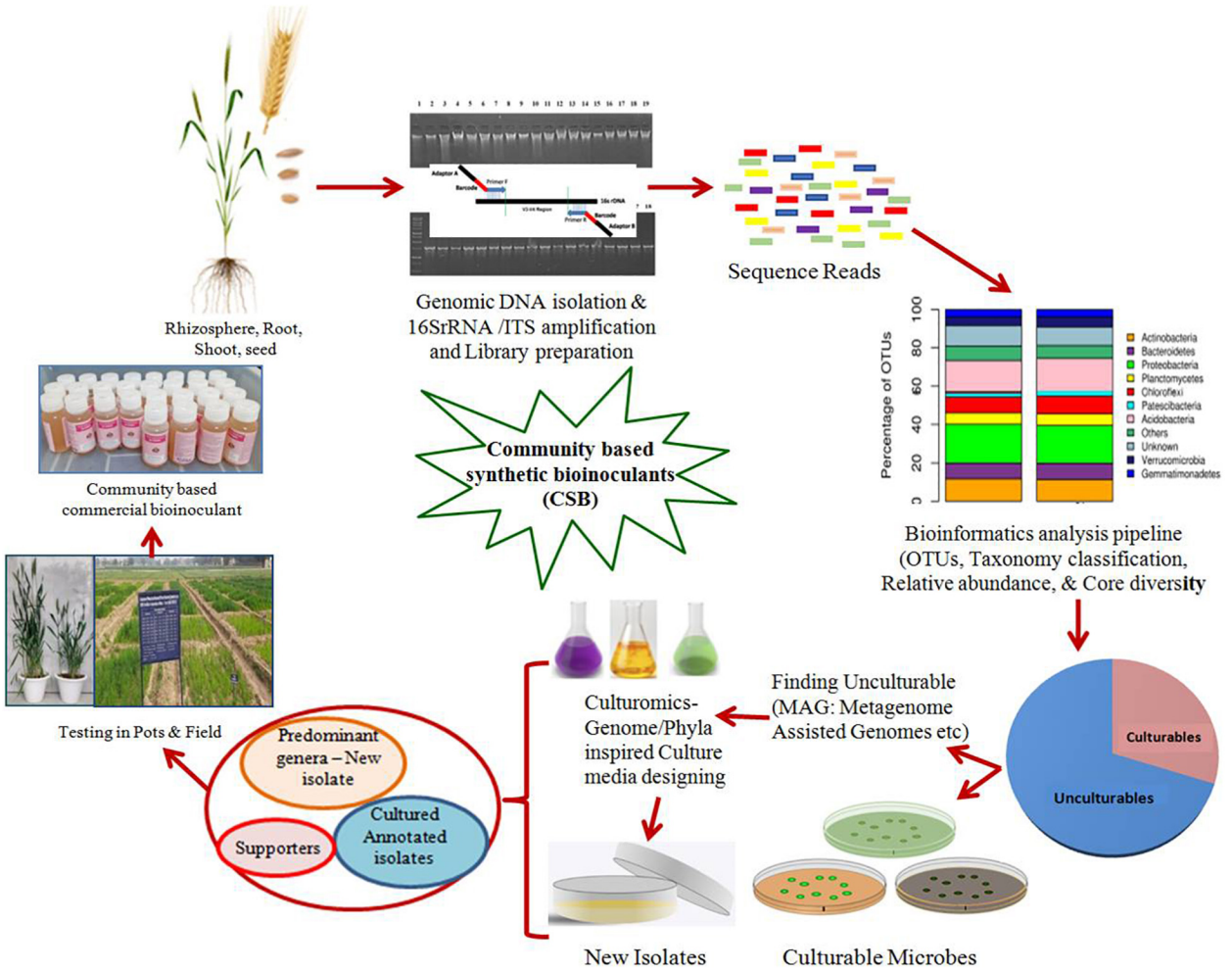


FIGURE 3 | Schematic depiction of different steps for the development of microbial community based synthetic bioinoculants (CSB) by employing metagenomic and bioinformatic techniques.

scope for culturomics to identify, culture, and include important taxa for their beneficial exploitation (Sarhan et al., 2019). Few commercial products have emerged that take advantage of combining different biofertility products. A bacterial consortium Mammoth P^{TM} consisting of *Comamonas testosteroni*, *P. putida*, *E. cloacae*, and *Citrobacter freundii* has been reported to enhance phosphate mobility and improve crop productivity twofold (Baas et al., 2016). The combined abilities of *Bacillus amyloliquefaciens* and the filamentous fungus *Trichoderma virens* marketed under the trade name QuickRoots® (Monsanto BioAgAlliance, 2015), when applied to field corn, show positive yield improvements ranging from 220 to 500 kg ha^{-1}. Similarly, several microbial consortia have been reported to improve host plants' nutrition (Shukla et al., 2008; Suman et al., 2008; Dal Cortivo et al., 2018). The synthetic microbial community of *P. putida* KT2440, *Sphingomonas* sp. OF178, *Azospirillum brasilense* Sp7, and *Acinetobacter* sp. EMM02 has been shown to improve drought stress tolerance in maize (Molina-Romero et al., 2017). Two synthetic microbial communities (SynComs 1 and 2) of known antagonistic *Bacillus* and other isolates from compost-rich soils inhibited *Fusarium* wilt symptoms and promoted tomato growth (Tsolakidou et al., 2019). Menéndez and Paço (2020) have explored synergies between rhizobial and non-rhizobial bacteria for beneficial effects on different crops. Woo and Pepe (2018) described *Trichoderma* and *Azotobacter* as anchorage microorganisms for developing their respective consortia for promoting plant health and mitigating stress conditions. The established arbuscular mycorrhizal fungi (AMF) system, mainly known for P transport, is also a carrier of endophytes in the plant system, can induce systemic resistance to pathogens, and assists in moisture conservation (Cameron et al., 2013; Rouphael et al., 2015). Through the genomic approach of using multiplex amplicon sequencing of the community-based culture collection, Xu et al. (2016) identified the four most representative genera, *Bacillus*, *Chitinophaga*, *Rhizobium*, and *Burkholderia*, for the development of bioinoculants. Armanhi et al. (2018) gave a novel methodology for developing a PGP community-based culture collection (CBC) from sugarcane microbiomes, particularly roots

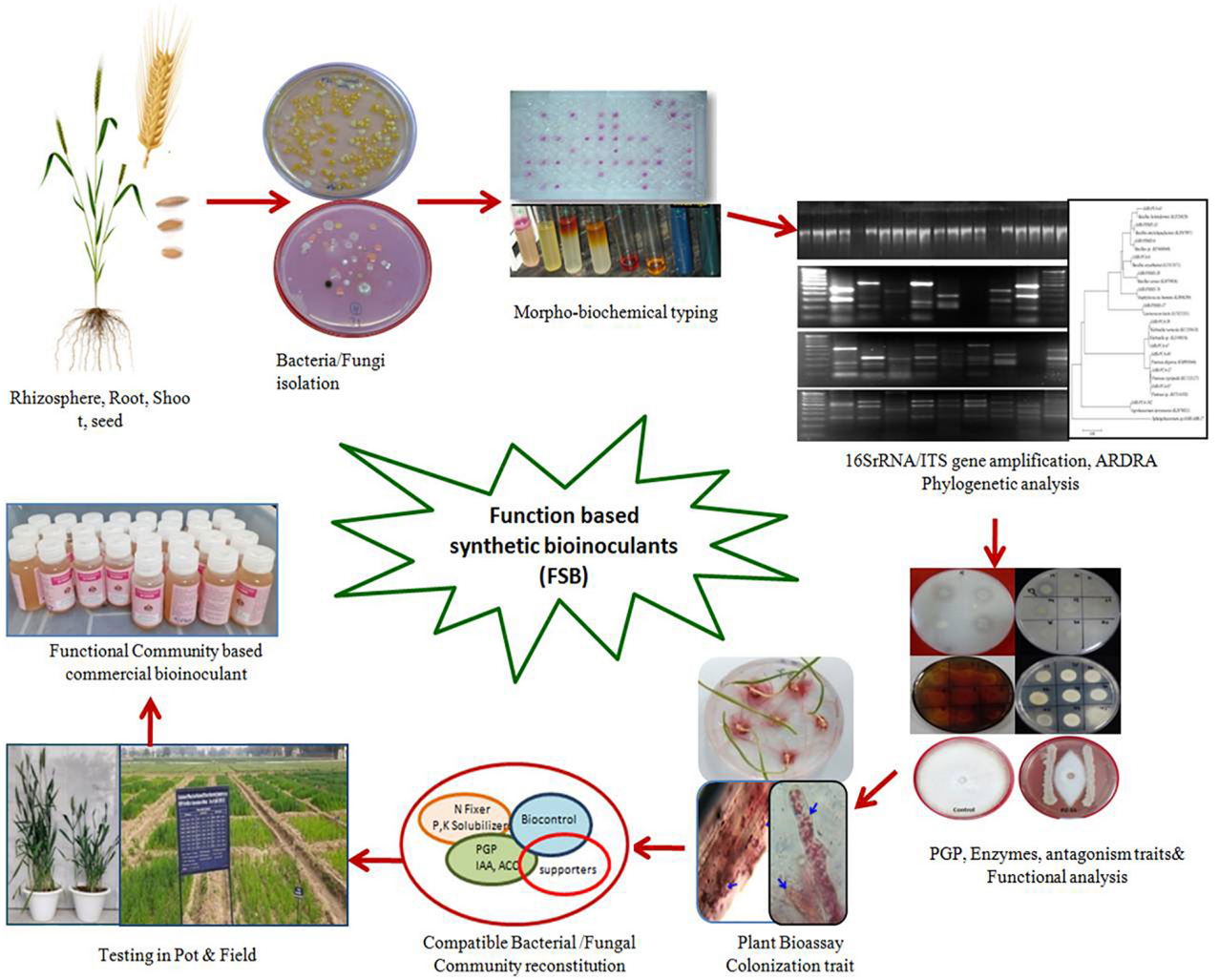


FIGURE 4 | Schematic depiction of different steps for the development of microbial Function based Synthetic Bioinoculants (FSB) using functional characteristics of cultured isolates.

and stalks. The CBC recovered 399 unique bacteria, representing 15.9% of the rhizosphere core microbiome and 61.6–65.3% of the endophytic core microbiomes of sugarcane stalks. This synthetic community of highly abundant genera was tested for colonization of maize as the test crop. The inoculated synthetic community efficiently colonized plant organs (53.9%) and improved plant biomass production, indicating their beneficial effects. Hence, the steps for designing Function-Based SB (FSB) essentially involve identifying and culturing the core microbes, selecting the microbes for plant growth functions, optimizing the microbial interactions according to their compatibility and suitable conditions, and assessing the efficacy of these FSBs under *in vitro* and *in vivo* conditions for the final release of the formulated product for farmers (**Figure 4**). Therefore, the FSBs can be foreseen as a small subset of the community from the natural existing microbial communities. Although the FSB may be similar to many other microbial consortia used in different crops, the fundamental difference lies in the functional analysis of the microbiome and the subsequent selection and formulation.

HARMONY OF BIOINOCULANTS WITH SUSTAINABLE AGRICULTURE GOALS

The UN framework of the "2030 Agenda" for 17 Sustainable Development Goals (SDGs) has been adopted by the 193 member states to develop their vision, strategy, and targets for achieving SDGs by effectively making them part of their policies. In its sustainability framework to realize the goal of ending hunger (SDG2), India has several initiatives that include the management of soil health. Successful organic cultivation and integrated agriculture will be highly dependent on the efficient microbiome-based bioinoculants for plant nutrient management and, more importantly, the recycling of crop residues for soil health (Vision 2030, DARE, India). In contrast, many other practices affect the

abundance of microbial taxa involved in pest and soil disease suppression and nutrient cycling (Lupatini et al., 2017). The importance of microbiome-based solutions is gaining attention in the interrelated systems of environmental management, sustainable food, and fuel production, and human/animal health (FAO, 2019). There is a strong need for integrated research among soil and microbial scientists, growers, extension clienteles, ecologists, and policymakers to develop strategies to preserve and utilize microbial resources for soil health and crop production (Saleem et al., 2019). The microbiome research also leads to a paradigm shift in preserving axenic samples in culture collections to preserving complex communities such as "microbiome biobanks" with their functional perspectives (Ryan et al., 2021). D'Hondt et al. (2021) have summarized the key role of microbiomes in contributing policies interfacing the SDGs globally and emphasized the investments, collaborations, regulatory changes, and public outreach for innovations in microbiome-based bioeconomies.

CONCLUSION

The sustainability of the modern agriculture system is critical to feed the continuously growing human and animal populations, wherein the guided use of microbiomes has an inevitable role in promoting plant growth, development, productivity, and nutrient value. The current biofertilizers are based on individual bacterial cultures with specific traits such as N fixation or the solubilization of P or K. But with the detailed diversity and functional analyses of plant-associated microorganisms, a better understanding has emerged that the plant-associated microbiomes have a tremendous and so-far untapped potential to improve the acquisition of nutrients and resilience to abiotic and biotic stresses and, ultimately, the crop yields. The options of generating synthetic communities using taxonomy abundance alone or with functionally annotated predominant taxa are now available for the improved use of microbial resources in crop cultivation. Nevertheless, developing any microbial community requires a collection of promising functionally annotated and compatible isolates in hand, rather than only microbiome data. Hence, it will be appropriate to holistically use the knowledge of unculturable microbiome generated through structural and functional genomics tools and culturable approaches to get the common and rare taxa for synthetic community preparations. The rational workflow for developing community and function-based bioinoculant preparations has been described, which can be used for developing formulations with the targeted functions of nutrient supplementation and stress management in sustainable agriculture.

AUTHOR CONTRIBUTIONS

AS conceptualized and wrote the manuscript. VG helped in the finalization of tables and figures. KAn and BR gave intellectual input and edited the manuscript. KAs, JS, PS, and DP contributed in data search for the content and table formulation. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

For this review formulation, authors acknowledge working in ICAR-IARI project "Integrated crop and resource management for enhanced productivity and profitability" under activity "Inquisition, functional annotation and evaluation of endophytic microflora for improved moisture and temperature stress tolerance" (Project 15610190062/1010512) and the DST-SERB project "Exploring seed microbiome of wheat from varied agro-climatic zones to develop synthetic microbials" (Project CRG/2019/002146). The authors acknowledge the NAHEP-CAAST for funding student work. AS acknowledges Joyce S. Khanuja for the figure drawings and the mentor "FloraFauna" foundation for critical suggestions.

REFERENCES

Abdul Rahman, N. S. N., Abdul Hamid, N. W., and Nadarajah, K. (2021). Effects of abiotic stress on soil microbiome. *Int. J. Mol. Sci.* 22:9036. doi: 10.3390/ijms22169036

Abiraami, T. V., Suman, A., Singh, B., Aswini, K., and Annapurna, K. (2021). Radiochemical evidence for the contribution of chemotyped siderophore producing bacteria towards plant iron nutrition. *Curr. Microbiol.* 78, 4072–4083. doi: 10.1007/s00284-021-02658-2658

Afzal, S., Tariq, S., Sultana, V., Ara, J., and Ehteshamul-Haque, S. (2013). Managing the root diseases of okra with endo-root plant growth promoting *Pseudomonas* and *Trichoderma viride* associated with healthy okra roots. *Pak. J. Bot.* 45, 1455–1460.

Ahemad, M., and Khan, M. S. (2009). Toxicity assessment of herbicides quizalafop-p-ethyl and clodinafop towards *Rhizobium pea* symbiosis. *Bull. Environ. Contam. Toxicol.* 82, 761–766. doi: 10.1007/s00128-009-9692-x

Ahemad, M., and Khan, M. S. (2010). Comparative toxicity of selected insecticides to pea plants and growth promotion in response to insecticide-tolerant and plant growth promoting *Rhizobium leguminosarum*. *Crop Protection* 29, 325–329.

Ahemad, M., and Khan, M. S. (2011a). Effect of tebuconazole-tolerant and plant growth promoting *Rhizobium* isolate MRP1 on pea-*Rhizobium symbiosis*. *Sci. Horticulturae* 129, 266–272. doi: 10.1016/j.scienta.2011.03.036

Ahemad, M., and Khan, M. S. (2011b). *Pseudomonas aeruginosa* strain PS1 enhances growth parameters of green gram [*Vigna radiata* (L.) Wilczek] in insecticide-stressed soils. *J. Pest Sci.* 84, 123–131. doi: 10.1007/s10340-010-0335-330

Ahemad, M., and Khan, M. S. (2012a). Alleviation of fungicide-induced phytotoxicity in greengram [*Vigna radiata* (L.) Wilczek] using fungicide-tolerant and plant growth promoting *Pseudomonas* strain. *Saudi J. Biol. Sci.* 19, 451–459. doi: 10.1016/j.sjbs.2012.06.003

Ahemad, M., and Khan, M. S. (2012b). Productivity of greengram in tebuconazole-stressed soil, by using a tolerant and plant growth-promoting *Bradyrhizobium sp*. MRM6 strain. *Acta Physiol. Plant.* 34, 245–254. doi: 10.1007/s11738-011-0823-828

Ahlawat, O. P., Tiwari, R., and Singh, G. P. (2018). Metagenomics of wheat rhizosphere for abiotic stress management. *Wheat Barley Res.* 10, 64–77. doi: 10.25174/2249-4065/2018/79291

Alves, G. C., Videira, S. S., Urquiaga, S., and Reis, V. M. (2015). Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several

Herbaspirillum inoculants. *Plant Soil* 387, 307–321. doi: 10.1007/s11104-014-2295-2292

Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L., and Young, V. B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infect. Immun.* 77, 2367–2375. doi: 10.1128/IAI.01520-1528

Armanhi, J. S. L., de Souza, R. S. C., Damasceno, N. D. B., de Araújo, L. M., Imperial, J., and Arruda, P. (2018). A community-based culture collection for targeting novel plant growth-promoting bacteria from the sugarcane microbiome. *Front. Plant Sci.* 8:2191. doi: 10.3389/fpls.2017.02191

Baas, P., Bell, C., Mancini, L. M., Lee, M. N., Conant, R. T., and Wallenstein, M. D. (2016). Phosphorus mobilizing consortium Mammoth P^{TM} enhances plant growth. *PeerJ* 4:e2121. doi: 10.7717/peerj.2121

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., et al. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* 528, 364–369. doi: 10.1038/nature16192

Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L., and Vivanco, J. M. (2012). Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360, 1–13. doi: 10.1007/s11104-012-1361-x

Banik, J. J., and Brady, S. F. (2010). Recent application of metagenomic approaches toward the discovery of antimicrobials and other bioactive small molecules. *Curr. Opin. Microbiol.* 13, 603–609. doi: 10.1016/j.mib.2010.08.012

Bao, Z., Sasaki, K., Okubo, T., Ikeda, S., Anda, M., Hanzawa, E., et al. (2013). Impact of *Azospirillum sp.* B510 inoculation on rice-associated bacterial communities in a paddy field. *Microbes Environ.* 28, 487–490. doi: 10.1264/jsme2.ME13049

Barra, P. J., Inostroza, N. G., Acuña, J. J., Mora, M. L., Crowley, D. E., and Jorquera, M. A. (2016). Formulation of bacterial consortia from avocado (*Persea americana* Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. *Appl. Soil Ecol.* 102, 80–91. doi: 10.1016/j.apsoil.2016.02.014

Basak, B. B., and Biswas, D. R. (2010). Co-inoculation of potassium solubilizing and nitrogen fixing bacteria on solubilization of waste mica and their effect on growth promotion and nutrient acquisition by a forage crop. *Biol. Fertility Soils* 46, 641–648. doi: 10.1007/s00374-010-0456-x

Bashan, Y., de-Bashan, L. E., Prabhu, S. R., and Hernandez, J. P. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). *Plant Soil* 378, 1–33. doi: 10.1007/s11104-013-1956-x

Beckers, B., Op De Beeck, M., Thijs, S., Truyens, S., Weyens, N., et al. (2016). Performance of 16s rDNA primer pairs in the study of rhizosphere and endosphere bacterial microbiomes in metabarcoding studies. *Front. Microbiol.* 7:650. doi: 10.3389/fmicb.2016.00650

Ben Said, S., and Or, D. (2017). Synthetic microbial ecology: engineering habitats for modular consortia. *Front. Microbiol.* 8:1125. doi: 10.3389/fmicb.2017.01125

Bender, S. F., Wagg, C., and van der Heijden, M. G. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol. Evol.* 31, 440–452. doi: 10.1016/j.tree.2016.02.016

Beneduzi, A., Moreira, F., Costa, P. B., Vargas, L. K., Lisboa, B. B., Favreto, R., et al. (2013). Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil. *Appl. Soil Ecol.* 63, 94–104. doi: 10.1016/j.apsoil.2012.08.010

Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., et al. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* 12, 1496–1507. doi: 10.1038/s41396-018-0093-91

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Berg, G., Kusstatscher, P., Abdelfattah, A., Cernava, T., and Smalla, K. (2021). Microbiome modulation—toward a better understanding of plant microbiome response to microbial inoculants. *Front. Microbiol.* 12:650610. doi: 10.3389/fmicb.2021.650610

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8:103. doi: 10.1186/s40168-020-00875-0

Berg, M., and Koskella, B. (2018). Nutrient-and dose-dependent microbiome-mediated protection against a plant pathogen. *Curr. Biol.* 28, 2487–2492. doi: 10.1016/j.cub.2018.05.085

Bhatt, K., and Maheshwari, D. K. (2020). Zinc solubilizing bacteria (*Bacillus megaterium*) with multifarious plant growth promoting activities alleviates growth in *Capsicum annuum* L. *3 Biotech* 10:36. doi: 10.1007/s13205-019-2033-2039

Bokhari, A., Essack, M., Lafi, F. F., Andres-Barrao, C., Jalal, R., Alamoudi, S., et al. (2019). Bioprospecting desert plant *Bacillus* endophytic strains for their potential to enhance plant stress tolerance. *Sci. Rep.* 9:18154. doi: 10.1038/s41598-019-54685-y

Bona, E., Cantamessa, S., Massa, N., Manassero, P., Marsano, F., Copetta, A., et al. (2017). Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study. *Mycorrhiza* 27, 1–11. doi: 10.1007/s00572-016-0727-y

Bradáčová, K., Florea, A. S., Bar-Tal, A., Minz, D., Yermiyahu, U., Shawahna, R., et al. (2019). Microbial consortia versus single-strain inoculants: an advantage in PGPM-assisted tomato production? *Agronomy* 9:105. doi: 10.3390/agronomy9020105

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Cameron, D. D., Neal, A. L., van Wees, S. C., and Ton, J. (2013). Mycorrhiza-induced resistance: more than the sum of its parts? *Trends Plant Sci.* 18, 539–545. doi: 10.1016/j.tplants.2013.06.004

Carlström, C. I., Field, C. M., Bortfeld-Miller, M., Müller, B., Sunagawa, S., and Vorholt, J. A. (2019). Synthetic microbiota reveal priority effects and keystone strains in the *Arabidopsis* phyllosphere. *Nat. Ecol. Evol.* 3, 1445–1454. doi: 10.1038/s41559-019-0994-z

Cassan, F., Perrig, D., Sgroy, V., Masciarelli, O., Penna, C., and Luna, V. (2009). *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). *Eur. J. Soil Biol.* 45, 28–35. doi: 10.1016/j.ejsobi.2008.08.005

Chakraborty, N., Ghosh, R., Ghosh, S., Narula, K., Tayal, R., Datta, A., et al. (2013). Reduction of oxalate levels in tomato fruit and consequent metabolic remodeling following overexpression of a fungal oxalate decarboxylase. *Plant Physiol.* 162, 364–378. doi: 10.1104/pp.112.209197

Chakraborty, U., Chakraborty, B., and Basnet, M. (2006). Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. *J. Basic Microbiol.* 46, 186–195. doi: 10.1002/jobm.200510050

Christy, C. (2021). *Unearthing the Soil Microbiome, Climate Change, Carbon Storage Nexus*. Available online at: https://asm.org/Articles/2021/May (accessed September 25, 2021).

Ciccillo, F., Fiore, A., Bevivino, A., Dalmastri, C., Tabacchioni, S., and Chiarini, L. (2002). Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizospheric bacterial diversity. *Environ. Microbiol.* 4, 238–245. doi: 10.1046/j.1462-2920.2002.00291.x

Clayton, G. W., Rice, W. A., Lupwayi, N. Z., Johnston, A. M., Lafond, G. P., Grant, C. A., et al. (2004). Inoculant formulation and fertilizer nitrogen effects on field pea: nodulation, N2 fixation and nitrogen partitioning. *Canadian J. Plant Sci.* 84, 79–88. doi: 10.4141/P02-089

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004

Cong, P. T., Dung, T. D., Hien, T. M., Hien, N. T., Choudhury, A. T., Kecskés, M. L., et al. (2009). Inoculant plant growth-promoting microorganisms enhance utilisation of urea-N and grain yield of paddy rice in southern Vietnam. *Eur. J. Soil Biol.* 45, 52–61. doi: 10.1016/j.ejsobi.2008.06.006

Conrad, R., Erkel, C., and Liesack, W. (2006). Rice cluster I methanogens, an important group of *Archaea* producing greenhouse gas in soil. *Curr. Opin. Biotechnol.* 17, 262–267. doi: 10.1016/j.copbio.2006.04.002

Culligan, E. P., and Sleator, R. D. (2016). From genes to species: novel insights from metagenomics. *Front. Microbiol.* 7:1181. 01181 doi: 10.3389/fmicb.2016

Curá, J. A., Franz, D. R., Filosofía, J. E., Balestrasse, K. B., and Burgueño, L. E. (2017). Inoculation with *Azospirillum sp*. and *Herbaspirillum sp*. bacteria increases the tolerance of maize to drought stress. *Microorganisms* 5:41. doi: 10.3390/microorganisms5030041

Dal Cortivo, C., Barion, G., Ferrari, M., Visioli, G., Dramis, L., Panozzo, A., et al. (2018). Effects of field inoculation with VAM and bacteria consortia on root growth and nutrients uptake in common wheat. *Sustainability* 10:3286. doi: 10.3390/su10093286

Dastogeer, K. M. G., Tumpa, F. H., Sultana, A., Akter, M. A., and Chakraborty, A. (2020). Plant microbiome: an account of the factors that shape community composition and diversity. *Curr. Plant Biol.* 23:100161. doi: 10.1016/j.cpb.2020.100161

De Souza, R. S. C., Okura, V. K., Armanhi, J. S. L., Jorrín, B., Lozano, N., Da Silva, M. J., et al. (2016). Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci. Rep.* 6:28774. doi: 10.1038/srep28774

Desbrosses, G. J., and Stougaard, J. (2011). Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways. *Cell Host Microbe* 10, 348–358. doi: 10.1016/j.chom.2011.09.005

Dey, R. K. K. P., Pal, K. K., Bhatt, D. M., and Chauhan, S. M. (2004). Growth promotion and yield enhancement of peanut (*Arachis hypogea* L.) by application of plant growth-promoting rhizobacteria. *Microbiol. Res.* 159, 371–394. doi: 10.1016/j.micres.2004.08.004

D'Hondt, K., Kostic, T., McDowell, R., Eudes, F., Singh, B. K., Sarkar, S., et al. (2021). Microbiome innovations for a sustainable future. *Nat. Microbiol.* 6, 138–142. doi: 10.1038/s41564-020-00857

Díaz-Zorita, M., and Fernández-Canigia, M. V. (2009). Field performance of a liquid formulation of *Azospirillum* brasilense on dryland wheat productivity. *Eur. J. Soil Biol.* 45, 3–11. doi: 10.1016/j.ejsobi.2008.07.001

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U. S. A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Egamberdiyeva, D. (2007). The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Appl. Soil Ecol.* 36, 184–189. doi: 10.1016/j.apsoil.2007.02.005

Elias, F., Woyessa, D., and Muleta, D. (2016). Phosphate solubilization potential of rhizosphere fungi isolated from plants in Jimma Zone, Southwest Ethiopia. *Int. J. Microbiol.* 2016:5472601. doi: 10.1155/2016/5472601

Elkoca, E., Kantar, F., and Sahin, F. (2007). Influence of nitrogen fixing and phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea. *J. Plant Nutrition* 31, 157–171. doi: 10.1080/01904160701742097

FAO (2018). *Trade and Nutrition Technical Note. Trade Policy Technical Notes 21. Trade and Food Security*. Rome: Markets and Trade Division, Food and Agriculture Organization of the United Nations.

FAO (2019). *The Biodiversity That is Crucial for Our Food and Agriculture is Disappearing by the Day*. Rome: FAO.

Fatima, I. S., Jamil, M. O., Hussain, A. Z., Mumtaz, M. Z., Luqman, M., Hussain, S., et al. (2018). Zinc solubilizing *Bacillus sp*. ZM20 and *Bacillus aryabhattai* ZM31 promoted the productivity in Okra (*Abelmoschus esculentus* L.). *Biologia* 64, 179–185. doi: 10.1016/j.micres.2017.06.001

Faust, K., and Raes, J. (2012). Microbial interactions: from networks to models. *Nat. Rev. Microbiol.* 10, 538–550. doi: 10.1038/nrmicro2832

Ferreira, A. S., Pires, R. R., Rabelo, P. G., Oliveira, R. C., Luz, J. M. Q., and Brito, C. H. (2013). Implications of *Azospirillum brasilense* inoculation and nutrient addition on maize in soils of the Brazilian Cerrado under greenhouse and field conditions. *Appl. Soil Ecol.* 72, 103–108. doi: 10.1016/j.apsoil.2013.05.020

Gandhi, A., and Muralidharan, G. (2016). Assessment of zinc solubilizing potentiality of *Acinetobacter* sp. isolated from rice rhizosphere. *Eur. J. Soil Biol.* 76, 1–8. doi: 10.1016/j.ejsobi.2016.06.006

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Glick, B. R., Liu, C., Ghosh, S., and Dumbroff, E. B. (1997). Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. *Soil Biol. Biochem.* 29, 1233–1239. doi: 10.1016/S0038-0717(97)00026-26

Govindarajan, M., Balandreau, J., Kwon, S. W., Weon, H. Y., and Lakshminarasimhan, C. (2008). Effects of the inoculation of *Burkholderia vietnamensis* and related endophytic diazotrophic bacteria on grain yield of rice. *Microb. Ecol.* 55, 21–37. doi: 10.1007/s00248-007-9247-9249

Govindasamy, V., George, P., Aher, L., Ramesh, S. V., Thangasamy, A., Anandan, S., et al. (2017). Comparative conventional and phenomics approach to assess symbiotic effectiveness of bradyrhizobia strains in soybean [*Glycine max* (L.) Merrill] to drought. *Sci. Rep.* 7:6958. doi: 10.1038/s41598-017-06441-3

Govindasamy, V., George, P., Kumar, M., Aher, L., Raina, S. K., Rane, J., et al. (2020). Multi-trait PGP rhizobacterial endophytes alleviate drought stress in a senescent genotype of sorghum [*Sorghum bicolor* (L.) Moench]. *3 Biotech* 10:13. doi: 10.1007/s13205-019-2001-2004

Großkopf, T., and Soyer, O. S. (2014). Synthetic microbial communities. *Curr. Opin. Microbiol.* 18, 72–77. doi: 10.1016/j.mib.2014.02.002

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., and Singh, V. (2015). Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J. Microbial Biochem. Technol.* 7, 096–102. doi: 10.1007/978-981-15-6949-4_9

Gurdeep, K. A. U. R., and Reddy, M. S. (2015). Effects of phosphate-solubilizing bacteria, rock phosphate and chemical fertilizers on maize-wheat cropping cycle and economics. *Pedosphere* 25, 428–437. doi: 10.1016/S1002-0160(15)30010-30012

Hernandez, J. P., de-Bashan, L. E., Rodriguez, D. J., Rodriguez, Y., and Bashan, Y. (2009). Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *Eur. J. Soil Biol.* 45, 88–93. doi: 10.1016/j.ejsobi.2008.08.004

Herrera Paredes, S., Gao, T., Law, T. F., Finkel, O. M., Mucyn, T., Teixeira, P. J. P. L., et al. (2018). Design of synthetic bacterial communities for predictable plant phenotypes. *PLoS Biol.* 16:e2003962. doi: 10.1371/journal.pbio.2003962

Hu, J., Wei, Z., Friman, V. P., Gu, S. H., Wang, X. F., Eisenhauer, N., et al. (2016). Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *mBio* 7:e001790-16. doi: 10.1128/mBio.01790-1716

Huang, L. H., Yuan, M. Q., Ao, X. J., Ren, A. Y., Zhang, H. B., and Yang, M. Z. (2018). Endophytic fungi specifically introduce novel metabolites into grape flesh cells *in vitro*. *PLoS One* 13:e0196996. doi: 10.1371/journal.pone.0196996

Hungria, M., Campo, R. J., Souza, E. M., and Pedrosa, F. O. (2010). Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant Soil* 331, 413–425. doi: 10.1007/s11104-009-0262-260

Hungria, M., Nogueira, M. A., and Araujo, R. S. (2013). Co-inoculation of soybeans and common beans with Rhizobia and Azospirilla: strategies to improve sustainability. *Biol. Fertility Soils* 49, 791–801. doi: 10.1007/s00374-012-0771-775

Imran, A., Mirza, M. S., Shah, T. M., Malik, K. A., and Hafeez, F. Y. (2015). Differential response of kabuli and desi chickpea genotypes toward inoculation with PGPR in different soils. *Front. Microbiol.* 6:859. doi: 10.3389/fmicb.2015.00859

Isawa, T., Yasuda, M., Awazaki, H., Minamisawa, K., Shinozaki, S., and Nakashita, H. (2009). *Azospirillum sp*. strain B510 enhances rice growth and yield. *Microbes Environ.* 25, 58–61. doi: 10.1007/s00248-002-2022-z

Jahanian, A., Chaichi, M. R., Rezaei, K., Rezayazdi, K., and Khavazi, K. (2012). The effect of plant growth promoting rhizobacteria (PGPR) on germination and primary growth of artichoke (*Cynara scolymus*). *Int. J. Agriculture Crop Sci.* 4, 923–929.

Jain, A., Singh, A., Singh, S., and Singh, H. B. (2015). Phenols enhancement effect of microbial consortium in pea plants restrains *Sclerotinia sclerotiorum*. *Biol. Control* 89, 23–32. doi: 10.1016/j.biocontrol.2015.04.013

John Bullied, W., Buss, T. J., and Kevin Vessey, J. (2002). Bacillus cereus UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: field studies. *Canadian J. Plant Sci.* 82, 291–298. doi: 10.4141/P01-048

Katiyar, V., and Goel, R. (2003). Solubilization of inorganic phosphate and plant growth promotion by cold tolerant mutants of *Pseudomonas fluorescens*. *Microbiol. Res.* 158, 163–168. doi: 10.1078/0944-5013-5188

Kong, Z., and Glick, B. R. (2017). The role of plant growth-promoting bacteria in metal phytoremediation. *Adv. Microbial Physiol.* 71, 97–132. doi: 10.1016/bs.ampbs.2017.04.001

Kong, Z., Hart, M., and Liu, H. (2018). Paving the way from the lab to the field: using synthetic microbial consortia to produce high-quality crops. *Front. Plant Sci.* 9:1467. doi: 10.3389/fpls.2018.01467

Kopittke, P. M., Menzies, N. W., Wang, P., McKenna, B. A., and Lombi, E. (2019). Soil and the intensification of agriculture for global food security. *Environ. Int.* 132:105078. doi: 10.1016/j.envint.2019.105078

Krithika, S., and Balachandar, D. (2016). Expression of zinc transporter genes in rice as influenced by zinc-solubilizing *Enterobacter cloacae* strain ZSB14. *Front. Plant Sci.* 7:446. doi: 10.3389/fpls.2016.00446

Kumar, P., Dubey, R. C., and Maheshwari, D. K. (2012). *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiol. Res.* 167, 493–499. doi: 10.1016/j.micres.2012.05.002

Kumar, P., Pandey, P., Dubey, R. C., and Maheshwari, D. K. (2016). Bacteria consortium optimization improves nutrient uptake, nodulation, disease suppression and growth of the common bean (*Phaseolus vulgaris*) in both pot and field studies. *Rhizosphere* 2, 13–23. doi: 10.1016/j.rhisph.2016.09.002

Kumawat, K. C., Sharma, P., Sirari, A., Singh, I., Gill, B. S., Singh, U., et al. (2019). Synergism of *Pseudomonas aeruginosa* (LSE-2) nodule endophyte with *Bradyrhizobium* sp. (LSBR-3) for improving plant growth, nutrient acquisition and soil health in soybean. *World J. Microbiol. Biotechnol.* 35:47. doi: 10.1007/s11274-019-2622-2620

Lally, R. D., Galbally, P., Moreira, A. S., Spink, J., Ryan, D., Germaine, K. J., et al. (2017). Application of endophytic *Pseudomonas fluorescens* and a bacterial consortium to *Brassica napus* can increase plant height and biomass under greenhouse and field conditions. *Front. Plant Sci.* 8:2193. doi: 10.3389/fpls.2017.02193

Levy, A., Gonzalez, I. S., Mittelviefhaus, M., Clingenpeel, S., Paredes, S. H., Miao, J., et al. (2018a). Genomic features of bacterial adaptation to plants. *Nat. Genet.* 50, 138–150. doi: 10.1038/s41588-017-0012-19

Levy, M. M., Evans, L. E., and Rhodes, A. (2018b). The surviving sepsis campaign bundle: 2018 update. *Intensive Care Med.* 44, 925–928. doi: 10.1007/s00134-018-5085-5080

Lucas, J. A., Solano, B. R., Montes, F., Ojeda, J., Megias, M., and Manero, F. G. (2009). Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain. *Field Crops Res.* 114, 404–410. doi: 10.1016/j.fcr.2009.09.013

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Lupatini, M., Korthals, G. W., de Hollander, M., Janssens, T. K. S., and Kuramae, E. E. (2017). Soil microbiome is more heterogeneous in organic than in conventional farming system. *Front. Microbiol.* 7:2064. doi: 10.3389/fmicb.2016.02064

Ma, Y., Oliveira, R. S., Wu, L., Luo, Y., Rajkumar, M., Rocha, I., et al. (2015). Inoculation with metal-mobilizing plant-growth-promoting rhizobacterium *Bacillus* sp. SC2b and its role in rhizoremediation. *J. Toxicol. Environ. Health Part A* 78, 931–944. doi: 10.1080/15287394.2015.1051205

Ma, Y., Rajkumar, M., Luo, Y., and Freitas, H. (2011). Inoculation of endophytic bacteria on host and non-host plants—effects on plant growth and Ni uptake. *J. Hazard. Mater.* 195, 230–237. doi: 10.1016/j.jhazmat.2011.08.034

Mäder, P., Kaiser, F., Adholeya, A., Singh, R., Uppal, H. S., Sharma, A. K., et al. (2011). Inoculation of root microorganisms for sustainable wheat-rice and wheat-black gram rotations in India. *Soil Biol. Biochem.* 43, 609–619. doi: 10.1016/j.soilbio.2010.11.031

Mahmood, S., Daur, I., Al-Solaimani, S. G., Ahmad, S., Madkour, M. H., Yasir, M., et al. (2016). Plant growth promoting rhizobacteria and silicon synergistically enhance salinity tolerance of mung bean. *Front. Plant Sci.* 7:876. doi: 10.3389/fpls.2016.00876

Marag, P. S., and Suman, A. (2018). Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (*Zea mays* L.). *Microbiol. Res.* 214, 101–113. doi: 10.1016/j.micres.2018.05.016

Marimuthu, S., Ramamoorthy, V., Samiyappan, R., and Subbian, P. (2013). Intercropping system with combined application of *Azospirillum* and *Pseudomonas fluorescens* reduces root rot incidence caused by *Rhizoctonia* bataticola and increases seed cotton yield. *J. Phytopathol.* 161, 405–411. doi: 10.1111/jph.12084

Martínez-Hidalgo, P., Maymon, M., Pule-Meulenberg, F., and Hirsch, A. M. (2019). Engineering root microbiomes for healthier crops and soils using beneficial, environmentally safe bacteria. *Can. J. Microbiol.* 65, 91–104. doi: 10.1139/cjm-2018-2315

Marulanda-Aguirre, A., Azcón, R., Ruiz-Lozano, J. M., and Aroca, R. (2008). Differential effects of a *Bacillus megaterium* strain on *Lactuca sativa* plant growth depending on the origin of the arbuscular mycorrhizal fungus coinoculated: physiologic and biochemical traits. *J. Plant Growth Regulation* 27:10. doi: 10.1007/s00344-007-9024-5

Mehnaz, S., and Lazarovits, G. (2006). Inoculation effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. *Microb. Ecol.* 51, 326–335. doi: 10.1007/s00248-006-9039-9037

Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37, 634–663. doi: 10.1111/1574-6976.12028

Menéndez, E., and Paço, A. (2020). Is the application of plant probiotic bacterial consortia always beneficial for plants? exploring synergies between rhizobial and non-rhizobial bacteria and their effects on agro-economically valuable crops. *Life* 10:24. doi: 10.3390/life10030024

Mitri, S., and Richard Foster, K. (2013). The genotypic view of social interactions in microbial communities. *Annu. Rev. Genet.* 47, 247–273. doi: 10.1146/annurev-genet-111212-133307

Molina-Romero, D., Baez, A., Quintero-Hernández, V., Castañeda-Lucio, M., Fuentes-Ramírez, L. E., Bustillos-Cristales, M. D. R., et al. (2017). Compatible bacterial mixture, tolerant to desiccation, improves maize plant growth. *PLoS One* 12:e0187913. doi: 10.1371/journal.pone.0187913

Monsanto BioAgAlliance (2015). *QuickRoots: Bacillus Amyloliquefaciens and Trichoderma Virens Based Inoculant for Corn (ProductInformation)*. Belgium: Monsantobioag.

Moreira, H., Pereira, S. I., Marques, A. P., Rangel, A. O., and Castro, P. M. (2016). Mine land valorization through energy maize production enhanced by the application of plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi. *Environ. Sci. Pollut. Res.* 23, 6940–6950. doi: 10.1007/s11356-015-5914-4

Mourad, E. F., Sarhan, M. S., Daanaa, H. S. A., Abdou, M., Morsi, A. T., Abdelfadeel, M. R., et al. (2018). Plant materials are sustainable substrates supporting new technologies of plant-only-based culture media for in vitro culturing of the plant microbiota. *Microbes Environ.* 33, 40–49. doi: 10.1264/jsme2.ME17135

Niu, B., Paulson, J. N., Zheng, X., and Kolter, R. (2017). Simplified and representative bacterial community of maize roots. *Proc. Natl. Acad. Sci. U. S. A.* 114, E2450–E2459. doi: 10.1073/pnas.1616148114

Otieno, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., et al. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Front. Microbiol.* 6:745. doi: 10.3389/fmicb.2015.00745

Padda, K. P., Puri, A., and Chanway, C. P. (2016). Plant growth promotion and nitrogen fixation in canola (*Brassica napus*) by an endophytic strain of *Paenibacillus polymyxa* and its GFP-tagged derivative in a long-term study. *Botany* 94, 1209–1217. doi: 10.1139/cjb-2016-2075

Pandey, A., Trivedi, P., Kumar, B., and Palni, L. M. S. (2006). Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. *Curr. Microbiol.* 53, 102–107. doi: 10.1007/s00284-006-4590-4595

Pandey, P., and Maheshwari, D. K. (2007). Bioformulation of *Burkholderia sp*. MSSP with a multispecies consortium for growth promotion of *Cajanus cajan*. *Canadian J. Microbiol.* 53, 213–222. doi: 10.1139/w06-118

Parnell, J. J., Berka, R., Young, H. A., Sturino, J. M., Kang, Y., Barnhart, D. M., et al. (2016). From the lab to the farm: an industrial perspective of plant beneficial microorganisms. *Front. Plant Sci.* 7:1110. doi: 10.3389/fpls.2016.01110

Patel, P., Trivedi, G., and Saraf, M. (2018). Iron biofortification in mungbean using siderophore producing plant growth promoting bacteria. *Environ. Sustainabil.* 1, 357–365. doi: 10.1007/s42398-018-00031-33

Paz, I. C. P., Santin, R. C. M., Guimarães, A. M., Rosa, O. P. P., Dias, A. C. F., Quecine, M. C., et al. (2012). Eucalyptus growth promotion by endophytic *Bacillus* spp. *Genet. Mol. Res.* 11, 3711–3720. doi: 10.4238/2012.August.17.9

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U. S. A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. a review. *Biol. Fertility Soils* 51, 403–415. doi: 10.1007/s00374-015-0996-991

Qi, W., and Zhao, L. (2013). Study of the siderophore-producing *Trichoderma asperellum* Q1 on cucumber growth promotion under salt stress. *J. Basic Microbiol.* 53, 355–364. doi: 10.1002/jobm.201200031

Rajkumar, M., and Freitas, H. (2008). Effects of inoculation of plant-growth promoting bacteria on N uptake by Indian mustard. *Bioresour. Technol.* 99, 3491–3498. doi: 10.1016/j.biortech.2007.07.046

Ramachandran, K., Srinivasan, V., Hamza, S., and Anandaraj, M. (2007). "Phosphate solubilizing bacteria isolated from the rhizosphere soil and its growth promotion on black pepper (*Piper nigrum* L.) cuttings," in *Proceedings of the 1st International Meeting on Microbial Phosphate Solubilization* (Dordrecht: Springer), 325–331. doi: 10.1007/978-1-4020-5765-6_51

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014). Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Appl. Soil Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Reyes, I., Bernier, L., and Antoun, H. (2002). Rock phosphate solubilization and colonization of maize rhizosphere by wild and genetically modified strains of *Penicillium rugulosum*. *Microb. Ecol.* 44, 39–48. doi: 10.1007/s00248-002-1001-8

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Horticulturae* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ryan, M. J., Schloter, M., Berg, G., Kostic, T., Kinkel, L. L., Eversole, K., et al. (2021). Development of microbiome biobanks-challenges and opportunities. *Trends Microbiol.* 29, 89–92. doi: 10.1016/j.tim.2020.06.009

Sai Prasad, J., Aswini, K., Sharma, P., Gond, S., and Suman, A. (2021). A Novel Wheat Matrix Medium (WMM) For rapid isolation of hydrolytic enzyme producing bacterial seed endophytes. *Int. J. Curr. Microbiol. Appl. Sci.* 9, 2181–2197. doi: 10.20546/ijcmas.2020.912.258

Saleem, M., Hu, J., and Jousset, A. (2019). More than the sum of its parts: microbiome biodiversity as a driver of plant growth and soil health. *Ann. Rev. Ecol. Evol. Systemat.* 50, 145–168. doi: 10.1146/annurev-ecolsys-110617-062605

Santhanam, R., Baldwin, I. T., and Groten, K. (2015). In wild tobacco, *Nicotiana attenuata*, variation among bacterial communities of isogenic plants is mainly shaped by the local soil microbiota independently of the plants' capacity to produce jasmonic acid. *Commun. Integrative Biol.* 8:e1017160. doi: 10.1080/19420889.2015.1017160

Sarhan, M. S., Hamza, M. A., Youssef, H. H., Patz, S., Becker, M., ElSawey, H., et al. (2019). Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media-a review. *J. Adv. Res.* 19, 15–27. doi: 10.1016/j.jare.2019.04.002

Selvakumar, G., Kundu, S., Gupta, A. D., Shouche, Y. S., and Gupta, H. S. (2008). Isolation and characterization of nonrhizobial plant growth promoting bacteria from nodules of Kudzu (*Pueraria thunbergiana*) and their effect on wheat seedling growth. *Curr. Microbiol.* 56, 134–139. doi: 10.1007/s00284-007-9062-z

Sessitsch, A., Pfaffenbichler, N., and Mitter, B. (2019). Microbiome applications from lab to field: facing complexity. *Trends Plant Sci.* 24, 194–198. doi: 10.1016/j.tplants.2018.12.004

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Shaikh, S., and Saraf, M. (2017). Biofortification of *Triticum aestivum* through the inoculation of zinc solubilizing plant growth promoting rhizobacteria in field experiment. *Biocatalysis Agricultural Biotechnol.* 9, 120–126. doi: 10.1016/j.bcab.2016.12.008

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* 2:587. doi: 10.1186/2193-1801-2-587

Shukla, S. K., Yadav, R. L., Suman, A., and Singh, P. N. (2008). Improving rhizospheric environment and sugarcane ratoon yield through bioagents amended farm yard manure in udic ustochrept soil. *Soil Tillage Res.* 99, 158–168. doi: 10.1016/j.still.2008.02.007

Siddiqui, Y., Meon, S., Ismail, M. R., and Ali, A. (2008). *Trichoderma*-fortified compost extracts for the control of *Choanephora* wet rot in okra production. *Crop Prot.* 27, 385–390. doi: 10.1016/j.cropro.2007.07.002

Singh, A. V., Chandra, R., and Goel, R. (2013). Phosphate solubilization by *Chryseobacterium* sp. and their combined effect with N and P fertilizers on plant growth promotion. *Arch. Agronomy Soil Sci.* 59, 641–651. doi: 10.1080/03650340.2012.664767

Singh, S., and Kapoor, K. K. (1999). Inoculation with phosphate-solubilizing microorganisms and a vesicular-arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. *Biol. Fertil. Soils* 28, 139–144. doi: 10.1007/s003740050475

Singh, S. R., Kundu, D. K., Dey, P., Singh, P., and Mahapatra, B. S. (2018). Effect of balanced fertilizers on soil quality and lentil yield in *Gangetic alluvial* soils of India. *J. Agricultural Sci.* 156:225. doi: 10.1017/S0021859618000254

Soto-Giron, M. J., Kim, J. N., Schott, E., Tahmin, C., Ishoey, T., Mincer, T. J., et al. (2021). The edible plant microbiome represents a diverse genetic reservoir with functional potential in the human host. *Sci. Rep.* 11:24017. doi: 10.1016/j.tplants.2021.09.001

Souza, R. D., Ambrosini, A., and Passaglia, L. M. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genet. Mol. Biol.* 38, 401–419. doi: 10.1590/S1415-475738420150053

Sruthilaxmi, C. B., and Babu, S. (2017). Microbial bio-inoculants in Indian agriculture: ecological perspectives for a more optimized use. *Agriculture Ecosystems Environ.* 242, 23–25. doi: 10.1016/j.agee.2017.03.019

Stephen, J., Shabanamol, S., Rishad, K. S., and Jisha, M. S. (2015). Growth enhancement of rice (*Oryza sativa*) by phosphate solubilizing *Gluconacetobacter* sp. (MTCC 8368) and *Burkholderia* sp. (MTCC 8369) under greenhouse conditions. *3 Biotech* 5, 831–837. doi: 10.1007/s13205-015-0286-5

Stewart, E. J. (2012). Growing unculturable bacteria. *J. Bacteriol.* 194, 4151–4160. doi: 10.1128/JB.00345-312

Stockwell, V. O., Johnson, K. B., Sugar, D., and Loper, J. E. (2011). Mechanistically compatible mixtures of bacterial antagonists improve biological control of fire blight of Pear. *Phytopathology* 101, 299–299. doi: 10.1094/PHYTO-03-10-0098

Suman, A., Gaur, A., Shrivastava, A. K., and Yadav, R. L. (2005). Improving sugarcane growth and nutrient uptake by inoculating *Gluconacetobacter diazotrophicus*. *Plant Growth Regulation* 47, 155–162. doi: 10.1007/s10725-005-2847-9

Suman, A., Shrivastava, A. K., Gaur, A., Singh, P., Singh, J., and Yadav, R. L. (2008). Nitrogen use efficiency of sugarcane in relation to its BNF potential and population of endophytic diazotrophs at different N levels. *Plant Growth Regulation* 54, 1–11. doi: 10.1007/s10725-007-9219-9216

Suman, A., Shukla, L., Marag, P. S., Verma, P., Gond, S., And Sai, et al. (2020). Potential use of plant colonizing *Pantoea* as generic plant growth promoting bacteria for cereal crops. *J. Environ. Biol.* 41, 987–994.

Suman, A., Verma, P., Yadav, A. N., Srinivasamurthy, R., Singh, A., and Prasanna, R. (2016). Development of hydrogel based bio-inoculant formulations and their impact on plant biometric parameters of wheat (*Triticum aestivum* L.). *Int. J. Curr. Microbiol. Appl. Sci.* 5, 890–901.

Tank, N., and Saraf, M. (2010). Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. *J. Plant Interact.* 5, 51–58. doi: 10.1080/17429140903125848

Taye, Z. M., Helgason, B. L., Bell, J. K., Norris, C. E., Vail, S., Robinson, S. J., et al. (2020). Core and differentially abundant bacterial taxa in the rhizosphere of field grown *Brassica napus* genotypes: implications for canola breeding. *Front. Microbiol.* 10:3007. doi: 10.3389/fmicb.2019.03007

Thilagar, G., Bagyaraj, D. J., and Rao, M. S. (2016). Selected microbial consortia developed for chilly reduces application of chemical fertilizers by 50% under field conditions. *Sci. Hortic.* 198, 27–35. doi: 10.1016/j.scienta.2015.11.021

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A. C. (2017). Perspectives and challenges of microbial application for crop improvement. *Front. Plant Sci.* 8:49. doi: 10.3389/fpls.2017.00049

Trivedi, P., and Pandey, A. (2008). Plant growth promotion abilities and formulation of *Bacillus megaterium* strain B 388 (MTCC6521) isolated from a temperate Himalayan location. *Indian J. Microbiol.* 48, 342–347. doi: 10.1007/s12088-008-0042-41

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., and Singh, B. K. (2021). Plant-microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol*. 19:72.

Tsolakidou, M. D., Stringlis, I. A., Fanega-Sleziak, N., Papageorgiou, S., Tsalakou, A., and Pantelides, I. S. (2019). Rhizosphere-enriched microbes as a pool to design synthetic communities for reproducible beneficial outputs. *FEMS Microbiol. Ecol.* 95:fiz138. doi: 10.1093/femsec/fiz138

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14, 1–10. doi: 10.3390/microorganisms9010188

Ullah, A., Farooq, M., Nadeem, F., Rehman, A., Hussain, M., Nawaz, A., et al. (2020). Zinc application in combination with zinc solubilizing *Enterobacter sp*. MN17 improved productivity, profitability, zinc efficiency, and quality of *Desi chickpea*. *J. Soil Sci. Plant Nutrition* 20, 2133–2144. doi: 10.1007/s42729-020-00281-283

Upadhyay, S. K., Singh, J. S., Saxena, A. K., and Singh, D. P. (2012). Impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions. *Plant Biol.* 14, 605–611. doi: 10.1111/j.1438-8677.2011.00533.x

Valverde, A., Burgos, A., Fiscella, T., Rivas, R., Velazquez, E., Rodríguez-Barrueco, C., et al. (2007). "Differential effects of coinoculations with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacterium) and *Mesorhizobium ciceri* C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions," in *Proceedings of the 1st International Meeting on Microbial Phosphate Solubilization* (Dordrecht: Springer), 43–50. doi: 10.1007/978-1-4020-5765-6_5

Verma, J. P., Yadav, J., Tiwari, K. N., and Kumar, A. (2013). Effect of indigenous *Mesorhizobium spp*. and plant growth promoting rhizobacteria on yields and nutrients uptake of chickpea (*Cicer arietinum* L.) under sustainable agriculture. *Ecol. Eng.* 51, 282–286. doi: 10.1016/j.ecoleng.2012.12.022

Verma, P., and Suman, A. (2018). Wheat microbiomes: ecological significances, molecular diversity and potential bioresources for sustainable agriculture. *EC Microbiol.* 14, 641–665.

Verma, P., Yadav, A. N., Khannam, K. S., Kumar, S., Saxena, A. K., and Suman, A. (2016). Molecular diversity and multifarious plant growth promoting attributes of Bacilli associated with wheat (*Triticum aestivum* L.) rhizosphere from six diverse agro-ecological zones of India. *J. Basic Microbiol.* 56, 44–58. doi: 10.1002/jobm.201500459

Verma, P., Yadav, A. N., Khannam, K. S., Mishra, S., Kumar, S., Saxena, A. K., et al. (2019). Appraisal of diversity and functional attributes of thermotolerant wheat associated bacteria from the peninsular zone of India. *Saudi J. Biol. Sci.* 26, 1882–1895. doi: 10.1016/j.sjbs.2016.01.042

Vorholt, J. A., Vogel, C., Carlström, C. I., and Müller, D. B. (2017). Establishing causality: opportunities of synthetic communities for plant microbiome research. *Cell Host Microbe* 22, 142–155. doi: 10.1016/j.chom.2017.07.004

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., and SkZ, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* 184, 13–24. doi: 10.1016/j.micres.2015.12.003

Vyas, P., Rahi, P., Chauhan, A., and Gulati, A. (2007). Phosphate solubilization potential and stress tolerance of *Eupenicillium parvum* from tea soil. *Mycol. Res.* 111, 931–938. doi: 10.1016/j.mycres.2007.06.003

Wagner, M. R., Lundberg, D. S., Tijana, G., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nat. Commun.* 7:12151. doi: 10.1038/ncomms12151

Waigi, M. G., Sun, K., and Gao, Y. (2017). Sphingomonads in microbe-assisted phytoremediation: tackling soil pollution. *Trends Biotechnol.* 35, 883–899. doi: 10.1016/j.tibtech.2017.06.014

Wei, Z., Yang, X., Yin, S., Shen, Q., Ran, W., and Xu, Y. (2011). Efficacy of Bacillus-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. *Appl. Soil Ecol.* 48, 152–159. doi: 10.1016/j.apsoil.2011.03.013

White, J. F., Kingsley, K. L., Zhang, Q., Verma, R., Obi, N., Dvinskikh, S., et al. (2019). Endophytic microbes and their potential applications in crop management. *Pest. Manag. Sci.* 75, 2558–2565. doi: 10.1002/ps.5527

Williams, S. C. (2013). The other microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 110, 2682–2684. doi: 10.1073/pnas.1300923110

Woo, S. L., and Pepe, O. (2018). Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1801. doi: 10.3389/fpls.2018.01801

Xu, W., Liu, L., He, T., Cao, M., Sha, L., Hu, Y., et al. (2016). Soil properties drive a negative correlation between species diversity and genetic diversity in a tropical seasonal rainforest. *Sci. Rep.* 6:20652. doi: 10.1038/srep20652

Yadav, R. L., Suman, A., Prasad, S. R., and Prakash, O. (2009). Effect of *Gluconacetobacter diazotrophicus* and *Trichoderma viride* on soil health, yield and N-economy of sugarcane cultivation under subtropical climatic conditions of India. *Eur. J. Agron.* 30, 296–303. doi: 10.1016/j.eja.2009.01.002

Yuan, S., Li, M., Fang, Z., Liu, Y., Shi, W., Pan, B., et al. (2016). Biological control of tobacco bacterial wilt using *Trichoderma harzianum* amended bioorganic fertilizer and the arbuscular mycorrhizal fungi *Glomus mosseae. Biol. Control* 92, 164–171. doi: 10.1016/j.biocontrol.2015.10.013

Zahir, Z. A., Munir, A., Asghar, H. N., Shaharoona, B., and Arshad, M. (2008). Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. *J. Microbiol. Biotechnol.* 18, 958–963.

Zhang, H., and Wang, X. (2016). Modular co-culture engineering, a new approach for metabolic engineering. *Metab. Eng.* 37, 114–121. doi: 10.1016/j.ymben.2016.05.007

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/fmicb.2021.798525

Bacillus amyloliquefaciens Rescues Glycyrrhizic Acid Loss Under Drought Stress in *Glycyrrhiza uralensis* by Activating the Jasmonic Acid Pathway

*Liang Yue[1,2,3], Constantine Uwaremwe[3,4], Yuan Tian[1,2,3], Yang Liu[1,2,3], Xia Zhao[1,2,3], Qin Zhou[1,2,3], Yun Wang[3,5], Yubao Zhang[1,2,3], Bailong Liu[6], Zengtuan Cui[7], Chengchao Dun[8] and Ruoyu Wang[1,2,3]**

[1] Key Laboratory of Stress Physiology and Ecology in Cold and Arid Regions of Gansu Province, Northwest Institute of Eco-Environment and Resources, Chinese Academy of Sciences, Lanzhou, China, [2] Gansu Gaolan Field Scientific Observation and Research Station for Agricultural Ecosystem, Northwest Institute of Eco-Environment and Resources, Chinese Academy of Sciences, Lanzhou, China, [3] University of Chinese Academy of Sciences, Beijing, China, [4] CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, China, [5] Key Laboratory of Desert and Desertification, Northwest Institute of Eco-Environment and Resources, Chinese Academy of Sciences, Lanzhou, China, [6] Gansu Institute for Drug Control, Lanzhou, China, [7] The General Station of Construction and Protection for The Cultivated Land and Quality of Gansu Province, Lanzhou, China, [8] School of Management, Lanzhou University, Lanzhou, China

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Jorge Poveda, Public University of Navarre, Spain
Hayssam M. Ali, King Saud University, Saudi Arabia

***Correspondence:**
Ruoyu Wang
wangruoyu@lzb.ac.cn

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *20 October 2021*
Accepted: *16 December 2021*
Published: *16 March 2022*

Citation:
Yue L, Uwaremwe C, Tian Y, Liu Y, Zhao X, Zhou Q, Wang Y, Zhang Y, Liu B, Cui Z, Dun C and Wang R (2022) Bacillus amyloliquefaciens Rescues Glycyrrhizic Acid Loss Under Drought Stress in Glycyrrhiza uralensis by Activating the Jasmonic Acid Pathway. Front. Microbiol. 12:798525. doi: 10.3389/fmicb.2021.798525

Drought is a major factor limiting the production of the perennial medicinal plant *Glycyrrhiza uralensis* Fisch. (Fabaceae) in Northwest China. In this study, 1-year-old potted plants were inoculated with the strain *Bacillus amyloliquefaciens* FZB42, using a gradient of concentrations (CFU), to test for microbe-induced host tolerance to drought condition treatments in a greenhouse experiment. At the concentration of 10^8 CFU ml^{-1}, FZB42 had significant growth-promoting effect on *G. uralensis*: the root biomass was 1.52, 0.84, 0.94, and 0.38 times that under normal watering and mild, moderate, and severe drought stress conditions, respectively. Under moderate drought, the positive impact of FZB42 on *G. uralensis* growth was most pronounced, with both developing axial and lateral roots strongly associated with indoleacetic acid (IAA) accumulation. An untargeted metabolomic analysis and physiological measurements of mature roots revealed that FZB42 improved the antioxidant system of *G. uralensis* through the accumulation of proline and sucrose, two osmotic adjustment solutes, and by promoting catalase (CAT) activity under moderate drought stress. Furthermore, significantly higher levels of total flavonoids, liquiritin, and glycyrrhizic acid (GA), the pharmacologically active substances of *G. uralensis*, were found in the roots of inoculated plants after FZB42 inoculation under all imposed drought conditions. The jasmonic acid (JA) content, which is closely related to plant defense responses and secondary metabolites' production, was greatly increased in roots after the bacterial inoculations, indicating that FZB42 activated the JA pathway. Taken together, our

results demonstrate that inoculation with FZB42 alleviates the losses in production and pharmacological metabolites of *G. uralensis* caused by drought *via* the JA pathway's activation. These results provide a developed prospect of a microbial agent to improve the yield and quality of medical plants in arid and semi-arid regions.

Keywords: PGPR, *Glycyrrhiza uralensis*, glycyrrhizic acid (GA), drought, jasmonic acid (JA)

INTRODUCTION

Licorice (*Glycyrrhiza uralensis* Fisch.) is an "essential herbal medicine" in China, whose roots harbor plentiful pharmacologically active substances, such as saponins and flavonoids (Schrofelbauer et al., 2009). Glycyrrhizic acid (GA), the most bioactive component of licorice root, is a triterpenoid saponin that has been extensively studied not only for its multiple health benefits, namely, its anti-viral, anti-inflammatory, and anti-cancer effects, but also other pharmacological activities (Lin, 2003; Matsumoto et al., 2013; Su et al., 2017). Moreover, GA can be utilized as a multifunctional drug carrier (Selyutina and Polyakov, 2019). Another bioactive component is liquiritin (LIQ), it being the predominant flavonoid in licorice root and known to have a variety of pharmacological activities, including anti-inflammatory, antitussive, anti-asthmatic, analgesic, anti-cancer, and neuroprotective effects (Liu Z.et al., 2020).

Wild *G. uralensis* plants, in populations distributed in arid and semi-arid regions of East Asia including Northwest China, vary in their tolerance to abiotic stress factors, including drought (Pan et al., 2006). Due to dwindling natural resources, now scarce, and concern over vegetation degradation, wild *G. uralensis* individuals are no longer the main source sustaining the market supply of this medicinal plant. Accordingly, to meet rising demand for it, cultivated *G. uralensis* has since become the paramount source sold in the current market; however, compared with the traits of wild stocks, both adequate soil fertility and fertilizer supplementation have probably weakened its tolerance to abiotic stresses (Liu et al., 2014; Li et al., 2016). Improving the ability of *G. uralensis* to withstand droughts in arid and semi-arid region where it is now *G. uralensis* mainly cultivated is imperative for securing the production and satisfying its burgeoning market demand worldwide.

Drought stress conditions prevail in drylands, including arid and semi-arid regions, where the scarcity of water limits the distribution and survival of plants and even local crop production (Lu and Zhang, 1998; Dekankova et al., 2004). A recent report concluded that half (50%) of all crop losses are now caused by abiotic stresses, most of which is from drought (10%) and heat (20%) (Zia et al., 2021). In plants incurring drought stress, typically their photosynthetic machinery is impaired and photorespiration increases, thus disrupting their cells' homeostasis; this then elicits the abundant generation of reactive oxygen species (ROS), the hallmark of and foremost plant response to stress. These ROS can cause much damage because they are highly reactive and toxic to proteins, lipids, and nucleic acids, which, if not dealt with by plants, eventually prolongs cellular damage and culminates in death (Moller et al., 2007). Plants under such stress will usually experience many metabolic, physiological, and biochemical changes. Organic solutes, however, such as soluble sugar, soluble protein, proline, and other low-molecular-weight metabolites, play key roles in how plants adjust to stress (Casanovas et al., 2002). Furthermore, antioxidant enzymes, such as superoxide, dismutase (SOD), peroxidase (POD), and catalase (CAT), can scavenge for ROS in plants (Zhu et al., 2009).

Plant-growth-promoting rhizobacteria (PGPR) can enhance plant growth directly, by attaching themselves onto roots, a feat possible because of their colonizing and biofilm-forming abilities (Bhattacharyya and Jha, 2012). In particular, PGPR could do more to help plants growing under drought conditions in several ways: optimizing the root environment and its water-absorbing capacity, accumulating antioxidant and osmolytes, and modifying phytohormones—the latter being a key player in plant defense responses (Naseem and Bano, 2014; Cohen et al., 2015; Poveda, 2020). For instance, the jasmonic acid (JA)/ethylene (ET) pathway usually involved in plant resistance can be induced by PGPR (Barnawal et al., 2017; Vries et al., 2020). For example, *Pseudomonas putida* H-2-3 increased the JA concentration and augmented antioxidant activity to enhance soybean (*Glycine max*. L. cv. Taekwang) plants' growth under saline and drought conditions (Kang et al., 2014).

Bacillus is one of the predominant genera of PGPR. It shows their plant-promoting effect as a complex mix of modes of action: produce phytohormones with beneficial effects for plant growth and tolerance; produce volatile organic compounds (VOCs) capable of modulating growth and inducing resistance in plant; and active systemic plant responses involving phenolic compounds, genetic and structural modifications, plant resistance activators, and the activation of enzymatic weapons (Hashem et al., 2019; Poveda, 2020, 2021; Poveda and Gonzalez-Andres, 2021). Our research showed that the strain *Bacillus amyloliquefaciens* FZB42, a typical PGPR, is capable of conferring greater growth and stress tolerance to *Arabidopsis* through rhizosphere inoculation and volatiles emitted from the JA pathway (Hao et al., 2016; Liu et al., 2017; Lu et al., 2018; Liu S.et al., 2020). In addition, PGPR inoculations can also increase the content of secondary metabolites in host plants. Zhao et al. (2016) found that *B. amyloliquefaciens* GB03 stimulated accumulation of secondary metabolites in *C. pilosula*. Moreover, it was reported that pennyroyal (*Mentha pulegium* L.) reduced the damage to its physio-biochemical characteristics and production of secondary metabolites normally caused by drought (Asghari et al., 2020).

In this study, the effects of *B. amyloliquefaciens* FZB42 inoculation for improving *G. uralensis* tolerance to a range of realistic drought conditions were experimentally investigated. We measured plant physiological traits and carried out an

untargeted metabolomic analysis of the root organ to elucidate bacterial-induced drought tolerance in *G. uralensis* mediated by osmotic solutes, antioxidase, and phytohormones, and the secondary metabolites GA and LIQ. Meanwhile, plant defense hormone JA plays a critical role in FZB42-induced drought tolerance of *G. uralensis*. The purpose is to provide a better understanding about the significant effect and mechanism of FZB42 on alleviating the adverse effect caused by drought stress on *G. uralensis*.

MATERIALS AND METHODS

Plants and Bacteria

One-year-old cultivated *G. uralensis* plants were collected from a single location in Yuzhong County, Gansu Province, China (36°17″ N 104°33″ E), a typical loess hilly region; mean annual average temperature and annual precipitation were 6.9°C and 350 mm, respectively. The soil mainly consists of subalpine meadow soil and gray cinnamon soil (Lv et al., 2020; Wei et al., 2021).

Bacillus amyloliquefaciens FZB42 deposited as strain 10A6 in the culture collection at the Bacillus Genetic Stock Center (BGSC). The FZB42 strain was cultured overnight, on Luria–Bertani liquid medium, at 37°C with shaking (200 rpm). Then, its cells were obtained by centrifugation (10,000 × *g* for 6 min) and re-suspended in sterile water to yield four concentrations, 1.0×10^7, 10^8, and 10^9 CFU ml^{-1}, for use as inocula in the experiment.

Plant-Growing Conditions and Treatments (Drought and Plant-Growth-Promoting Rhizobacteria)

The factorial pot experiment was conducted in a greenhouse at the Gansu Gaolan Field Scientific Observation and Research Station (36°13″ N 103°47″ E), in Lanzhou, China. The mean day and night temperatures were, respectively, 28 and 16°C, with a light/dark cycle of 14 h:10 h. On April 30, 2019, every *G. uralensis* plant was transplanted into a pot (30-cm diameter × 35-cm depth; 480 plants in total), each containing 20 kg of soil, which had been taken from the surface layer (0–30 cm depth) of a field at the station. The pH, C/N, TP, and TK of this soil was 8.54, 25.35, 1.00, and 19.13, respectively.

The experiment used a split plot design to test the effects of two crossed treatment factors (drought and inoculation), each with four levels. The drought conditions consisted of a (i) normal watering, that is, plants were grown in soil with the field water capacity (FWC) maintained between 60 and 65%, and likewise; (ii) mild drought stress, with an FWC of 55–60%; (iii) moderate drought stress, with an FWC of 45–50%; and (iv) severe drought stress, with an FWC of 35–40%. Then, in each of these four drought groups, their plants were rhizosphere inoculated with the FZB42 bacteria suspension at concentrations of 0, 1.0×10^7, 10^8, or 10^9 CFU ml^{-1}. In this experiment, the volume of water required to maintain the pot-level field capacity of each drought treatment corresponded to the amount drawn from soil by plants in previous days, assessed using the weighing method (i.e., weighing and watering the pots every 5 days). At 2 months post-transplanting, for the inoculations, 1,000 ml of the bacteria suspension for a given CFU was applied to the soil around each plant's main root (∼10 cm); the non-inoculated plants (CFU = 0) received the same volume of water by similar methods. In addition, the greenhouse was covered with a transparent plastic canopy to avoid interference from rain.

Three months after imposing the drought treatments, all the experimental plants were removed from their potted soil and separated into shoot and root parts using shears, cleaned with distilled water, then immediately flash frozen in liquid nitrogen and stored at −80°C to preserve the ROS and antioxidant components and prevent lipid peroxidation, for their use in the later metabolomic analysis. Each treatment combination had 30 replicates.

Plant Growth Response Variables and Biomass

At the experiment's end—before removing them from their pots—the plants' height, basal stem diameter, and the total biomass in each treatment combination were measured. Plant height was measured from the bottom of the stem to the terminal bud of the main stem. For basal diameter, the stem diameter of a plant was measured with a digital Vernier caliper at the soil surface. To determine biomass, plants were cleaned and divided into shoot and root tissues (see above), and these parts were weighed on an electronic balance (YP2001B, Lichen Tech, Nanjing, China).

Osmotic-Adjustment Solutes and Antioxidase Activity

The proline content in the plant shoots (stem + leaves) and roots was determined according to the method described previously (Bates et al., 1973). Briefly, 0.5 g of fresh leaves was frozen in liquid nitrogen, homogenized by vortex in 3% (w/v) sulfosalicylic acid (Merck KGaA, Hamburg, Germany) in 2-ml microtubes, and immediately centrifuged at 10,000 × *g* for 5 min (Eppendorf 5415C Centrifuge, Hamburg, Germany); the pellet was then discarded. Next, 1 ml of supernatant was taken and mixed with a solution of 1.25 g ninhidrin (Merck KGaA, Hamburg, Germany) dissolved in 30 ml of acetic acid (Merck KGaA, Hamburg, Germany) and then mixed with 20 ml of 6 M phosphoric acid (Merck KGaA, Hamburg, Germany) for analysis. Its absorbance was measured immediately, at 520 nm, using a spectrometer (DU 530, Beckman Coulter, Brea, CA, United States) at room temperature (20 ± 2°C); the calibration curve was determined using pure L-proline (Merck KGaA, Hamburg, Germany) as the standard reference.

Superoxide dismutase (SOD) and catalase (CAT) activities were determined as described previously (Qiu et al., 2008). SOD activity was estimated spectrophotometrically as the inhibition of the nitroblue tetrazolium (NBT) photochemical reduction at 560 nm. CAT activity was determined based on the decrease in the level of H_2O_2. Each sample comprising 0.2 g of roots tissues was homogenized in 2 ml of 50 mM ice-cold phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 15,000 × *g* for

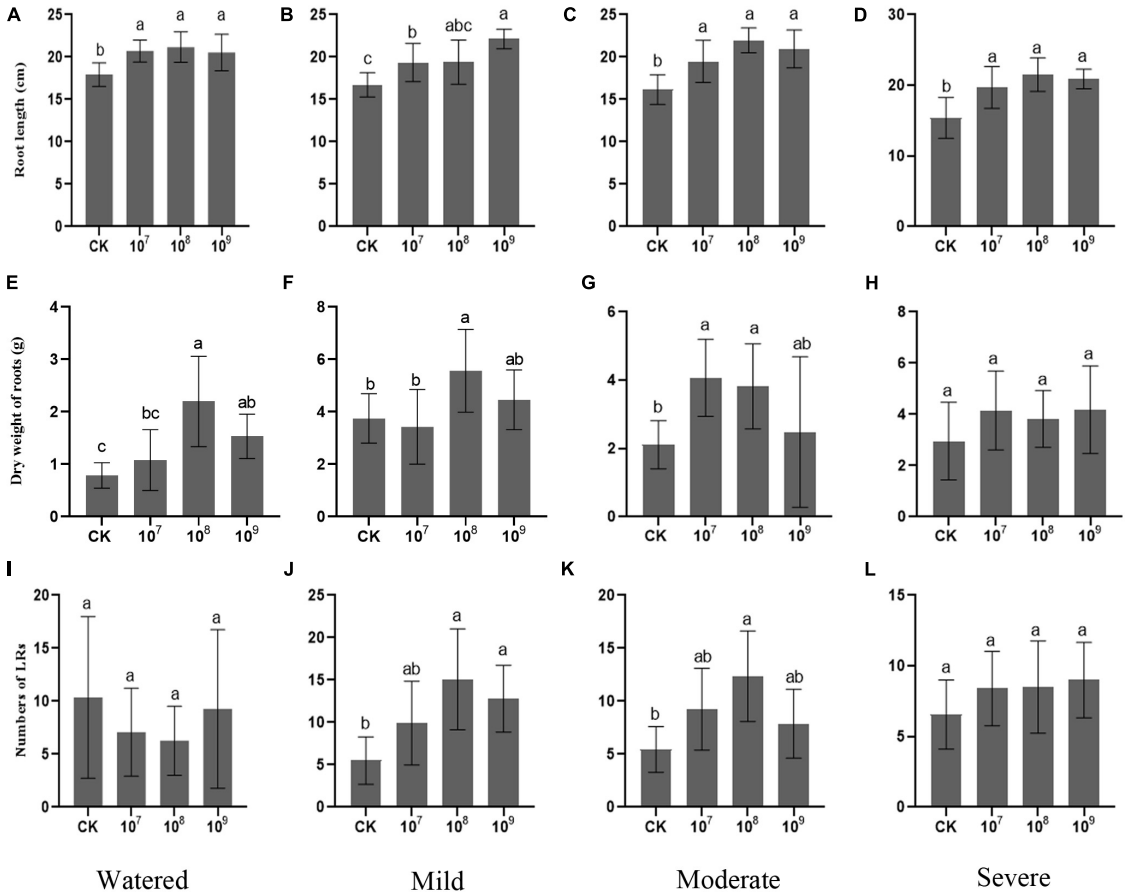


FIGURE 1 | *Bacillus amyloliquefaciens* FZB42 improves the root growth of *Glycyrrhiza uralensis* under drought. The far-left column is the control group (watered condition) followed to the right by increasing drought stress conditions (mild, moderate, severe). The panel rows show growth responses in terms of **(A–D)** root length (*F*-value = 8.1, 9.6, 15.5, 11.5), **(E–H)** dry weight of roots (*F*-value = 13.8, 8.0, 5.9, 1.4), and **(I–L)** counts of lateral roots (LRs) (*F*-value = 1.1, 8.3, 7.4, 1.4). Bars are the mean ± SE (*n* = 15). CK, zero inoculum.

15 min at 4°C. The supernatant comprised an enzyme extract containing both SOD and CAT.

The Glycyrrhizic Acid, Liquiritin, Indoleacetic Acid, Jasmonic Acid, and Total Flavonoid Contents of Roots

To determine the total flavonoid content, 0.02 g of root powder was placed in 25 ml of 50% methanol for the extraction, followed by its ultrasonication at room temperature for 1.5 h; then, it was filtered and diluted to 25-ml volumetric flask, and set aside. Absorption measurements of these extracts for the determination of total flavonoids content were taken at 530 nm, using a UV–vis spectrophotometer and quantified with respect to the standard curve (Zou et al., 2012). The high-performance liquid chromatography (HPLC) analysis was conducted for GA, LIQ, indoleacetic acid (IAA), and JA; all extracts were dissolved in a small volume of 70% ethyl alcohol and filtered through a 0.22-μm microporous membrane. A 20-μl aliquot of each sample extract was analyzed, at 25°C, by high-performance liquid chromatography (HPLC) (Agilent1260 Infinity II, CA, United States). The GA and LIQ were measured as described previously; the detected wavelength was 254 nm (17.340 and 10.817 min, respectively) (Zheng et al., 2013). The IAA and JA were detected at 254 nm (14.900 min) and 210 nm (10.117 min), respectively.

Metabolomic Analysis

All metabolite profiling analyses were performed independently at the Metware Biotechnology Co., Ltd. (Beijing, China). To do this, the company followed a standardized protocol [metabolites extraction, liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, data preprocessing and annotation] exactly as described in Smith et al. (2006), Dunn et al. (2011), Kuhl et al. (2012), and Wang et al. (2016). Full details on the methodology used by that company can be found in **Supplementary Material 1**.

Statistical Analysis

All experimental data were analyzed by ANOVA using SPSS 17.0 software (SPSS Inc., Chicago, IL, United States). After a

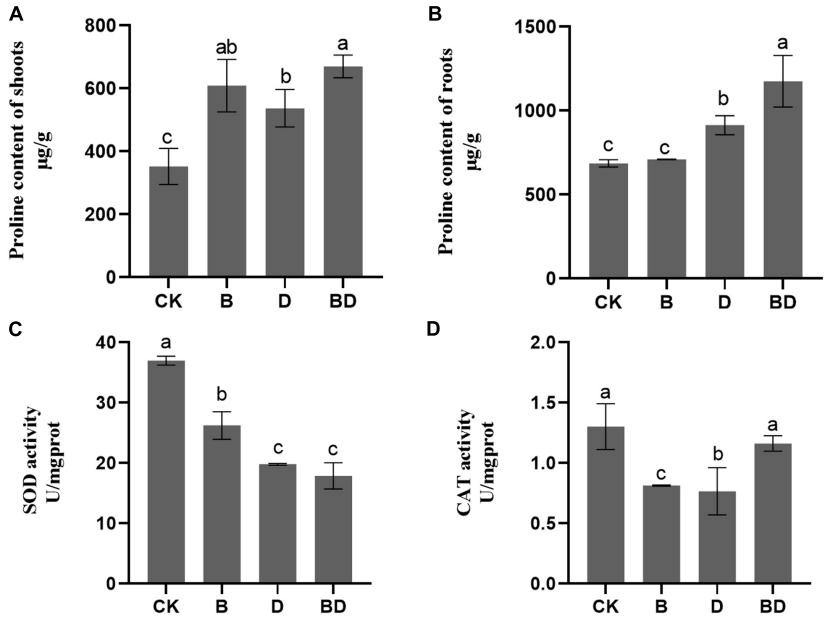


FIGURE 2 | Plant osmotic adjustment solutes' content and antioxidase activities. The proline content of **(A)** shoot and **(B)** root tissues, and the **(C)** SOD and **(D)** CAT activities of *Glycyrrhiza uralensis* (*F*-value = 15.1, 24.1, 85.9, 10.5). Bars are the mean ± SE (n = 3).

significant *F*-value, differences between means were assessed on a pairwise basis using the Tukey's honestly significant difference (HSD), at $p < 0.05$. Mean values and their standard errors (SE) are presented.

RESULTS

Bacillus amyloliquefaciens FZB42 Enhances Plant Growth Under Drought

Under the watered condition, varied concentrations of FZB42 differentially improved both shoot and root growth. When inoculated with 10^8 CFU ml^{-1}, the root biomass increased substantially, to 2.5 times that of CK (control: zero inoculum), with a 16.9% longer root length. Although not as pronounced for root length, both biomass were also significantly promoted by the inoculation with FZB42 at a concentration of 10^9 CFU ml^{-1}. However, applying 10^7 CFU ml^{-1} FZB42 can only significantly improve the root length but not biomass (**Figures 1A,E**). These results indicated that, under drought-free conditions, initial root inoculation with FZB42 improved the growth of *G. uralensis* roots for all three tested concentrations, but that of 10^8 CFU ml^{-1} FZB42 was optimal. Moreover, this root promotion effect mainly impacted the axial root, where the numbers of lateral roots were similar irrespective of the inoculation treatments (**Figure 1I**).

Drought significantly affected the growth of *G. uralensis*, reducing this plant's biomass (**Figure 1**). However, administering 10^8 CFU ml^{-1} of FZB42 was beneficial to *G. uralensis*, resulting in this plant's higher biomass accumulation (**Figures 1B–D**) and a longer root elongation under the three drought stress conditions while also enabling it to form more lateral roots (LRs) under the mild and moderate drought conditions (**Figures 1J,K**). Hence, these results indicated that 10^8 CFU ml^{-1} FZB42 promoted the development of both axial and lateral roots in drought-stressed *G. uralensis*. The inocula of 10^7 and 10^9 CFU ml^{-1} FZB42 significantly promoted root elongation under all drought conditions, yet 10^7 CFU ml^{-1} FZB42 was more effective for biomass accumulation under moderate and severe drought conditions, while using 10^9 CFU ml^{-1} FZB42 did not increase the biomass in any drought treatment (**Figures 1B–D,F–H**). Taken together, 10^8 CFU ml^{-1} FZB42 is evidently optimal for root growth promotion in *G. uralensis* under either normal (watered) or drought conditions. Under the watered condition, the FZB42 could only stimulate root's axial elongation, but the bacterial strain was able stimulate both axial and lateral root growth in drought-stressed plants.

Bacillus amyloliquefaciens FZB42 Improves the Accumulation Osmotic Adjustment Solutes and Antioxidase Activity in *Glycyrrhiza uralensis*

These analyses focused on the samples from plants inoculated with 10^8 CFU ml^{-1} given its strong positive effects on root growth and drought tolerance. Here, we abbreviated the 10^8 CFU ml^{-1} FZB42 under the normal watering condition treatment as "B" (i.e., 10^8 CFU ml^{-1} FZB42 applied, with zero drought stress to plants) and, conversely, moderate drought without the FZB42 treatment as "D" (i.e., 0 CFU ml^{-1} FZB42 applied, with drought stress to plants). Correspondingly, to infer the joint effect, 10^8 CFU ml^{-1} FZB42 under moderate drought treatment was designated as "BD" (i.e., 10^8 CFU ml^{-1} FZB42

applied, with drought stress to plants). Hence, treatment B, D, and BD values could be compared to each other, as well to the CK (i.e., 0 CFU ml^{-1} FZB42 applied, with zero drought stress to plants). The proline content of both the shoot and root parts were generally increased by drought (**Figure 2**). However, these proline levels were significantly higher in BD than D at 3 months post-treatment. These results suggested that the FZB42 inoculation fostered proline accumulation in *G. uralensis* plant tissues, above and belowground, so as to restore their cell osmotic equilibrium disrupted by water deficits.

Two months after FZB42 inoculation, we found markedly decreased SOD and CAT activities under the watered condition, with no significant difference detected in SOD between D and BD. Yet intriguingly, a significant increase in CAT activity occurred in BD compared with B, which indicated that the bacterial strained improved oxidation resistance of *G. uralensis* by enhancing the antioxidant enzyme CAT's activity in the roots.

Multivariate Analysis and Identified Metabolites

To further investigate the effects of FZB42 inoculation on metabolism in *G. uralensis*, we used the CK, B, D, and BD treatment combinations in the metabolomic analysis. The Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) models were used to analyze the metabolome data in the data matrix built. For the two comparative groupings of CK vs. B and B vs. BD, their score plots from the fitted OPLS-DA models and corresponding validation plots were constructed (**Figure 3**). To screen for differentially expressed metabolites in roots of either comparative grouping, we integrated the results of the multivariate and univariate to obtain suitable criteria for their designation: VIP > 1 for the first principal component in the OPLS-DA, p-value < 0.05, and fold-change (FC) > 1. Overall, 1,811 metabolites were identified, including 13 up- and 15 downregulated metabolites in the CK vs. B grouping. In the D vs. BD grouping, 79 metabolites were upregulated, while 24 were downregulated. Therefore, differential metabolites in the roots of *G. uralensis* with/without FZBZ42 inoculation under normal or drought conditions were identified.

Kyoto Encyclopedia of Genes and Genomes Functional Annotation and Enrichment Analysis of Differential Metabolites

Differential expressed metabolites were mapped using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database onto the KEGG pathways, for which those with increased or decreased differential metabolites (and enzymes) are presented in **Figure 4**. By comparing the metabolites of roots between CK and B, they could be mapped to five KEGG pathways and likewise to 22 KEGG pathways for those found between D and BD. In the CK vs. B comparative grouping, sphingolipid metabolism (ko00600), anthocyanin biosynthesis (ko00942), nicotinate and nicotinamide metabolism (ko00760), and flavone and flavonol biosynthesis (ko00944) all featured positive enrichment, whereas histidine metabolism (ko00340) underwent a negative enrichment (**Figure 4A**). In the D vs. BD comparative grouping, ABC transporters (ko00240), starch and sucrose metabolism (ko00500), fructose and mannose metabolism (ko00051), arginine and proline metabolism (ko00330), D-arginine and D-ornithine metabolism (ko00472), tryptophan metabolism (ko000380), and histidine metabolism (ko00340) were all significantly upregulated, while cutin, suberine, and wax biosynthesis (ko00073); carotenoid biosynthesis (ko00906); and plant hormone signal transduction (ko04075) were each downregulated (**Figure 4B**).

These results showed that FZB42 inoculation enhanced the biosynthesis of anthocyanin, which functions to mitigate stress in plants by ameliorating oxidative damage, as demonstrated in *Arabidopsis* and tomato (Tohge and Fernie, 2017). In addition, nicotinate and nicotinamide metabolism are positively related to nicotinamide adenine dinucleotide (NAD^+), a redox carrier and a signal molecule. Accordingly, we speculate that FZB42 contributes to maintaining sufficient NAD^+ pools in *G. uralensis*, which could protect this plant's cells from energy depletion caused by adverse environmental conditions, thereby avoiding cell death and plant productivity loss as posited by Gakiere et al. (2018). Altogether, our results suggested the FZB42 treatment (10^8 CFU ml^{-1}) positively enriched proline and sucrose synthesis under moderate drought stress.

Bacillus amyloliquefaciens FZB42 Augmented the Indoleacetic Acid and Jasmonic Acid Contents of Roots to Improve the Drought Stress Tolerance of *Glycyrrhiza uralensis*

For the metabolism of tryptophan, the precursor of IAA, it was positively enriched after FZB42 under the drought condition (**Figure 4B**), and the IAA content of the root tissue was determined by HPLC. Compared with the CK, the IAA content of plants that received the FZB42 treatment B decreased significantly under the watered condition, and conversely, it increased under the moderate drought condition. IAA accumulation might thus be an essential factor for sustaining root and shoot biomass production; stated differently, the mechanism by which FZB42 promoted plant growth under drought stress could involve the maintenance of IAA biosynthesis.

Furthermore, FZB42 inoculation increased the JA content of *G. uralensis* roots, under both watered and drought conditions. This result is consistent with the findings of similar studies on *Arabidopsis* previously reported (Hao et al., 2016; Liu et al., 2017; Lu et al., 2018; Liu S.et al., 2020). Hence, FZB42 may likewise activate the JA pathway in *G. uralensis*.

Bacillus amyloliquefaciens FZB42 Improves Secondary Metabolites' Accumulation Under Drought Stress

The positive enrichment of flavone and flavonol biosynthesis (ko00944) suggests that FZB42 is able to promote flavonol biosynthesis in *G. uralensis* under

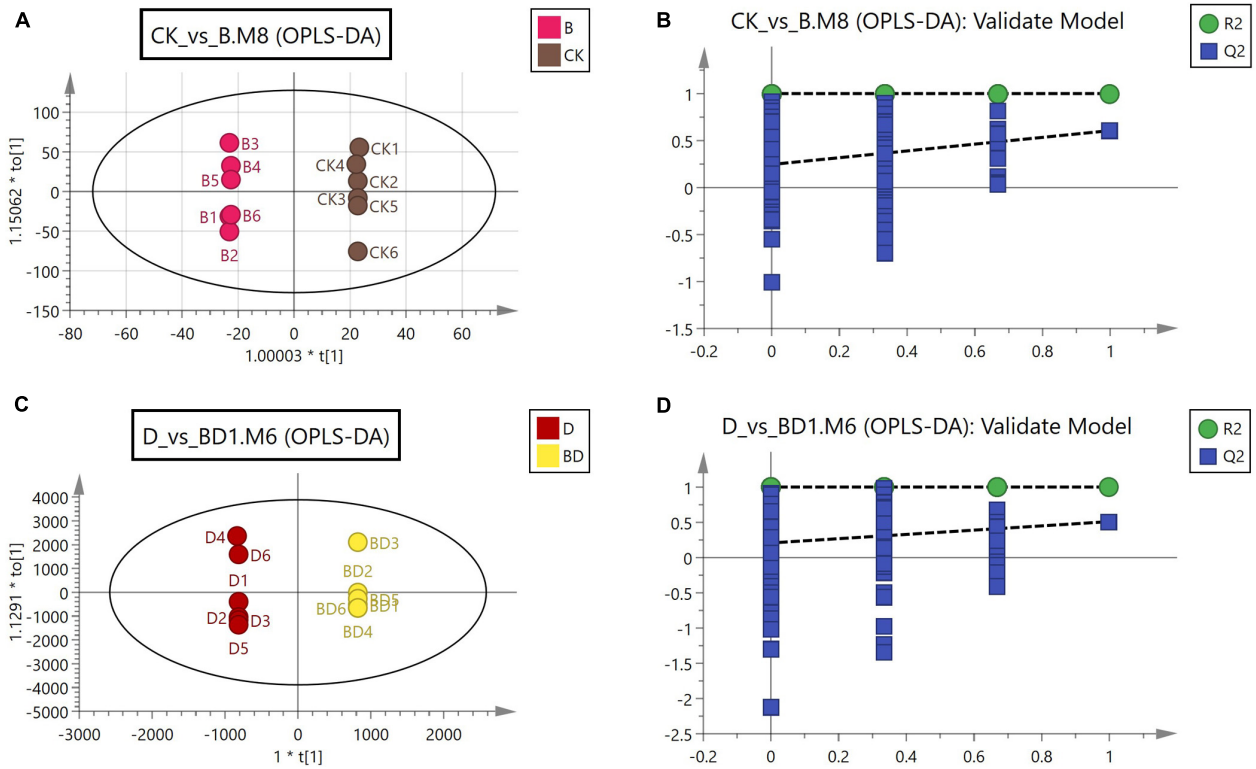


FIGURE 3 | Score scatterplots of OPLS-DA **(A,C)** and validation plots **(B,D)** for two comparative groupings: CK vs. B, and D vs. DB.

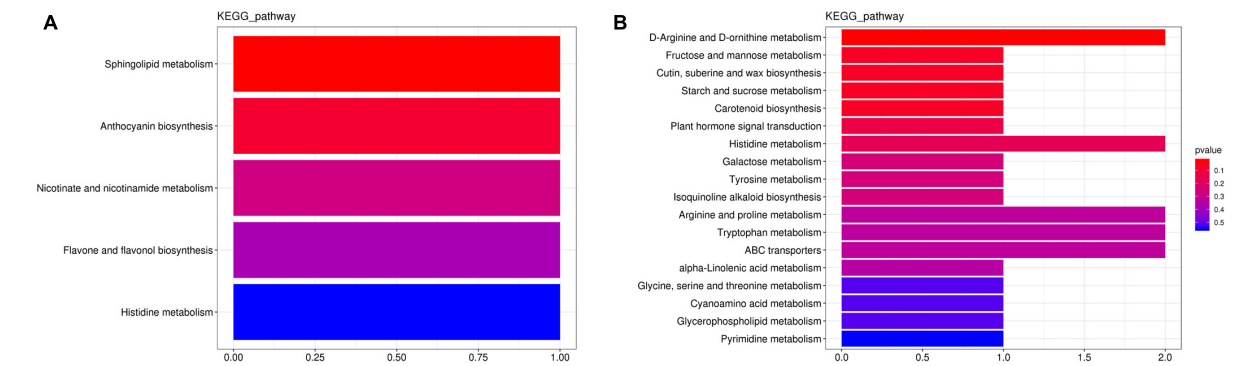


FIGURE 4 | Kyoto Encyclopedia of Genes and Genomes pathways' enrichment for **(A)** the CK vs. B comparative grouping and **(B)** the D vs. DB comparative grouping.

the normal condition (**Figure 4A**). The GA, LIQ, and total flavonoid content were determined to confirm this speculation.

Regarding GA and LIQ, their contents were generally decreased after the plants were subjected to drought stress, which indicated that the accumulation of secondary metabolites would be inhibited when plants were subjected to long-term moderate drought. Compared with CK, the FZB42 inoculation treatment (B) diminished the GA and LIQ contents but increased that of its total flavonoid content. This demonstrated that FZB42 can improve flavonoids' biosynthesis, corroborating the results for the metabolites analysis, but did not enable GA and LIQ to accumulate (**Figures 5A,C**). However, the contents of GA, LIQ, and total flavonoids were significantly higher in BD than D and on par with those of B, which suggested that the FZB42 inoculation seems to limit losses of GA and LIQ accumulation in plants caused by drought stress (**Figure 5**). Interestingly, we uncovered a strong positive correlation between total flavonoids content and the JA content in roots (Pearson's $r = 0.629$, $p = 0.028$), which pointed to the JA pathway's involvement in the flavonoids' accumulation in *G. uralensis*.

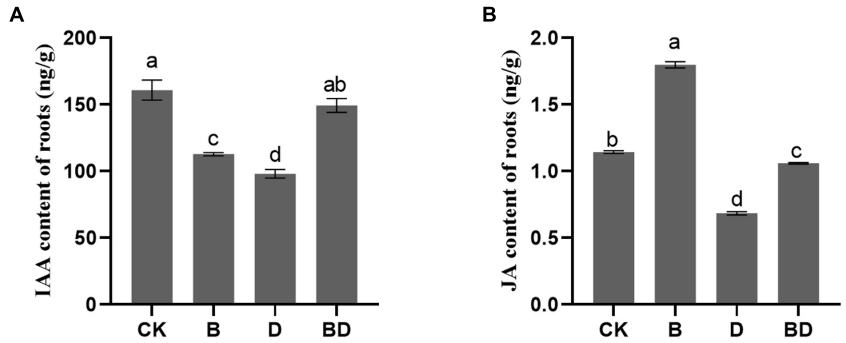


FIGURE 5 | Phytohormones in the roots of *Glycyrrhiza uralensis*. The **(A)** indoleacetic acid content and **(B)** jasmonic acid content (*F*-value = 109.9, 3,153.8). Bars are the mean ± SE (*n* = 3).

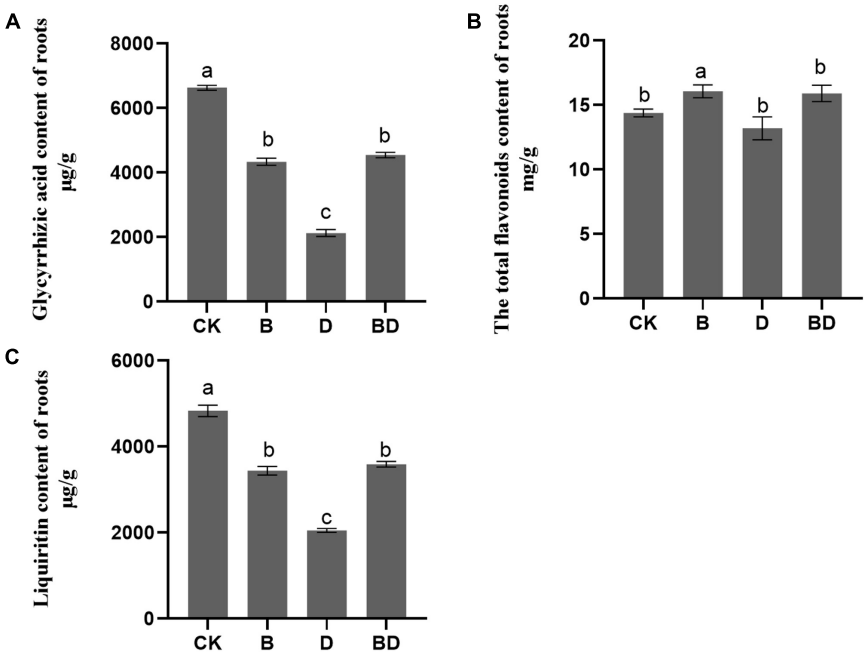


FIGURE 6 | Secondary metabolites of *Glycyrrhiza uralensis*. The **(A)** GA content, **(B)** total flavonoids content, and **(C)** LIQ content of roots (*F*-value = 1,114.8, 465.8, 14.5). Bars are the mean ± SE (*n* = 3).

DISCUSSION

Effect of *Bacillus amyloliquefaciens* Upon Plant Growth

Water is essential for plant survival, and chronic water deficits constrain plant growth and fitness (Gupta et al., 2020). Studies have shown that inoculations with PGPR could improve drought stress tolerance of various plant species (Chanway and Holl, 1994; Saravanakumar et al., 2010; Marasco et al., 2012). In this study, the drought tolerance ability of *G. uralensis* was significantly enhanced after inoculating its rhizosphere with the strain *B. amyloliquefaciens* FZB42; specifically, it tempered the drought-induced reductions the length of roots, the number of lateral roots, and the dry weight of root tissue (**Figure 1**). This result is consistent with several previous studies (Kang et al., 2013; Tiwari et al., 2016; Xie et al., 2019). Greater water and nutrition acquisition by roots is crucial for sustaining plants' performance in the face of drought stress. Auxin plays an important role in the molecular mechanisms of action of PGPR on root architecture, which including directly synthesis from tryptophan in plant root exudates and indirectly activated by various signaling chemicals (Kalyanasundaram et al., 2021). Therefore, our results provide strong evidence that inoculation with *B. amyloliquefaciens* could play an important role in improving the growth and biomass of *G. uralensis* through promoting root growth and altering root architecture under drought stress conditions.

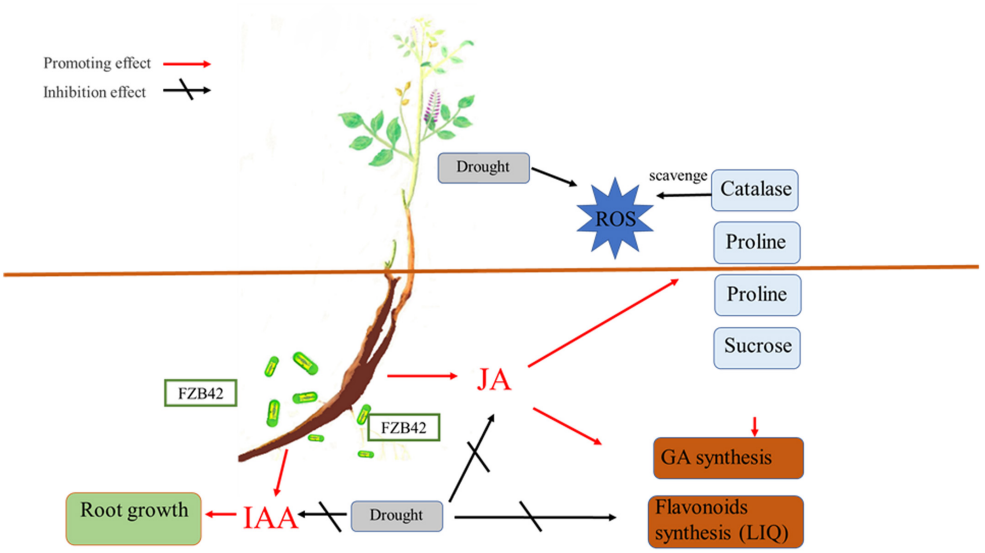


FIGURE 7 | Postulated mechanism by which the *Bacillus amyloliquefaciens* FZB42 strain induces drought tolerance and rescues glycyrrhizic acid (GA) loss in drought-stressed *Glycyrrhiza uralensis* plants *via* the JA pathway's activation. IAA, indoleacetic acid; JA, jasmonic acid; ROS, reactive oxygen species.

Effect of *Bacillus amyloliquefaciens* on Tryptophan Metabolism and Indoleacetic Acid's Accumulation

Plant hormones are among the most critical growth regulators. The plant growth and stress tolerance induced by beneficial microorganisms partly depends on their ability to facilitate synthesis of phytohormones in the rhizosphere or root tissue network (Davies et al., 1996; Bano et al., 2013). Being the principal auxin naturally occurring in most plants, IAA figures prominently in many key biological processes, including cell division, elongation, differentiation, and leaf expansion (Estelle, 1992). Furthermore, higher levels of IAA are expected to lower intracellular ROS levels (Guan and Scandalios, 2002; Fei et al., 2016; Khaksar et al., 2017). A work by Ma et al. (2011) highlighted the positive role of plant-exuded IAA in activating those bacterial genes responsible for the colonization of host plants' roots and adaptation to them. For example, the *Bacillus subtilis* strain GB03 promotes *Arabidopsis* growth *via* upregulating transcripts for auxin homeostasis (Zheng et al., 2013). Tryptophan is the precursor of IAA; we found that tryptophan metabolism (ko000380) upregulated, along with the content of IAA augmented after FZB42 inoculation of *G. uralensis* plants under drought stress (**Figure 6**). In addition, analogously to other PGPR, FZB42 owns the ability of IAA production that might have positive effects on root architecture alterations, which could promote plant growth as a whole (Helmut and Rainer, 2004). The IAA accumulation results in more root tips and a larger root surface area (**Figure 1**), thereby enabling greater water and nutrient acquisition under drought conditions. In sum, FZB42 inoculation induced IAA accumulation, which can ensure the growth of drought-stricken *G. uralensis* plants—all of which survived the experiment—and generally enhanced its drought tolerance.

Effect of *Bacillus amyloliquefaciens* on the Osmolytes and Antioxidants *via* Activation of the Jasmonic Acid Pathway

Osmotic adjustment is one of the key adaptations at the cellular level that helps plants tolerate drought-induced oxidative damage (Farooq et al., 2009; Huang et al., 2014). Under abiotic stress, plant accumulate solutes, including sugars (e.g., sucrose) and non-protein amino acids (e.g., proline), to maintain cellular turgor and help plants lower water potential without decreasing actual water content (Farooq et al., 2008). Proline acts as the key role for osmotic adjustment and also contributes to stabilizing subcellular structures, scavenging free radicals, and buffering cellular redox potential (Hayat et al., 2012). Treatment of plants with PGPR has been shown to increase proline levels (Casanovas et al., 2002; Sziderics et al., 2007; Bano et al., 2013; Sharma et al., 2013; Gusain et al., 2015). In accordance with this, the metabolome analysis and proline content results confirmed that inoculation with FZB42, a PGPR, induced proline accumulation under drought condition (**Figures 2B**, **4B**). In addition, our metabolomics results showed that soluble sugars-related metabolism, starch and sucrose metabolism (ko00500), and fructose and mannose metabolism (ko00051) were upregulated in plant roots (**Figure 4B**). During water loss, soluble sugars function critically as osmoprotectants in maintaining turgor pressure with a sufficient degree of hydration (Loutfy et al., 2011; Krasensky and Jonak, 2012). Moreover, we found that the FZB42 treatment also enhanced the accumulation of starch, which is directly linked to the photosynthetic capacity of plants under drought stress (Sharma et al., 2020). We, thus, may reasonably infer that FZB42 inoculation could induce a plant's tolerance of drought stress by modifying its osmotic adjustment.

Furthermore, plants can increase the activities of certain antioxidant enzymes, namely, CAT, SOD, GPX, APX, and GR,

to clear out H_2O_2 and O_2^- from their cells (Tian et al., 2012). Because SOD converts O_2^- to H_2O_2, it is thought to play a fundamental role in the antioxidant defense system, while CAT and POD destroy H_2O_2 in the cytoplasm and other cell components. In previous studies, drought stress altered the amount and activities of enzymes involved in scavenging for ROS in various species (Sankar et al., 2007; Uzilday et al., 2012; Akitha Devi and Giridhar, 2013). In our study, inoculation with *B. amyloliquefaciens* FZB42 was capable of increasing the activity of CAT, but that of SOD did not change much (**Figure 2**). These results suggest that the improved CAT was induced by PGPR *B. amyloliquefaciens* to remove H_2O_2 and produce H_2O, thereby keeping free radicals at a lower abundance and avoiding lipid peroxidation damage of cell membrane in *G. uralensis*. This is similar to findings of previous studies in basil (*Ocimum basilicum* L.), maize (*Zea mays* L.), and tomato (Heidari and Golpayegani, 2012; Naveed et al., 2014).

Jasmonic acid can contribute to tolerance of drought stress in plants by increasing the activity of their antioxidants (Bao et al., 2009). In a recent study, methylated JA (MeJA) was found able to alleviate oxidative stress caused by salt stress in *G. uralensis* seedlings *via* enhancing the activity of antioxidant enzymes and non-enzymatic antioxidants (Lang et al., 2020). PGPR was considering to have potential to enhance plant growth directly by improving nutrition, regulating stress phytohormone like JA, increasing antioxidants activity, and producing siderophores (Bukhat et al., 2020). Kang et al. (2014) reported that *P. putida* H-2-3 modulates JA and antioxidants expression of soybean to improve the plant growth under saline and drought conditions. Given that FZB42 induced JA's accumulation under drought stress (**Figure 6**), we speculate that both this PGPR strain and its volatile organic compounds (VOCs) were able to activate the JA pathway to defend against this abiotic stress, which is consistent with our previous work (Liu et al., 2017; Liu S.et al., 2020).

Bacillus amyloliquefaciens Affects the Production of Secondary Metabolites by Activating the Jasmonic Acid Pathway

Although moderate drought stress is reported to show beneficial effect on secondary metabolites accumulation in medicinal plants (Bloem et al., 2014), a previous study showed that the contents of total flavonoids, total polysaccharides, and glycyrrhizic acid were significantly decreased by drought stress (Xie et al., 2019). *Bacillus pumilus* inoculation could alleviate the abiotic-stress-induced negative effects on the secondary metabolite accumulation in *Mentha arvensis* and *G. uralensis* (Bharti et al., 2013; Xie et al., 2019). In this study, we found that FZB42 inoculation could interfere with the suppression of total flavonoid content and LIQ (liquiritin) and GA (glycyrrhizic acid) contents caused by drought stress (**Figure 5**).

Jasmonic acid and its related compounds are considered as transducers of elicitor signals for plant secondary metabolites (Devoto et al., 2005; Zhai et al., 2017). The role of MeJA in the secondary metabolites of *G. uralensis* has been considerable recently. Treatment with MeJA increased the GA content of *G. uralensis* but limited its root growth (Shabani et al., 2009; Li et al., 2016). In another experiment, the MeJA treatment spurred the production of total flavonoids in a suspension of cultured cells of the conspecific plant, *G. inflata* (Ying et al., 2008). In addition, MeJA improved metabolism and sucrose content of NaCl-stressed *G. uralensis* seedlings (Lang et al., 2020). Thus, MeJA exerts a beneficial effect on secondary metabolites production of *G. uralensis*. In addition, JA combined with other secondary messengers was reported to regulate the synthesis of secondary metabolites *via* microbial elicitors (Hu et al., 2006). Moreover, the increased sucrose content reportedly caused by JA rescued the GA loss caused by salt condition in *G. uralensis* (Fuzhi and Jun, 2015; Lang et al., 2020). Based on these results, we suppose that FZB42 activated JA pathway and promoted JA accumulation in *G. uralensis*; these excess JAs further enhanced the secondary metabolites accumulation. Meanwhile, the diminished root growth caused by JA was partly rescued by the accumulation of IAA.

CONCLUSION

In conclusion, our experimental results demonstrate that the drought stress tolerance of *G. uralensis* can be improved by inoculation with *B. amyloliquefaciens* FZB42 by modifying root architecture and increasing the antioxidant enzyme activities and proline and sucrose contents. *Bacillus amyloliquefaciens* FZB42 can also rescue the GA, LIQ, and total flavonoid losses caused by drought. The greater JA content, a consequence of FZB42 inoculation, likely fosters favorable physiological changes in plants under drought stress. These results suggest that inoculation with *B. amyloliquefaciens* strains may offer a promising and practical way to improve the drought tolerance of cultivated *G. uralensis* plants (summarized in **Figure 7**). Our results demonstrated that inoculation of FZB42 showed both significant plant growth promotion and accumulation of GA, LIQ, and total flavonoid in *G. uralensis* under drought condition. It will guide a new strategy for cultivating the Chinese herbal plant *G. uralensis*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LY and RW conceived and designed the experiments. LY and CU performed the experiments and wrote the manuscript. YT and YL analyzed the data. XZ, QZ, BL, ZC, and CD provided the technical assistance to LY. YW and YZ revised the language of the

manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was financially supported by the grants from National Key Research and Development Project of China (Grant No. 2018YFE0127200). The Science and Technology Planning Project of Gansu Province, major special project (Grant No. 21ZD4NA019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.798525/full#supplementary-material

REFERENCES

Akitha Devi, M. K., and Giridhar, P. (2013). Variations in Physiological Response, Lipid Peroxidation, Antioxidant Enzyme Activities, Proline and Isoflavones Content in Soybean Varieties Subjected to Drought Stress. *Proc. Natl. Acad. Sci. India Sect. B* 85, 35–44. doi: 10.1007/s40011-013-0244-0

Asghari, B., Khademian, R., and Sedaghati, B. (2020). Plant growth promoting rhizobacteria (PGPR) confer drought resistance and stimulate biosynthesis of secondary metabolites in pennyroyal (*Mentha pulegium* L.) under water shortage condition. *Sci. Hortic.* 263:109132. doi: 10.1016/j.scienta.2019.109132

Bano, Q., Ilyas, N., Bano, A., Zafar, N., Akram, A., and Hassan, F. U. L. (2013). Effect of Azospirillum inoculation on maize (Zea mays L.) under drought stress. *Pak. J. Bot.* 45, 13–20.

Bao, A. K., Wang, S. M., Wu, G. Q., Xi, J. J., Zhang, J. L., and Wang, C. M. (2009). Overexpression of the Arabidopsis H^+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Sci.* 176, 232–240. doi: 10.1016/j.plantsci.2008.10.009

Barnawal, D., Bharti, N., Pandey, S. S., Pandey, A., Chanotiya, C. S., and Kalra, A. (2017). Plant growth-promoting rhizobacteria enhance wheat salt and drought stress tolerance by altering endogenous phytohormone levels and TaCTR1/TaDREB2 expression. *Physiol. Plant.* 161, 502–514. doi: 10.1111/ppl.12614

Bates, L. S., Waldren, R. P., and Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant Soil* 39, 205–207. doi: 10.1007/bf00018060

Bharti, N., Barnawal, D., Awasthi, A., Yadav, A., and Kalra, A. (2013). Plant growth promoting rhizobacteria alleviate salinity induced negative effects on growth, oil content and physiological status in *Mentha arvensis*. *Acta Physiol. Plant.* 36, 45–60. doi: 10.1007/s11738-013-1385-8

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bloem, E., Haneklaus, S., Kleinwächter, M., Paulsen, J., Schnug, E., and Selmar, D. (2014). Stress-induced changes of bioactive compounds in *Tropaeolum majus* L. *Ind. Crops Prod.* 60, 349–359. doi: 10.1016/j.indcrop.2014.06.040

Bukhat, S., Imran, A., Javaid, S., Shahid, M., Majeed, A., and Naqqash, T. (2020). Communication of plants with microbial world: exploring the regulatory networks for PGPR mediated defense signaling. *Microbiol. Res.* 238:126486. doi: 10.1016/j.micres.2020.126486

Casanovas, E. M., Barassi, C. A., and Sueldo, R. J. (2002). Azospiriflum Inoculation Mitigates Water Stress Effects in Maize Seedlings. *Cereal Res. Commun.* 30, 343–350. doi: 10.1007/BF03543428

Chanway, C., and Holl, F. (1994). Growth of Outplanted Lodgepole Pine Seedlings One Year After Inoculation with Plant Growth Promoting Rhizobacteria. *For. Sci.* 40, 238–246. doi: 10.1093/forestscience/40.2.238

Cohen, A. C., Bottini, R., Pontin, M., Berli, F. J., Moreno, D., Boccanlandro, H., et al. (2015). Azospirillum brasilense ameliorates the response of *Arabidopsis thaliana* to drought mainly *via* enhancement of ABA levels. *Physiol. Plant.* 153, 79–90. doi: 10.1111/ppl.12221

Davies, P., Davies, P., and Krikorian, A. D. (1996). Plant Hormones: physiology, Biochemistry and Molecular Biology. *Sci. Hortic.* 66, 267–270. doi: 10.1016/S0304-4238(96)00922-3

Dekankova, K., Luxova, M., Gasparikova, O., and Kolarovic, L. (2004). Response of maize plants to water stress. *Bioligia* 5, 151–155.

Devoto, A., Ellis, C., Magusin, A., Chang, H. S., Chilcott, C., Zhu, T., et al. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Mol. Biol.* 58, 497–513. doi: 10.1007/s11103-005-7306-5

Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., et al. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 6, 1060–1083. doi: 10.1038/nprot.2011.335

Estelle, M. (1992). The plant hormone auxin: insight in sight. *Bioessays* 14, 439–444. doi: 10.1002/bies.950140703

Farooq, M., Basra, S. M. A., Wahid, A., Cheema, Z. A., Cheema, M. A., and Khaliq, A. (2008). Physiological Role of Exogenously Applied Glycinebetaine to Improve Drought Tolerance in Fine Grain Aromatic Rice (*Oryza sativa* L.). *J. Agron. Crop Sci.* 194, 325–333. doi: 10.1111/j.1439-037X.2008.00323.x

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., and Basra, S. M. A. (2009). "Plant Drought Stress: effects, Mechanisms and Management," in *Sustainable Agriculture*, ed E. Lichtfouse, M. Navarrete, P. Debaeke, S. Véronique and C. Alberola (Dordrecht: Springer), 153–188. doi: 10.1007/978-90-481-2666-8_12

Fei, Y., Xiao, B., Yang, M., Ding, Q., and Tang, W. (2016). MicroRNAs, polyamines, and the activities antioxidant enzymes are associated with *in vitro* rooting in white pine (*Pinus strobus* L.). *Springerplus* 5:416. doi: 10.1186/s40064-016-2080-1

Fuzhi, L., and Jun, Y. (2015). Effect of exogenous sucrose on growth and active ingredient content of licorice seedlings under salt stress conditions. *China J. Chin. Mater. Medica* 40:5. doi: 10.4268/cjcmm20152212

Gakiere, B., Fernie, A. R., and Petriacq, P. (2018). More to NAD(+) than meets the eye: a regulator of metabolic pools and gene expression in Arabidopsis. *Free Radic. Biol. Med.* 122, 86–95. doi: 10.1016/j.freeradbiomed.2018.01.003

Guan, L. M., and Scandalios, J. G. (2002). Catalase gene expression in response to auxin-mediated developmental signals. *Physiol. Plant.* 114, 288–295. doi: 10.1034/j.1399-3054.2002.1140215.x

Gupta, A., Rico-Medina, A., and Cao-Delgado, A. I. (2020). The physiology of plant responses to drought. *Science* 368, 266–269. doi: 10.1126/science.aaz7614

Gusain, Y. S., Singh, U. S., and Sharma, A. K. (2015). Bacterial mediated amelioration of drought stress in drought tolerant and susceptible cultivars of rice (*Oryza sativa* L.). *Afr. J. Biotechnol.* 14, 764–773. doi: 10.5897/ajb2015.14405

Hao, H. T., Zhao, X., Shang, Q. H., Wang, Y., and Wang, R. Y. (2016). Comparative Digital Gene Expression Analysis of the Arabidopsis Response to Volatiles Emitted by *Bacillus amyloliquefaciens*. *PLoS One* 11:e0158621. doi: 10.1371/journal.pone.0158621

Hashem, A., Tabassum, B., and Fathi Abd Allah, E. (2019). *Bacillus subtilis*: a plant-growth promoting rhizobacterium that also impacts biotic stress. *Saudi J. Biol. Sci.* 26, 1291–1297. doi: 10.1016/j.sjbs.2019.05.004

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal. Behav.* 7, 1456–1466. doi: 10.4161/psb.21949

Heidari, M., and Golpayegani, A. (2012). Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil (*Ocimum basilicum* L.). *J. Saudi Soc. Agric. Sci.* 11, 57–61. doi: 10.1016/j.jssas.2011.09.001

Helmut, B., and Rainer, B. (2004). Use of *Bacillus subtilis* as biocontrol agent. VI. Phytohormonelike action of culture filtrates prepared from plant growth promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. *J. Plant Dis. Prot.* 111:15.

Hu, F., Huang, J., Xu, Y., Qian, X., and Zhong, J. J. (2006). Responses of defense signals, biosynthetic gene transcription and taxoid biosynthesis to elicitation

by a novel synthetic jasmonate in cell cultures of *Taxus chinensis*. *Biotechnol. Bioeng.* 94, 1064–1071. doi: 10.1002/bit.20921

Huang, B., DaCosta, M., and Jiang, Y. (2014). Research Advances in Mechanisms of Turfgrass Tolerance to Abiotic Stresses: from Physiology to Molecular Biology. *Crit. Rev. Plant Sci.* 33, 141–189. doi: 10.1080/07352689.2014.870411

Kalyanasundaram, G. T., Syed, N., and Subburamu, K. (2021). "Recent developments in plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture," in *Recent Developments in Applied Microbiology and Biochemistry*, ed. B. Viswanath (Amsterdam: Elsevier), 181–192. doi: 10.1016/b978-0-12-821406-0.00017-5

Kang, S. M., Radhakrishnan, R., Khan, A. L., Kim, M. J., Park, J. M., Kim, B. R., et al. (2014). Gibberellin secreting rhizobacterium, *Pseudomonas putida* H-2-3 modulates the hormonal and stress physiology of soybean to improve the plant growth under saline and drought conditions. *Plant Physiol. Biochem.* 84, 115–124. doi: 10.1016/j.plaphy.2014.09.001

Kang, Y., Shen, M., Wang, H., and Zhao, Q. (2013). A possible mechanism of action of plant growth-promoting rhizobacteria (PGPR) strain *Bacillus pumilus* WP8 *via* regulation of soil bacterial community structure. *J. Gen. Appl. Microbiol.* 59, 267–277. doi: 10.2323/jgam.59.267

Khaksar, G., Treesubsuntorn, C., and Thiravetyan, P. (2017). Impact of endophytic colonization patterns on *Zamioculcas zamiifolia* stress response and in regulating ROS, tryptophan and IAA levels under airborne formaldehyde and formaldehyde-contaminated soil conditions. *Plant Physiol. Biochem.* 114, 1–9. doi: 10.1016/j.plaphy.2017.02.016

Krasensky, J., and Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J. Exp. Bot.* 63, 1593–1608. doi: 10.1093/jxb/err460

Kuhl, C., Tautenhahn, R., Bottcher, C., Larson, T. R., and Neumann, S. (2012). CAMERA: an integrated strategy for compound spectra extraction and annotation of liquid chromatography/mass spectrometry data sets. *Anal. Chem.* 84, 283–289. doi: 10.1021/ac202450g

Lang, D., Yu, X., Jia, X., Li, Z., and Zhang, X. (2020). Methyl jasmonate improves metabolism and growth of NaCl-stressed *Glycyrrhiza uralensis* seedlings. *Sci. Hortic.* 266:109287. doi: 10.1016/j.scienta.2020.109287

Li, Y. T., Zhang, W. J., Cui, J. J., Lang, D. Y., Li, M., Zhao, Q. P., et al. (2016). Silicon nutrition alleviates the lipid peroxidation and ion imbalance of *Glycyrrhiza uralensis* seedlings under salt stress. *Acta Physiol. Plant.* 38:96. doi: 10.1007/s11738-016-2108-8

Lin, J. C. (2003). Mechanism of action of glycyrrhizic acid in inhibition of Epstein-Barr virus replication in *vitro*. *Antivir. Res.* 59, 41–47. doi: 10.1016/s0166-3542(03)00030-5

Liu, S., Hao, H., Lu, X., Zhao, X., Wang, Y., Zhang, Y., et al. (2017). Transcriptome profiling of genes involved in induced systemic salt tolerance conferred by *Bacillus amyloliquefaciens* FZB42 in *Arabidopsis thaliana*. *Sci. Rep.* 7:10795. doi: 10.1038/s41598-017-11308-8

Liu, S., Tian, Y., Jia, M., Lu, X., Yue, L., Zhao, X., et al. (2020). Induction of Salt Tolerance in *Arabidopsis thaliana* by Volatiles From *Bacillus amyloliquefaciens* FZB42 *via* the Jasmonic Acid Signaling Pathway. *Front. Microbiol.* 11:562934. doi: 10.3389/fmicb.2020.562934

Liu, Y., Zhan, X.-J., Li, W.-D., Gao, Y., Wen, H., Chen, H.-H., et al. (2014). Copy number variations of functional genes influence contents of glycyrrhizic acid in *Glycyrrhiza uralensis*. *Acta Physiol. Plant.* 36, 1433–1440. doi: 10.1007/s11738-014-1521-0

Liu, Z., Wang, P., Lu, S., Guo, R., Gao, W., Tong, H., et al. (2020). Liquiritin, a novel inhibitor of TRPV1 and TRPA1, protects against LPS-induced acute lung injury. *Cell Calcium* 88:102198. doi: 10.1016/j.ceca.2020.102198

Loutfy, N., El-Tayeb, M. A., Hassanen, A. M., Moustafa, M. F. M., Sakuma, Y., and Inouhe, M. (2011). Changes in the water status and osmotic solute contents in response to drought and salicylic acid treatments in four different cultivars of wheat (*Triticum aestivum*). *J. Plant Res.* 125, 173–184. doi: 10.1007/s10265-011-0419-9

Lu, C., and Zhang, J. H. (1998). Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. *Aust. J. Plant Physiol.* 50, 1199–1206.

Lu, X., Liu, S. F., Yue, L., Zhao, X., Zhang, Y. B., Xie, Z. K., et al. (2018). Epsc Involved in the Encoding of Exopolysaccharides Produced by *Bacillus amyloliquefaciens* FZB42 Act to Boost the Drought Tolerance of *Arabidopsis thaliana*. *Int. J. Mol. Sci.* 19:3795. doi: 10.3390/ijms19123795

Lv, X. L., Liu, J. T., Zhu, L., Chen, X., and Li, H. J. (2020). Characteristics and controlling factors of chemical evolution of groundwater in Yuzhong basin. *J. Arid Land Resour. Environ.* 34:8. doi: 10.13448/j.cnki.jalre.2020.56

Ma, Y., Prasad, M. N., Rajkumar, M., and Freitas, H. (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnol. Adv.* 29, 248–258. doi: 10.1016/j.biotechadv.2010.12.001

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., et al. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PLoS One* 7:e48479. doi: 10.1371/journal.pone.0048479

Matsumoto, Y., Matsuura, T., Aoyagi, H., Matsuda, M., Hmwe, S. S., Date, T., et al. (2013). Antiviral activity of glycyrrhizin against hepatitis C virus in *vitro*. *PLoS One* 8:e68992. doi: 10.1371/journal.pone.0068992

Moller, I. M., Jensen, P. E., and Hansson, A. (2007). Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* 58, 459–481. doi: 10.1146/annurev.arplant.58.032806.103946

Naseem, H., and Bano, A. (2014). Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. *J. Plant Interact.* 9, 689–701. doi: 10.1080/17429145.2014.902125

Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., and Sessitsch, A. (2014). Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17. *Environ. Exp. Bot.* 97, 30–39. doi: 10.1016/j.envexpbot.2013.09.014

Pan, Y., Wu, L. J., and Yu, Z. L. (2006). Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis* Fisch). *Plant Growth Regul.* 49, 157–165. doi: 10.1007/s10725-006-9101-y

Poveda, J. (2020). Use of plant-defense hormones against pathogen-diseases of postharvest fresh produce. *Physiol. Mol. Plant Pathol.* 111:101521. doi: 10.1016/j.pmpp.2020.101521

Poveda, J. (2021). Beneficial effects of microbial volatile organic compounds (MVOCs) in plants. *Appl. Soil Ecol.* 168:104118. doi: 10.1016/j.apsoil.2021.104118

Poveda, J., and Gonzalez-Andres, F. (2021). Bacillus as a source of phytohormones for use in agriculture. *Appl. Microbiol. Biotechnol.* 105, 8629–8645. doi: 10.1007/s00253-021-11492-8

Qiu, Z. B., Liu, X., Tian, X. J., and Yue, M. (2008). Effects of CO2 laser pretreatment on drought stress resistance in wheat. *J. Photochem. Photobiol. B* 90, 17–25. doi: 10.1016/j.jphotobiol.2007.09.014

Sankar, B., Jaleel, C. A., Manivannan, P., Kishorekumar, A., Somasundaram, R., and Panneerselvam, R. (2007). Drought-induced biochemical modifications and proline metabolism in *Abelmoschus esculentus* (L.) Moench. *Acta Bot. Fenn.* 66, 43–56.

Saravanakumar, D., Kavino, M., Raguchander, T., Subbian, P., and Samiyappan, R. (2010). Plant growth promoting bacteria enhance water stress resistance in green gram plants. *Acta Physiol. Plant.* 33, 203–209. doi: 10.1007/s11738-010-0539-1

Schrofelbauer, B., Raffetseder, J., Hauner, M., Wolkerstorfer, A., Ernst, W., and Szolar, O. H. (2009). Glycyrrhizin, the main active compound in liquorice, attenuates pro-inflammatory responses by interfering with membrane-dependent receptor signalling. *Biochem. J.* 421, 473–482. doi: 10.1042/BJ20082416

Selyutina, O. Y., and Polyakov, N. E. (2019). Glycyrrhizic acid as a multifunctional drug carrier - From physicochemical properties to biomedical applications: a modern insight on the ancient drug. *Int. J. Pharm.* 559, 271–279. doi: 10.1016/j.ijpharm.2019.01.047

Shabani, L., Ehsanpour, A. A., Asghari, G., and Emami, J. (2009). Glycyrrhizin production by in *vitro* cultured Glycyrrhiza glabra elicited by methyl Jasmonate and salicylic acid. *Russ. J. Plant Physiol.* 56, 621–626. doi: 10.1134/s1021443709050069

Sharma, P., Khanna, V., and Kumari, P. (2013). Efficacy of aminocyclopropane-1-carboxylic acid (ACC)-deaminase-producing rhizobacteria in ameliorating water stress in chickpea under axenic conditions. *Afr. J. Microbiol. Res.* 7, 5749–5757. doi: 10.5897/ajmr2013.5918

Sharma, A., Wang, J., Xu, D., Tao, S., Chong, S., Yan, D., et al. (2020). Melatonin regulates the functional components of photosynthesis, antioxidant system, gene expression, and metabolic pathways to induce drought resistance in grafted *Carya cathayensis* plants. *Sci. Total Environ*. 713:136675. doi: 10.1016/j.scitotenv.2020.136675

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006). XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78, 779–787. doi: 10.1021/ac051437y

Su, X., Wu, L., Hu, M., Dong, W., Xu, M., and Zhang, P. (2017). Glycyrrhizic acid: a promising carrier material for anticancer therapy. *Biomed. Pharmacother.* 95, 670–678. doi: 10.1016/j.biopha.2017.08.123

Sziderics, A. H., Rasche, F., Trognitz, F., Sessitsch, A., and Wilhelm, E. (2007). Bacterial endophytes contribute to abiotic stress adaptation in pepper plants (*Capsicum annuum* L.). *Can. J. Microbiol*. 53, 1195–1202. doi: 10.1139/W07-082

Tian, Z., Wang, F., Zhang, W., Liu, C., and Zhao, X. (2012). Antioxidant mechanism and lipid peroxidation patterns in leaves and petals of marigold in response to drought stress. *Hortic. Environ. Biotechnol.* 53, 183–192. doi: 10.1007/s13580-012-0069-4

Tiwari, S., Lata, C., Chauhan, P. S., and Nautiyal, C. S. (2016). *Pseudomonas putida* attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum* L. during drought stress and recovery. *Plant Physiol. Biochem.* 99, 108–117. doi: 10.1016/j.plaphy.2015.11.001

Tohge, T., and Fernie, A. R. (2017). Leveraging Natural Variance towards Enhanced Understanding of Phytochemical Sunscreens. *Trends Plant Sci.* 22, 308–315. doi: 10.1016/j.tplants.2017.01.003

Uzilday, B., Turkan, I., Sekmen, A. H., Ozgur, R., and Karakaya, H. C. (2012). Comparison of ROS formation and antioxidant enzymes in Cleome gynandra (C(4)) and Cleome spinosa (C(3)) under drought stress. *Plant Sci.* 182, 59–70. doi: 10.1016/j.plantsci.2011.03.015

Vries, F., Griffiths, R., Knight, C., Nicolitch, O., and Williams, A. (2020). Harnessing rhizosphere microbiomes for drought-resilient crop production. *Science* 368, 270–274. doi: 10.1126/science.aaz5192

Wang, J., Zhang, T., Shen, X., Liu, J., Zhao, D., Sun, Y., et al. (2016). Serum metabolomics for early diagnosis of esophageal squamous cell carcinoma by UHPLC-QTOF/MS. *Metabolomics* 12, 1–10. doi: 10.1007/s11306-016-1050-5

Wei, T., Bu, L., Wu, D., Chen, H., Gao, D., Liu, H., et al. (2021). Temporal and Spatial Characteristics of Soil Nutrients under Different Artificial Vegetation Types in Yuzhong Basin, Gansu Province. *J. Henan Agric. Sci.* 50, 79–86.

Xie, Z., Chu, Y., Zhang, W., Lang, D., and Zhang, X. (2019). *Bacillus pumilus* alleviates drought stress and increases metabolite accumulation in *Glycyrrhiza uralensis* Fisch. *Environ. Exp. Bot.* 158, 99–106. doi: 10.1016/j.envexpbot.2018.11.021

Ying, Y., Hui, Z., Feng, H. E., Jia-Xing, J. I., and Long-Jiang, Y. U. (2008). The Effects of Methyl Jasmonate on the Flavonoids Synthesis in Cell Suspension Culture of *Glycyrrhiza inflata*(Leguminosae). *Acta Bot. Yunnan.* 5, 586–592.

Zhai, X., Jia, M., Chen, L., Zheng, C. J., Rahman, K., Han, T., et al. (2017). The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medical plants. *CRC Crit. Rev. Microbiol.* 43, 238–261. doi: 10.1080/1040841X.2016.1201041

Zhao, Q., Wu, Y. N., Fan, Q., Han, Q. Q., Pare, P. W., Xu, R., et al. (2016). Improved Growth and Metabolite Accumulation in *Codonopsis pilosula* (Franch.) Nannf. by Inoculation of *Bacillus amyloliquefaciens* GB03. *J. Agric. Food Chem.* 64, 8103–8108. doi: 10.1021/acs.jafc.6b03390

Zheng, Y. F., Wei, J. H., Qi, L. W., Cheng, J. M., and Peng, G. P. (2013). A green and efficient protocol for large-scale production of glycyrrhizic acid from licorice roots by combination of polyamide and macroporous resin adsorbent chromatography. *J. Sep. Sci.* 36, 809–816. doi: 10.1002/jssc.201200793

Zhu, Z., Liang, Z., and Han, R. (2009). Saikosaponin accumulation and antioxidative protection in drought-stressed *Bupleurum chinense* DC. plants. *Environ. Exp. Bot.* 66, 326–333. doi: 10.1016/j.envexpbot.2009.03.017

Zia, R., Nawaz, M. S., Siddique, M. J., Hakim, S., and Imran, A. (2021). Plant survival under drought stress: implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. *Microbiol. Res.* 242:126626. doi: 10.1016/j.micres.2020.126626

Zou, Y. H., Han, Q. X., and Guo, L. (2012). Study on Extraction of Total Flavonoids from Licorice with Ultrasonic Assistance and Antioxidant Activity. *Adv. Mater. Res.* 550-553, 1629–1632. doi: 10.4028/www.scientific.net/AMR.550-553.1629

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frontiers in Microbiology

REVIEW
published: 28 March 2022
doi: 10.3389/fmicb.2022.855473

Role of Ectomycorrhizal Symbiosis Behind the Host Plants Ameliorated Tolerance Against Heavy Metal Stress

*Eetika Chot and Mondem Sudhakara Reddy**

Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, India

Soil heavy metal (HM) pollution, which arises from natural and anthropogenic sources, is a prime threat to the environment due to its accumulative property and non-biodegradability. Ectomycorrhizal (ECM) symbiosis is highly efficient in conferring enhanced metal tolerance to their host plants, enabling their regeneration on metal-contaminated lands for bioremediation programs. Numerous reports are available regarding ECM fungal potential to colonize metal-contaminated lands and various defense mechanisms of ECM fungi and plants against HM stress separately. To utilize ECM–plant symbiosis successfully for bioremediation of metal-contaminated lands, understanding the fundamental regulatory mechanisms through which ECM symbiosis develops an enhanced metal tolerance in their host plants has prime importance. As this field is highly understudied, the present review emphasizes how plant's various defense systems and their nutrient dynamics with soil are affected by ECM fungal symbiosis under metal stress, ultimately leading to their host plants ameliorated tolerance and growth. Overall, we conclude that ECM symbiosis improves the plant growth and tolerance against metal stress by (i) preventing their roots direct exposure to toxic soil HMs, (ii) improving plant antioxidant activity and intracellular metal sequestration potential, and (iii) altering plant nutrient uptake from the soil in such a way to enhance their tolerance against metal stress. In some cases, ECM symbiosis promotes HM accumulation in metal stressed plants simultaneous to improved growth under the HM dilution effect.

Keywords: ectomycorrhizal fungi, heavy metal stress, host plants, metal tolerance, symbiosis, metal defense mechanisms

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Simone Cantamessa, Council for Agricultural and Economics Research (CREA), Italy
Hayssam M. Ali, King Saud University, Saudi Arabia

***Correspondence:**
Mondem Sudhakara Reddy
msreddy@thapar.edu

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *15 January 2022*
Accepted: *15 February 2022*
Published: *28 March 2022*

Citation:
Chot E and Reddy MS (2022) Role of Ectomycorrhizal Symbiosis Behind the Host Plants Ameliorated Tolerance Against Heavy Metal Stress. Front. Microbiol. 13:855473. doi: 10.3389/fmicb.2022.855473

INTRODUCTION

The advancements in human technologies such as industrialization and urbanization increase the soil heavy metal (HM) pollution. HMs can also leach down into groundwater or can be transferred to the successive levels of the food chain (Khosla and Reddy, 2014), causing a significant threat to living organisms and the environment (Vaclavikova et al., 2008; Nagajyoti et al., 2010). Both

Abbreviations: Al, Aluminium; Fe, Iron; Ni, Nickel; Pb, Lead; Zn, Zinc; Cd, Cadmium; H, Hydrogen; C, Carbon; Cu, Copper; Cr, Chromium; Ca, Calcium; K, Potassium; P, Phosphorus; N, Nitrogen; Na, Sodium; Mg, Magnesium; Mn, Manganese.

anthropogenic sources (such as industrialization, agriculture, sewage sludge, traffic emissions, and untreated wastewater) and natural sources (such as volcanic eruptions, rock weathering, and windblown dust) can contribute to the soil HM pollution (Srivastava et al., 2017). HMs are mainly categorized as essential or non-essential based on their role in various biological functions such as cell structure stabilization and enzyme catalysis (Bruins et al., 2000). The non-essential HMs are not required for cell metabolisms and are highly toxic for cells even in trace amounts (Haferburg and Kothe, 2007). HM toxicity in plants can reduce plant biomass, seed germination, fruit yield, nutrition content, and root and shoot length and induce chlorosis and mortality (Rai et al., 2021). The plant immune system, production of photosynthetic pigments such as carotenoids, chlorophylls, and reactive oxygen species (ROS) scavenging systems is also predominantly affected in the plants subjected to the high concentrations of HMs (Rastgoo and Alemzadeh, 2011; Ghnaya et al., 2013). HM toxicity can induce oxidative stress in plants (Khator and Shekhawat, 2020), further damaging cellular biomolecules such as protein and nucleic acids (Romero-Puertas et al., 2019). HM stress in plants leads to the impaired growth of primary root and root hairs (Broadley et al., 2007; Hayat et al., 2012; Feleafel and Mirdad, 2013), thus resulting in reduced water uptake efficiency of host plants (Rucińska-Sobkowiak, 2016). The content of HMs uptake varies with plant species and depends on environmental factors such as temperature, pH, nutrients, and moisture. For example, the accumulation of metal ions in *Beta vulgaris* is higher in summers than in winter due to the relatively high transpiration rates (Sharma et al., 2007). From plants, the metal can enter into higher trophic levels of food chains such as insects, herbivores, and humans, resulting in the ecosystem and food chains imbalance (Zhang et al., 2017). HM toxicity reduces the growth of plants in terms of dry weight and height, which can be improved by plant symbiotic association with ectomycorrhizal (ECM) fungi. The transmission of HMs from soil to plants is highly influenced by the presence of ECM fungal partners in the symbiosis with plant roots (Tang et al., 2019; Sun et al., 2021).

Ectomycorrhizal fungi are ubiquitous symbionts of plants, predominately found in Boreal and Temperate biomes. They colonize the roots of a wide range of woody plants such as *Eucalyptus*, *Pinus*, *Acacia*, and *Picea* (Smith and Read, 2010). ECM fungi play a critical role in nutrient dynamics of the terrestrial ecosystem by facilitating the mobilization of soil unavailable nutrients and water to host plants in return to their photosynthesis driven carbon (Smith and Read, 2010; Van der Heijden et al., 2015; Hodge, 2017). ECM fungi possess highly efficient and diverse defense mechanisms against HM stress, allowing them to thrive on metal-polluted lands (Khullar and Reddy, 2018). They enhance the tolerance of host plants against metal stress by various mechanisms and play a critical role in the bioremediation of metal-contaminated lands (Jourand et al., 2014; Reddy et al., 2016; Liu B. et al., 2020).

Ectomycorrhizal fungi in symbiosis develop into extramatrical mycelia growing in the soil surrounding the rhizosphere, aggregated fungal hyphae to ensheath lateral roots called as a mantle, and hyphae penetrating the apoplastic zones of cortical and epidermal cells of the host roots named as Hartig net

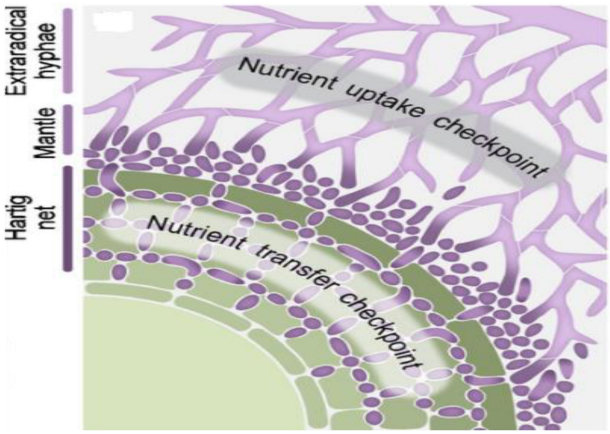


FIGURE 1 | Schematic view depicting various parts (Hartig net, mantle, and extraradical hyphae) of established Ectomycorrhizal (ECM) symbiosis with plant root cells and regulatory checkpoints of nutrient transfer that occurs between ECM fungi and plant in established symbiosis. Reproduced from the work of Garcia et al. (2015) with permission of John Wiley and Sons.

(**Figure 1**; Martin et al., 2016). The extramatrical hyphae, the potential sinks for host plant-derived carbon, act as an essential candidate for delivering the carbon to the soil. They also play a significant function in N dynamics (Wu, 2011), P uptake (Cairney, 2011), and mineral weathering (Landeweert et al., 2001; Rosling, 2009). The immense networks of ECM fungal mycelia in the soil can also link the root tips of different tree species. The labeled carbon (^{13}C) derived from tree *Picea* can transfer to the surrounding trees through ECM mycelia networks, where the exchange is found to be higher with phylogenetically related trees. The ECM communities among the phylogenetically related trees are very similar in composition (Rog et al., 2020). The diversity of ECM fungi is significantly determined by edaphic factors such as (i) soil moisture (Jarvis et al., 2015), pH, carbon, and N and P content (Veach et al., 2018); (ii) type of host (Tedersoo et al., 2014; Saitta et al., 2018), host age (Zhang et al., 2014), and host genotype (Wang et al., 2018); and (iii) environmental factors such as climatic gradients (Steidinger et al., 2020), location coordinates on mountain slopes (Wei et al., 2020), light availability (Kummel and Lostroh, 2011), and canopy and terrestrial soils (Nilsen et al., 2020).

It is well-known fact that ECM fungal symbiosis improves their host plants tolerance against metal stress (Jourand et al., 2010, 2014; Sun et al., 2021) and that ECM symbiosis application in plant regeneration on metal contaminated land has received considerable focus in global research (Wen et al., 2017; Shi et al., 2019; Dagher et al., 2020). Various reports concerning HM defense mechanisms in ECM fungi (Kalsotra et al., 2018; Khullar and Reddy, 2018; Shi et al., 2018, 2020) or plants (Hasan et al., 2017; Li et al., 2019; Schat et al., 2020) are available separately. Thus, understanding how ECM symbiosis affects and regulates the different plant defense mechanisms against metal stress has prime importance. This field is not extensively studied and reviewed but requires more focus to reinforce ECM symbiosis as

a bioremediation tool for rehabilitating metal contaminated lands with plants. The present review mainly focused on understanding how ECM symbiosis affects the various host plant defense systems against metal stress, which thus results in their enhanced metal tolerance.

ECTOMYCORRHIZAL SYMBIOSIS–DRIVEN MECHANISMS BEHIND ENHANCED METAL TOLERANCE OF THEIR HOST PLANTS

Plants have diverse molecular and physiological mechanisms to counteract the HM stress, which broadly includes HM exclusion, compartmentalization, chelation, sequestration, and mitigation of HM-induced oxidative stress (Joshi et al., 2019; Zhu et al., 2021). Various plant defense mechanisms against HM stress have been reported to be positively affected by ECM symbiosis for their improved tolerance against HM stress, which are discussed in the following (**Figure 2**).

Extracellular Secretion of Organic Acids by Ectomycorrhizal Plants Under Heavy Metal Stress

The assessment of bioavailable content forms a solid basis for predicting soil pollution and risk (Gonzalez-Chavez et al., 2004). Several environmental factors such as soil pH strongly affect the HM availability (Fässler et al., 2010; Bolan et al., 2014; Liu B. et al., 2020), with alkaline soil pH favoring metal unavailability (Fernández-Fuego et al., 2017). ECM-mediated reduction in bio-available or exchangeable soil HM content decreases metal toxicity on their host plants (Shi et al., 2019). The exudates of mycorrhizal fungi contain organic acids such as oxalic acid, succinic acid, malic acid, and formic acid, which chelate the metals and play an important role in metal detoxification (Meharg, 2003; Ray and Adholeya, 2009; Colpaert et al., 2011). The trivalent ions such as Fe^{3+} and Al^{3+} can form strong complexes with chelating agents like oxalate, citrate, and malate (Jones, 1998). The ECM fungal symbiosis induces the secretion of root exudates by host plants, which, in soil, chelates metal ions, thereby reducing their toxicity on plants (Gonzalez-Guerrero et al., 2008; Acosta et al., 2014). Many fungal species generally produce oxalic acid in large amounts (Dutton and Evans, 1996). Higher levels of oxalic acid production were reported in mycorrhizal plants of *Pinus sylvestris* in the presence of Al (Ahonen-Jonnarth et al., 2000). Contrary to this, a lower amount of oxalic acid production was recorded in mycorrhizal plants under high Ni stress, thus suggesting fungal sheath as a barrier restricting HM uptake in ECM roots, leading to reduced HM accumulation (Jourand et al., 2010). Although reduced organic acid production in metal stressed ECM plants relative to non-mycorrhizal plants has been reported, the ECM symbiosis enhances their host plant growth under metal stress (Fernández-Fuego et al., 2017). Plants have to pay high metabolic costs to survive the HM-induced oxidative stress, which could cause the reduced production of organic acids in plants under high HM stress (Iori et al., 2012). In support of this, Fernández-Fuego et al. (2017) showed that ECM-mediated enhanced HM accumulation in their metal stressed host plants reduces organic acid production compared to non-mycorrhizal control and *vice versa*. The utilization of plant high energy in expressing metallothioneins (MTs) (see section "Intracellular Heavy Metal Sequestration With Metal-Chelating Compounds") reduces the growth of Willow plants upon ECM inoculation in *Betulapubescens* (Lanfranco, 2007). Besides the variations in the conclusion of the different studies described above, Ahonen-Jonnarth et al. (2000) also reported the varying potential of ECM symbiotic *Pinus sylvestris* to produce organic acids under metal stress depending upon the different ECM fungal species, HM type, and its concentration. Other ECM fungal species–based variations suggest their different metal tolerance strategies (Ahonen-Jonnarth et al., 2000; Courbot et al., 2004), which could also be possible due to varying fungal cell wall efficiencies to bind with HMs (Johansson et al., 2008). Hence, on the basis of these contradictory results, the impact of ECM symbiosis on the secretion of plant root exudates under metal stress cannot be precisely commented on and requires more studies.

Ectomycorrhizal-Associated Plant Roots Against Heavy Metal Toxicity

The ECM fungus, *Paxillus involutus*, inoculated to *Pinus sylvestris* showed the localization of Pb aggregates in epidermis and cortex of roots but not in stem or roots endodermis. These results suggest that roots are significant plant defense operators against metal stress (Bizo et al., 2017). The number of electronegative sites present on the cell wall of fungal mycelium for HM binding and, further, the fungal potential of intracellular HM binding forms the firm basis of varying efficiency of different ECM fungi in providing HM tolerance to their host plants (Shi et al., 2019). HMs binding to the sulfhydryl and phosphate compounds intracellularly or by binding with electronegative sites on fungal cell wall confers that ECM fungi provide high tolerance toward HM stress (Bizo et al., 2017). Several past studies focused on determining the impact of ECM inoculation on HM transfer from soil to host plants had generated diverse and contradictory results from each other. (i) Some studies reported ECM fungi as a physical barrier between soil HM content and plants, thereby reducing metal accumulation in plants (Colpaert and van Assche, 1993; Baum et al., 2006; Reddy et al., 2016). Under increasing Pb stress, ECM plants accumulate comparatively fewer Pb than non-mycorrhizal plants due to the fungal mantle-mediated reduction in roots exposure with Pb, eliminating the requirement of high energy-consuming plant defense metabolism against HM in ECM plants (Szuba et al., 2020). (ii) In contrast, other studies showed an increase of HM absorption and accumulation in plants upon ECM fungal inoculation (Fernández et al., 2008; Wen et al., 2017; Tang et al., 2019). Furthermore, ECM fungi can enhance the HM accumulation in plant roots but alleviate its transport into shoots (Kozdrój et al., 2007; Mrnka et al., 2012; Yu et al., 2020). Zong et al. (2015) reported that the extent of HM uptake by ECM roots and further transfer to shoots varies according to different ECM species inoculated with the same host

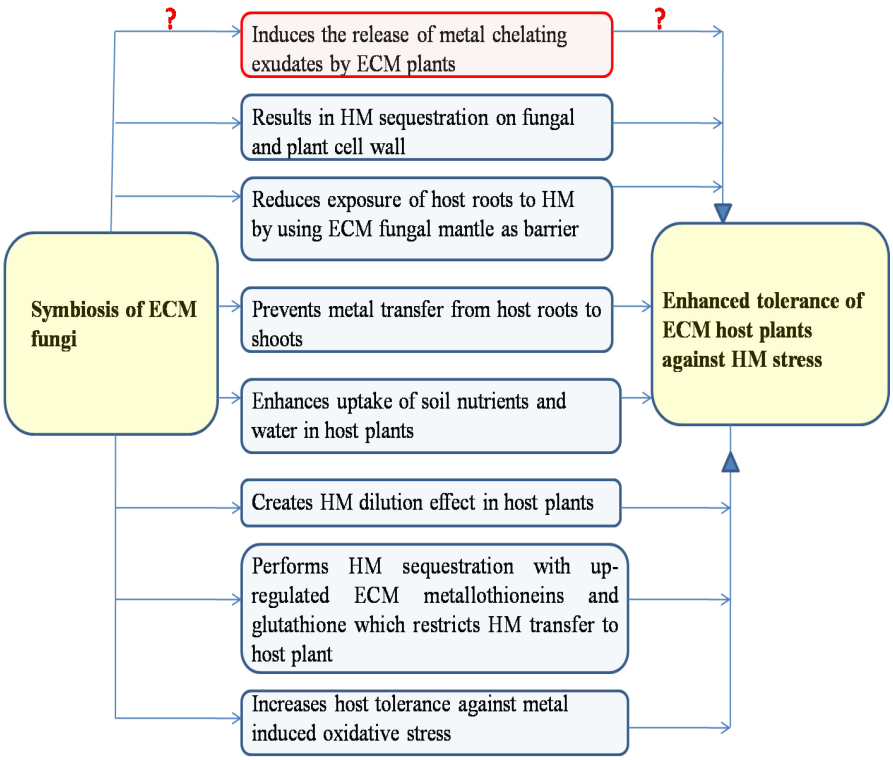


FIGURE 2 | Ectomycorrhizal (ECM) symbiosis mediated different mechanisms conferring their host plants an increased tolerance against heavy metal stress (ECM symbiosis induces or represses the release of organic acids from ECM plants remain unclear).

separately and with other host plants. The soil HMs taken up by mycorrhizal fungi are loaded from their Hartig net to host roots. Still, the restricted apoplastic pathway of root endoderm due to Casparian strips causes the higher HM accumulation in roots than plant other tissues (Luo et al., 2014). (iii) A few studies have reported that ECM fungi could both increase and inhibit HM transport to the host plants (Marschner et al., 1996; Zimmer et al., 2009). In the case of plant nutrient metals such as Zn, the ECM fungi can perform the dual function of promoting and inhibiting HM accumulation in host plants depending upon its low and high toxic concentration present in an external environment, respectively (Zhang et al., 2021). The enhanced uptake of essential and non-essential HM by ECM roots was reported under low HM concentration, whereas the metal uptake got restricted under high HM concentrations, keeping plants healthy in both cases (Bojarczuk and Kieliszewska-Rokicka, 2010; Fernández-Fuego et al., 2017). The variability, in conclusions, derived from various studies could be due to several factors such as differences in culture medium used, period of HM treatment, and mycorrhization rates (Tang et al., 2019). While increasing the HM content of their host plants under HM stress, ECM fungi also improve plant growth, which the HM dilution effect can describe. The inoculation of ECM fungi promotes the nutrients, water, and HM uptake in host plants, which results in improved plant growth and dilution of HM content in metal stressed plants, thus reducing the HM-mediated toxicity in plants (Shi et al., 2019; Tang et al., 2019). Under Cd stress, the ECM plants can couple the net Cd influx with net H^+ efflux through H^+-ATPases, causing a higher Cd influx than non-ECM plant roots. Although Cd accumulates higher in ECM plant roots and leaves than non-ECM plants, the improved C assimilation, growth, and nutrition status of ECM plants provide them an enhanced growth compared to non-ECM plants under Cd stress (**Table 1**; Ma et al., 2014). The ECM inoculation increases the host plant biomass on metal-contaminated land. It improves the phytoextraction of HM in the host plant, resulting in reduced soil content compared to non-mycorrhizal plants (Dagher et al., 2020). To enhance the phytoremediation of HM pollution, the increase in HM mobilization can also lead to HM leaching to groundwater, which thus requires careful monitoring (Bolan et al., 2014).

Intracellular Heavy Metal Sequestration With Metal-Chelating Compounds

Metallothioneins are cysteine-rich low–molecular weight proteins that strongly bind to metal ions through their thiol groups of cysteine residues, thus playing an essential role in metal sequestration and detoxification (Cobbett and Goldsbrough, 2002; Zhu et al., 2009). Besides the metal chelation, plant MTs function in scavenging accumulated ROS under oxidative stress (Xue et al., 2009: Hu et al., 2011; Ansarypour and Shahpiri, 2017; Malekzadeh and Shahpiri, 2017). The plant MT activity in metal detoxification varies with different valence states of metal (Zeng et al., 2011; Yu et al., 2019) and various plant tissues such

TABLE 1 | Ectomycorrhizal (ECM) symbiosis–based altered parameters of metal stressed host plants, which confers them a better HM tolerance than non-ECM plants [upward (↑) and downward (↓) arrows show activity increase and decrease, respectively].

ECM fungi	Host plant	Metal stress	Metal exposure period	Parameters of plants	Activity level in ECM plants relative to non-ECM plants	Conclusions	References
Pisolithus albus	*Eucalyptus globulus*	60 mg Ni kg^{-1} of substrate	12 weeks	• Root Ni content • Shoot Ni content	↓	ECM fungal as barrier in plant HM uptake from soil	Jourand et al., 2010
Paxillus involutus	*Populus* × *canescens*	0.75 mM $Pb(NO_3)_2$	6 weeks	• Pb root content • ROS burst in roots H^+ ATPases • Pb sequestration • Cytoskeleton modifications	↓ ↓ ↓ ↓	Pb biofiltering effect by ECM fungi	Szuba et al., 2020
Paxillus Involutus	*Populus* × *canescens*	50 μM $CdSO_4$	40 days	• Cd influx in plant • Plant growth and nutritional status	↑ ↑	Improved host growth under HM dilution effect	Ma et al., 2014
Pisolithus albus	*Eucalyptus globulus* and *Acacia spirorbis*	Ultramafic substrate rich in heavy metals	Pre-contaminated site	• Uptake of soil deficient essential nutrients • Barrier to toxic metals	↑ ↑	Increases growth by enhanced nutrient uptake and metal avoidance	Jourand et al., 2014
Pisolithus sp.	*Pinus thunbergii*	100–800 mg Cr kg^{-1} of soil	5 months	• Percentage content of bioavailable or exchangeable Cr in soil	↑	Relieving HM toxicity on host plants	Shi et al., 2019
Pisolithus albus	*Eucalyptus tereticornis*	150 μM Cu 40 μM Cd	4 weeks	• Metal uptake in host plant	↓	Metal immobilization in fungal extraradical mycelium by upregulated fungal metallothionein	Reddy et al., 2016
Suillus luteus	*Quercus acutissima*	0.1 mg/kg and 5 mg/kg Cd	14 days	• Catalase • MDA content • Glutathione	↑ ↓ ↑	Mitigation of metal-induced oxidative stress by ECM symbiosis	Sun et al., 2021
Paxillus ammoniavirescens	*Betulapubescens*	Multi metal stress	Pre-contaminated soil samples	• Production of antioxidants • Host plant biomass • Metal accumulation in host	↑ ↑ ↓	Enhanced tolerance to oxidative stress and barrier to HM uptake	Fernández-Fuego et al., 2017
Pisolithus tinctorius	*Eucalyptus*	1,000 μM Mn	90 days	• Mg content	↑	Metal dilution effect	Canton et al., 2016
Suillus luteus	*Pinus massoniana*	0.4 mmol L^{-1} Al	59 days	• Malondialdehyde content (indicator of oxidative stress)	↓	Reduction in HM induced oxidative stress	Liu H. et al., 2020
Sphaerosporella brunnea	*Salix miyabeana*	Decommissioned industrial land (Multimetal stress)	Pre-contaminated industrial plots	• Host plant biomass • Soil Cu, Pb, and Sn content after ∼4 years of inoculation	↑ ↓	Enhanced plant growth and phytoextraction under metal stress	Dagher et al., 2020

as roots or shoots (Yu et al., 2019). Numerous reports about the differential expression of plant MTs under metal stress are available, which functions in metal detoxification; for example, *Oryza sativa OsMT1b* and *OsMT2c* under Cr stress (Yu et al., 2019); *Oryza sativa OsMT1e* under Cd stress (Rono et al., 2021); *Physcomitrella patens PpMT2* under Cd and Cu stress (Liu Y. et al., 2020); and *Phytolacca americana PaMT3-1* under Cd stress (Zhi et al., 2020). On the other hand, in the case of ECM fungi, the differential expression of MTs: *LbMT1* and *LbMT2* in *Laccaria bicolor* under Cd and Cu stress, respectively (Reddy et al., 2014); *PaMT1* in *Pisolithus albus* under excess Cd and Cu stress (Reddy et al., 2016); HmMT3 in *Hebeloma mesophaeum* under Cd stress; and *SuiMT1* and *SuiMT2* in *Suillus indicus* under Cu stress (Shikha et al., 2019), leads to the detoxification of respective metal ions. The increased expression of symbiotic ECM MTs under metal stress causes the immobilization of metal ions in their extraradical mycelium and reduces the metal content in ECM roots compared to non-ECM roots (Reddy et al., 2016). Although not many studies are available regarding ECM effects on MT content of metal stressed host plants, past reports have determined the enhanced expression of host plant MTs upon their inoculation with arbuscular mycorrhizal (AM) fungi as compared to non-mycorrhizal plants under metal stress, ultimately leading to the host ameliorated metal tolerance (Cicatelli et al., 2010; Shabani and Sabzalian, 2016).

Glutathiones are well known to prevent oxidative stress and xenobiotics detoxification in cells (Sheehan et al., 2001). The upregulation of ECM fungal genes associated with glutathione biosynthesis under metal stress causes enhanced complexing of HM ions with glutathione and further sequestration of HM-glutathione complexes in their vacuoles. This process limits the HM transfer to their host plants, reducing HM toxicity (Khullar and Reddy, 2020). Similarly, the enhanced glutathione in ECM plants has been reported under metal stress compared to non-ECM plants, as shown in **Table 1**.

Host Plant Tolerance Against Heavy Metal Stress-Induced Oxidative Burst

The excess concentration of plant nutrient Zn can lead to increased production of ROS, which, if it breaks its balance with ROS destruction, results in oxidative stress (Bartoli et al., 2013; Mohammadhasani et al., 2017). With evolution, plants acquire native defense mechanisms against oxidative stress, including antioxidant enzymes such as superoxide, catalase, glutathione peroxidase, ascorbate peroxidase, and guaiacol peroxidase (Wu et al., 2014). Plants under HM stress can increase their antioxidant activities as a defense mechanism, and mycorrhizal symbiosis further enhances this activity (Fernández-Fuego et al., 2017; Mohammadhasani et al., 2017). Catalase, peroxidase, ascorbate peroxidase, and superoxide dismutase are the antioxidants that play an essential role in alleviating the HM-induced oxidative stress in plants. The superoxide dismutase mediates the ROS species conversion to H_2O_2, whereas peroxidase and catalase mediate the H_2O_2 conversion to H_2O (Li et al., 2015). The activities of these antioxidants in ECM fungi rise with increasing HM concentration upto certain levels, after which their activities decreases (Dachuan and Jinyu, 2021). The ECM symbiosis mediates the increase in antioxidant activities in their host plants against HM stress as shown by enhanced catalase, glutathione activity, and reduced malondialdehyde (MDA) content in metal stressed *Quercus acutissima* roots inoculated with *Suillus luteus* compared to non-ECM plants (**Table 1**; Sun et al., 2021). The lower MDA content corresponds to the stronger antioxidant activities of an organism. The reduction in MDA content of host *Pinus massoniana* under Al stress has also been reported upon their inoculation with *Suillus luteus* compared to non-ECM plants (Liu H. et al., 2020). It thus suggests the ECM-mediated improved antioxidant machinery of the host plant as one of the essential mechanisms for their enhanced HM tolerance.

Alterations in the Nutrient Status of the Host Plants

The resource allocation between ECM fungal and plant partners strongly regulates the maintenance of long-term symbiotic association among both the partners. This long-term cooperation of nutrient transfer is maintained by transcriptional and translational regulations of transporter systems at regulatory checkpoints as shown in **Figure 1** (Garcia et al., 2015). The presence of ECM symbiosis alters the transfer of nutrients from the soil to their host plants under HM stress (**Table 2**). The plant minerals such as Fe, Ca, N, P, and K content in roots and shoots get improved by ECM symbiosis in Pb-, Zn-, and Cd-contaminated soils (Hachani et al., 2020). The mycorrhiza-mediated N and P uptake in plants provides an ameliorated tolerance against oxidative stress (Begum et al., 2019). The P content is well correlated with the Cd concentration. The enhanced P content increases Cd accumulation in plants roots and significantly reduces Cd translocation upward in plants (Kong et al., 2020). At low Cd stress, the P content decreased in leaves and got doubled in the roots of ECM fungal plants. At high Cd stress, P content doubled in leaves and reduced in roots significantly compared with non-mycorrhizal plants under Cd stress, suggesting the vital role of ECM fungi in Cd retention of their host plants by regulating their P content (Sun et al., 2021). N and P enrichment can relieve Cd-induced oxidative stress in plants, possibly by increasing proline content (Kong et al., 2020). Proline acts as a potent non-enzymatic antioxidant and metal chelating agent (Sharma and Dietz, 2006).

Further, the Na and P enrichment highly promotes Cd uptake and its sequestration with proline in plant roots which further decreases Cd transfer from sources to stem and helps to enhance the phytoextraction potential of the plant (Kong et al., 2020). On the other hand, the high external concentration of Zn in ECM plants negatively correlates with Fe accumulation in ECM roots. Ca uptake was enhanced under initial low external Zn and reduced under high Zn stress (Zhang et al., 2021). The antagonism between external Zn concentrations and Fe accumulation could be due to Fe displacement by Zn on ligand binding sites of metal transporters or siderophores (Suzuki et al., 2008; Hussein and Joo, 2019). Similarly, Langer et al. (2012)

TABLE 2 | Alteration in the nutrition status of host plants by Ectomycorrhizal (ECM) symbiosis under metal stress.

ECM fungi	Host plant	Metal stress	Metal exposure period	Effects on ECM leaves	Effects on ECM roots	References
Suillus luteus	*Quercus acutissima*	Low Cd, 0.1 mg/kg	14 days	Ca, Mg, P, and K increased and Zn decreased	Ca, Mg, Zn and P increased; K decreased	Sun et al., 2021
Suillus luteus	*Quercus acutissima*	High Cd, 5 mg/kg	14 days	Ca, Mg, P, K, and Zn increased	K and P decreased	Sun et al., 2021
Rhizopogon sp.	*Pinus halepensis*	High Pb, Zn, and Cd stress	12 months	N, P, K, Ca, and Fe enhanced	N, P, K, Ca, and Fe Enhanced	Hachani et al., 2020
Suillus luteus	*Pinus sylvestris*	Zn, 0–1 mM	4 weeks	–	• Increase in Zn stress negatively relates to Fe content • Low and high Zn stress enhances and reduced Ca accumulation, respectively	Zhang et al., 2021
Pisolithus sp., *Laccaria* sp., and *Cenococcum* sp.	*Pinus sylvestris*	Zn-, Cd-, and Pb-contaminated tailing pond soil	Pre-contaminated soil samples	Mg and Ca increased, whereas Fe content reduced	Pi, Mg, Ca, and Fe accumulation increased	Liu B. et al., 2020
Pisolithus albus	*Eucalyptus globulus* *Acacia spirorbis*	Ultramafic substrate	Pre-contaminated site	• N, P, K, and Ca increased • Mg content decreased	-	Jourand et al., 2014

showed that the Fe uptake by ECM plants reduces under Zn stress compared to non-mycorrhizal plants, suggesting the competitive uptake among Fe, Zn, and Cd based on congruent ionic radii (Marschner, 1995). Among HM defense systems of ECM plants, the ion dilution effect is the mechanism under which the uptake and accumulation of nutrient minerals such as P and Mg got enhanced in plants to counteract the toxicity of harmful HMs to create HM dilution effects (Malcová et al., 2002; Canton et al., 2016). The increase in Ca and Mg content in ECM plants under HM stress suggests their role in improved plant biomass and further HM dilution effect in ECM plants as their tolerance mechanism (Sun et al., 2021).

On the other hand, *P. albus* symbiosis reduces Mg uptake in their host plants growing on ultramafic substrate rich in HMs to check excess Mg transfer. In contrast, host uptake for ultramafic soil deficient in N, P, K, and Ca increased, thus improving plant growth and creating a barrier for HMs present in excess (Jourand et al., 2014). The enhanced content of P, Mg, Ca, and Fe in *Pinus sylvestris* roots inoculated with different ECM fungal species under HM stress was reported compared to non-mycorrhizal plants. In shoots, reduced Fe content and increased Mg and Ca content were observed upon ECM symbiosis compared to non-mycorrhizal plants under metal stress (Liu B. et al., 2020). The varying uptake capacity of ECM plants for different nutrients could be due to the other kind of ECM fungi and their host plant species used and their tolerance potential for excess nutrients (Teotia et al., 2017).

At present, the restoration of abandoned mining lands is highly required to improve soil quality, microorganisms and plants growth for ecological rehabilitation (Wani et al., 2017). The use of traditional physical and chemical technologies for restoration programs can result in secondary pollution and high cost (Ayangbenro and Babalola, 2017).

The different species of ECM fungi affect host tolerance efficiency against metal stress differently (Sousa et al., 2012), thereby highlighting the need to optimize the best ECM fungal partner for the host before large-scale afforestation programs. In the reforestation of mine wastelands, the ECM infection rates in ECM fungal inoculated plant seedlings reduce after 6 months of their growth in mining lands (Chappelka et al., 1991; Hartley-Whitaker et al., 2000; Huang et al., 2012; Zong et al., 2015). The decline in ECM fungal colonization rate on land with high HM concentrations is associated with poor soil properties such as low organic matter, soil texture (Guo et al., 2007), macronutrients deficiency, and high processed residues content (Huang et al., 2012). The different species combination of ECM fungi and host plants gives different mycorrhization rates (Zong et al., 2015). On Pb-, Zn-, and Cd-polluted land, ECM fungal community richness and diversity have been correlated with soil N content but not with Pb, Zn, and Cd concentrations. For example, the dominant ECM fungal species obtained on N deficient tailing are mostly found on N-poor soils (Huang et al., 2012). By contrast, the diversity of ECM fungal community associated with white oak and Mason pine on Mn mine site correlated with less toxic Mn concentration (Huang et al., 2014). The Cd hyperaccumulator ecotypes of *Sedum alfredii* have more Cd accumulation in roots than non-hyperaccumulators (Sun et al., 2013). Among

the different strains of the same ECM fungal species, the HM tolerant ecotypes of species are reported to be more potent for enhancing metal tolerance and growth in their host plants. Hence, this depicts ECM fungal selection's importance for the phytoremediation programs of HM contaminated lands (Szuba et al., 2017). It is necessary to focus future studies on determining the ECM-mediated antagonist or synergistic effects of external HM stress with the dynamics of different nutrients in host plants, which may participate in plant response to HM stress. As different species of ECM fungi affect plant HM defense systems differently, further studies are required to explore the factors playing a role behind this. Furthermore, plant phytohormones such as auxin, ethylene, and abscisic acid are widely reported for their important roles in plant defense response against HM stress conditions (Emamverdian et al., 2021). Inoculating AM fungi to *Robinia pseudoacacia* seedlings under As stress enhanced the Indole-3-acetic acid and abscisic acid content, decreasing the zeatin riboside gibberellic acid concentrations and altering the ratios of various phytohormones in the host plant. These results suggest that the mycorrhiza-mediated phytohormones are essential factors behind host-alleviated metal toxicity (Zhang et al., 2020). Although, exogenously applying phytohormones and manipulating the plant's endogenous level of phytohormones are reported as promising ways to enhance plants tolerance against metal stress (Saini et al., 2021). Several past studies reported that the ECM symbiosis significantly alters the hormonal status of their host plants (Felten et al., 2009; Basso et al., 2020). It would be interesting to explore the impact of ECM inoculation on hormones derived metal tolerance in host plants, which is not yet extensively reported. Besides the few lighter elements possessing naturally occurring radioisotopes, all elements with an atomic number more than 83 are considered radioactive (Thompson et al., 1949). To these natural radionuclides, several anthropogenic activities, such as nuclear industries, mining, and nuclear weapon trials, further add up radionuclides concentration in environment (Hain et al., 2020). The bioavailability and mobility of radionuclides significantly influence their deleterious impacts on environment and human health (Lopez-Fernandez et al., 2021). The AM symbiosis significantly affects the transfer of soil ribonuclides to their host plants. When inoculated with the same host plant, further ribonuclease retention in roots or shoot transfer varies depending upon different AM fungal species. For bioremediation purpose, the utilization of mycorrhizal symbiosis in enhancing phytoaccumulation of ribonuclides requires further studies (Rosas-Moreno et al., 2021). Similarly, the impact of ECM symbiosis over radionuclides accumulation in their host plants is reported in a few studies but remains unclear and needs focus studies (Ogo et al., 2018).

CONCLUSION

The ECM symbiosis improves the HM tolerance and growth of their metal stressed host plants through several mechanisms, which thus help in regenerating the metal-contaminated lands with plants. First, the ECM fungi can either act as a physical barrier between soil HM and plant roots or enhance HM accumulation in host plants depending on several factors such as HM type, external concentrations, and plant and fungal species. ECM fungi can also prevent HM transfer to plant roots by sequestering them on fungal cell walls or intracellularly with MTs and glutathione. ECM symbiosis ameliorates plant growth and their tolerance to oxidative stress under HM stress. The ECM fungi can change the nutrition dynamic of plants with soil in such a way to create HM dilution effects and to prevent HM transfer from roots to shoots. The role of ECM promoted or inhibited release plant root exudates in HM stress tolerance needs more studies for clarification. On the basis of the collected evidences, the ECM symbiosis proves to be beneficial for promoting plant growth on metal-contaminated lands and enhancing soil HM phytoextraction in host plants to reduce soil HM content. This field requires more extensive studies to understand the nutrient dynamics of ECM plants under metal stress and how it affects their tolerance.

AUTHOR CONTRIBUTIONS

EC conducted the literature review and designed and wrote the manuscript. MR supervised, corrected, improved, and accepted the final version of the manuscript. Both authors contributed to the article and approved the submitted version.

REFERENCES

Acosta, J. A., Faz, A., Kalbitz, K., Jansen, B., and Martínez-Martínez, S. (2014). Partitioning of heavy metals over different chemical fraction in street dust of Murcia (Spain) as a basis for risk assessment. *J. Geochem. Explor.* 144, 298–305. doi: 10.1016/j.gexplo.2014.02.004

Ahonen-Jonnarth, U. L. L. A., Van Hees, P. A., Lundström, U. S., and Finlay, R. D. (2000). Organic acids produced by mycorrhizal Pinus sylvestris exposed to elevated aluminium and heavy metal concentrations. *New Phytol.* 146, 557–567. doi: 10.1046/j.1469-8137.2000.00653.x

Ansarypour, Z., and Shahpiri, A. (2017). Heterologous expression of a rice metallothionein isoform (OsMTI-1b) in Saccharomyces cerevisiae enhances cadmium, hydrogen peroxide and ethanol tolerance. *Braz. J. Microbiol.* 48, 537–543. doi: 10.1016/j.bjm.2016.10.024

Ayangbenro, A. S., and Babalola, O. O. (2017). A new strategy for heavy metal polluted environments: a review of microbial biosorbents. *Int. J. Environ. Res. Public Health* 14:94. doi: 10.3390/ijerph14010094

Bartoli, C. G., Casalongué, C. A., Simontacchi, M., Marquez-Garcia, B., and Foyer, C. H. (2013). Interactions between hormone and redox signalling pathways in the control of growth and cross tolerance to stress. *Environ. Exp. Bot.* 94, 73–88. doi: 10.1016/j.envexpbot.2012.05.003

Basso, V., Kohler, A., Miyauchi, S., Singan, V., Guinet, F., Šimura, J., et al. (2020). An ectomycorrhizal fungus alters sensitivity to jasmonate, salicylate, gibberellin, and ethylene in host roots. *Plant Cell Environ.* 43, 1047–1068. doi: 10.1111/pce.13702

Baum, C., Hrynkiewicz, K., Leinweber, P., and Meißner, R. (2006). Heavy-metal mobilization and uptake by mycorrhizal and nonmycorrhizal willows (Salix× dasyclados). *J. Plant Nutr. Soil Sci.* 169, 516–522. doi: 10.1002/jpln.200521925

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10:1068. doi: 10.3389/fpls.2019.01068

Bizo, M. L., Nietzsche, S., Mansfeld, U., Langenhorst, F., Majzlan, J., Göttlicher, J., et al. (2017). Response to lead pollution: mycorrhizal Pinus sylvestris forms the biomineral pyromorphite in roots and needles. *Environ. Sci. Pollut. Res. Int.* 24, 14455–14462. doi: 10.1007/s11356-017-9020-7

Bojarczuk, K., and Kieliszewska-Rokicka, B. (2010). Effect of ectomycorrhiza on Cu and Pb accumulation in leaves and roots of silver birch (Betula pendula Roth.) seedlings grown in metal-contaminated soil. *Water Air Soil Pollut.* 207, 227–240. doi: 10.1007/s11270-009-0131-8

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., et al. (2014). Remediation of heavy metal (loid) s contaminated soils–to mobilize or to immobilize? *J. Hazard. Mater.* 266, 141–166. doi: 10.1016/j.jhazmat.2013.12.018

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., and Lux, A. (2007). Zinc in plants. *New Phytol.* 173, 677–702. doi: 10.1111/j.1469-8137.2007.01996.x

Bruins, M. R., Kapil, S., and Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicol. Environ. Saf.* 45, 198–207. doi: 10.1006/eesa.1999.1860

Cairney, J. W. (2011). Ectomycorrhizal fungi: the symbiotic route to the root for phosphorus in forest soils. *Plant Soil* 344, 51–71. doi: 10.1007/s11104-011-0731-0

Canton, G. C., Bertolazi, A. A., Cogo, A. J., Eutrópio, F. J., Melo, J., de Souza, S. B., et al. (2016). Biochemical and ecophysiological responses to manganese stress by ectomycorrhizal fungus Pisolithus tinctorius and in association with Eucalyptus grandis. *Mycorrhiza* 26, 475–487. doi: 10.1007/s00572-016-0686-3

Chappelka, A. H., Kush, J. S., Runion, G. B., Meier, S., and Kelley, W. D. (1991). Effects of soil-applied lead on seedling growth and ectomycorrhizal colonization of loblolly pine. *Environ. Pollut.* 72, 307–316. doi: 10.1016/0269-7491(91)90004-G

Cicatelli, A., Lingua, G., Todeschini, V., Biondi, S., Torrigiani, P., and Castiglione, S. (2010). Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and polyamine biosynthetic gene expression. *Ann. Bot.* 106, 791–802. doi: 10.1093/aob/mcq170

Cobbett, C., and Goldsbrough, P. (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* 53, 159–182. doi: 10.1146/annurev.arplant.53.100301.135154

Colpaert, J. V., and van Assche, J. A. (1993). The effects of cadmium on ectomycorrhizal Pinus sylvestris L. *New Phytol.* 123, 325–333. doi: 10.1111/j.1469-8137.1993.tb03742.x

Colpaert, J. V., Wevers, J. H., Krznaric, E., and Adriaensen, K. (2011). How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. *Ann. Sci.* 68, 17–24. doi: 10.1007/s13595-010-0003-9

Courbot, M., Diez, L., Ruotolo, R., Chalot, M., and Leroy, P. (2004). Cadmium-responsive thiols in the ectomycorrhizal fungus Paxillus involutus. *Appl. Environ. Microbiol.* 70, 7413–7417. doi: 10.1128/AEM.70.12.7413-7417.2004

Dachuan, Y., and Jinyu, Q. (2021). The physiological response of Ectomycorrhizal fungus Lepista sordida to Cd and Cu stress. *PeerJ* 9:e11115. doi: 10.7717/peerj.11115

Dagher, D. J., Pitre, F. E., and Hijri, M. (2020). Ectomycorrhizal fungal inoculation of sphaerosporella brunnea significantly increased stem biomass of Salix miyabeana and decreased lead, tin, and zinc, soil concentrations during the phytoremediation of an industrial landfill. *J. Fungi.* 6:87. doi: 10.3390/jof6020087

Dutton, M. V., and Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* 42, 881–895. doi: 10.1139/m96-114

Emamverdian, A., Ding, Y., Mokhberdoran, F., and Ahmad, Z. (2021). Mechanisms of selected plant hormones under heavy metal stress. *Pol. J. Environ. Stud.* 30, 497–508. doi: 10.15244/pjoes/122809

Fässler, E., Robinson, B. H., Stauffer, W., Gupta, S. K., Papritz, A., and Schulin, R. (2010). Phytomanagement of metal-contaminated agricultural land using sunflower, maize and tobacco. *Agric. Ecosyst. Environ.* 136, 49–58. doi: 10.1016/j.agee.2009.11.007

Feleafel, M. N., and Mirdad, Z. M. (2013). Hazard and effects of pollution by lead on vegetable crops. *J. Agric. Environ. Ethics.* 26, 547–567. doi: 10.1007/s10806-012-9403-1

Felten, J., Kohler, A., Morin, E., Bhalerao, R. P., Palme, K., Martin, F., et al. (2009). The ectomycorrhizal fungus Laccaria bicolor stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signaling. *Plant Physiol.* 151, 1991–2005. doi: 10.1104/pp.109.147231

Fernández, R., Bertrand, A., Casares, A., García, R., González, A., and Tamés, R. S. (2008). Cadmium accumulation and its effect on the in vitro growth of woody fleabane and mycorrhized white birch. *Environ. Pollut.* 152, 522–529. doi: 10.1016/j.envpol.2007.07.011

Fernández-Fuego, D., Bertrand, A., and González, A. (2017). Metal accumulation and detoxification mechanisms in mycorrhizal Betula pubescens. *Environ. Pollut.* 231, 1153–1162. doi: 10.1016/j.envpol.2017.07.072

Garcia, K., Delaux, P. M., Cope, K. R., and Ané, J. M. (2015). Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. *New Phytol.* 208, 79–87. doi: 10.1111/nph.13423

Ghnaya, T., Zaier, H., Baioui, R., Sghaier, S., Lucchini, G., Sacchi, G. A., et al. (2013). Implication of organic acids in the long-distance transport and the accumulation of lead in Sesuvium portulacastrum and Brassica juncea. *Chemosphere* 90, 1449–1454. doi: 10.1016/j.chemosphere.2012.08.061

Gonzalez-Chavez, M. C., Carrillo-Gonzalez, R., Wright, S. F., and Nichols, K. A. (2004). The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. *Environ. Pollut.* 130, 317–323. doi: 10.1016/j.envpol.2004.01.004

Gonzalez-Guerrero, M., Melville, L. H., Ferrol, N., Lott, J. N., Azcon-Aguilar, C., and Peterson, R. L. (2008). Ultrastructural localization of heavy metals in the extraradical mycelium and spores of the arbuscular mycorrhizal fungus Glomus intraradices. *Can. J. Microbiol.* 54, 103–110. doi: 10.1139/W07-119

Guo, J. P., Wu, F. C., Xie, S. R., Yao, L. L., and Xie, Y. H. (2007). Environmental conditions and exploitation of lead–zinc tailings in Linxiang County, Hunan Province. *Chin. J. Soil Sci.* 38, 553–557.

Hachani, C., Lamhamedi, M. S., Cameselle, C., Gouveia, S., Zine El Abidine, A., Khasa, D. P., et al. (2020). Effects of ectomycorrhizal fungi and heavy metals (Pb, Zn, and Cd) on growth and mineral nutrition of Pinus halepensis seedlings in North Africa. *Microorganisms* 8:2033. doi: 10.3390/microorganisms8122033

Haferburg, G., and Kothe, E. (2007). Microbes and metals: interactions in the environment. *J. Basic Microbiol.* 47, 453–467. doi: 10.1002/jobm.200700275

Hain, K., Steier, P., Froehlich, M. B., Golser, R., Hou, X., Lachner, J., et al. (2020). 233U/236U signature allows to distinguish environmental emissions of civil nuclear industry from weapons fallout. *Nat. Commun.* 11, 1–11. doi: 10.1038/s41467-020-15008-2

Hartley-Whitaker, J., Cairney, J. W., and Meharg, A. A. (2000). Sensitivity to Cd or Zn of host and symbiont of ectomycorrhizal Pinus sylvestris L.(Scots pine) seedlings. *Plant Soil* 218, 31–42. doi: 10.1023/A:1014989422241

Hasan, M., Cheng, Y., Kanwar, M. K., Chu, X. Y., Ahammed, G. J., and Qi, Z. Y. (2017). Responses of plant proteins to heavy metal stress—a review. *Front. Plant Sci.* 8:1492. doi: 10.3389/fpls.2017.01492

Hayat, S., Khalique, G., Irfan, M., Wani, A. S., Tripathi, B. N., and Ahmad, A. (2012). Physiological changes induced by chromium stress in plants: an overview. *Protoplasma* 249, 599–611. doi: 10.1007/s00709-011-0331-0

Hodge, A. (2017). *Accessibility of inorganic and organic nutrients for mycorrhizas in Mycorrhizal mediation of soil*. Amsterdam: Elsevier, 129–148.

Hu, L., Liang, W., Yin, C., Cui, X., Zong, J., Wang, X., et al. (2011). Rice MADS3 regulates ROS homeostasis during late anther development. *Plant Cell* 23, 515–533. doi: 10.1105/tpc.110.074369

Huang, J., Nara, K., Lian, C., Zong, K., Peng, K., Xue, S., et al. (2012). Ectomycorrhizal fungal communities associated with Masson pine (Pinus massoniana Lamb.) in Pb–Zn mine sites of central south China. *Mycorrhiza* 22, 589–602. doi: 10.1007/s00572-012-0436-0

Huang, J., Nara, K., Zong, K., Wang, J., Xue, S., Peng, K., et al. (2014). Ectomycorrhizal fungal communities associated with Masson pine (Pinus massoniana) and white oak (Quercus fabri) in a manganese mining region in Hunan Province. *China Fungal Ecol.* 9, 1–10. doi: 10.1016/j.funeco.2014.01.001

Hussein, K. A., and Joo, J. H. (2019). Zinc ions affect siderophore production by fungi isolated from the Panax ginseng rhizosphere. *J. Microbiol. Biotechnol.* 29, 105–113. doi: 10.4014/jmb.1712.12026

Iori, V., Pietrini, F., Massacci, A., and Zacchini, M. (2012). Induction of metal binding compounds and antioxidative defence in callus cultures of two black poplar (P. nigra) clones with different tolerance to cadmium. *Plant Cell Tissue Organ. Cult.* 108, 17–26. doi: 10.1007/s11240-011-0006-8

Jarvis, S. G., Woodward, S., and Taylor, A. F. (2015). Strong altitudinal partitioning in the distributions of ectomycorrhizal fungi along a short (300 m) elevation gradient. *New Phytol.* 206, 1145–1155. doi: 10.1111/nph.13315

Johansson, E. M., Fransson, P. M., Finlay, R. D., and van Hees, P. A. (2008). Quantitative analysis of root and ectomycorrhizal exudates as a response to Pb, Cd and as stress. *Plant Soil* 313, 39–54. doi: 10.1007/s11104-008-9678-1

Jones, D. L. (1998). Organic acids in the rhizosphere–a critical review. *Plant Soil* 205, 25–44. doi: 10.1023/A:1004356007312

Joshi, R., Dkhar, J., Singla-Pareek, S. L., and Pareek, A. (2019). *Molecular mechanism and signaling response of heavy metal stress tolerance in plants in Plant-metal interactions*. Cham: Springer, 29–47. doi: 10.1007/978-3-030-20732-8_2

Jourand, P., Ducousso, M., Reid, R., Majorel, C., Richert, C., Riss, J., et al. (2010). Nickel-tolerant ectomycorrhizal Pisolithus albus ultramafic ecotype isolated from nickel mines in New Caledonia strongly enhance growth of the host plant Eucalyptus globulus at toxic nickel concentrations. *Tree Physiol.* 30, 1311–1319. doi: 10.1093/treephys/tpq070

Jourand, P., Hannibal, L., Majorel, C., Mengant, S., and Ducousso, M. (2014). Ectomycorrhizal Pisolithus albus inoculation of Acacia spirorbis and Eucalyptus globulus grown in ultramafic topsoil enhances plant growth and mineral nutrition while limits metal uptake. *J. Plant Physiol.* 171, 164–172. doi: 10.1016/j.jplph.2013.10.011

Kalsotra, T., Khullar, S., Agnihotri, R., and Reddy, M. S. (2018). Metal induction of two metallothionein genes in the ectomycorrhizal fungus Suillus himalayensis and their role in metal tolerance. *Microbiology* 164, 868–876. doi: 10.1099/mic.0.000666

Khator, K., and Shekhawat, G. S. (2020). Cd-and Cu-induced phytotoxicity on 2–3 leaf stage of Cyamopsis tetragonoloba and its regulation by nitrate reductase and ROS quenching enzyme. *Acta Physiol.* 42, 1–14. doi: 10.1007/s11738-020-03105-0

Khosla, B., and Reddy, M. S. (2014). Mycorrhizal fungi in extreme environments and their impact on plant growth. *Kavaka* 42, 123–130.

Khullar, S., and Reddy, M. S. (2018). Ectomycorrhizal fungi and its role in metal homeostasis through metallothionein and glutathione mechanisms. *Curr. Biotechnol.* 7, 231–241. doi: 10.2174/2211550105666160531145544

Khullar, S., and Reddy, M. S. (2020). Arsenic toxicity and its mitigation in ectomycorrhizal fungus Hebeloma cylindrosporum through glutathione biosynthesis. *Chemosphere* 240:124914. doi: 10.1016/j.chemosphere.2019.124914

Kong, X., Zhao, Y., Tian, K., He, X., Jia, Y., He, Z., et al. (2020). Insight into nitrogen and phosphorus enrichment on cadmium phytoextraction of hydroponically grown Salix matsudana Koidz cuttings. *Environ. Sci. Pollut. Res. Int.* 27, 8406–8417. doi: 10.1007/s11356-019-07499-4

Kozdrój, J., Piotrowska-Seget, Z., and Krupa, P. (2007). Mycorrhizal fungi and ectomycorrhiza associated bacteria isolated from an industrial desert soil protect pine seedlings against Cd (II) impact. *Ecotoxicology* 16, 449–456. doi: 10.1007/s10646-007-0149-x

Kummel, M., and Lostroh, P. (2011). Altering light availability to the plant host determined the identity of the dominant ectomycorrhizal fungal partners and mediated mycorrhizal effects on plant growth. *Botany* 89, 439–450. doi: 10.1139/b11-033

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T. W., and van Breemen, N. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol. Evol.* 16, 248–254. doi: 10.1016/S0169-5347(01)02122-X

Lanfranco, L. (2007). The fine-tuning of heavy metals in mycorrhizal fungi. *New Phytol.* 174, 3–6. doi: 10.1111/j.1469-8137.2007.02029.x

Langer, I., Santner, J., Krpata, D., Fitz, W. J., Wenzel, W. W., and Schweiger, P. F. (2012). Ectomycorrhizal impact on Zn accumulation of Populus tremula L. grown in metalliferous soil with increasing levels of Zn concentration. *Plant Soil* 355, 283–297. doi: 10.1007/s11104-011-1098-y

Li, D. Q., Chen, G. K., Zheng, H., Li, H. S., and Li, X. B. (2015). Effects of cadmium on growth and antioxidant enzyme activities of two kidney bean (Phaseolus vulgaris L.) cultivars. *J. Agro-Environ. Sci.* 34, 221–226.

Li, J., Jia, Y., Dong, R., Huang, R., Liu, P., Li, X., et al. (2019). Advances in the mechanisms of plant tolerance to manganese toxicity. *Int. J. Mol. Sci.* 20:5096. doi: 10.3390/ijms20205096

Liu, B., Wang, S., Wang, J., Zhang, X., Shen, Z., Shi, L., et al. (2020). The great potential for phytoremediation of abandoned tailings pond using ectomycorrhizal Pinus sylvestris. *Sci. Total Environ.* 719:137475. doi: 10.1016/j.scitotenv.2020.137475

Liu, H., Chen, H., Ding, G., Li, K., and Ren, Q. (2020). Identification of candidate genes conferring tolerance to aluminum stress in Pinus massoniana inoculated with ectomycorrhizal fungus. *BMC Plant Biol.* 20:1–13. doi: 10.1186/s12870-020-02719-3

Liu, Y., Kang, T., Cheng, J. S., Yi, Y. J., Han, J. J., Cheng, H. L., et al. (2020). Heterologous expression of the metallothionein PpMT2 gene from Physcomitrella patens confers enhanced tolerance to heavy metal stress on transgenic Arabidopsis plants. *Plant Growth Regul.* 90, 63–72. doi: 10.1007/s10725-019-00558-3

Lopez-Fernandez, M., Jroundi, F., Ruiz-Fresneda, M. A., and Merroun, M. L. (2021). Microbial interaction with and tolerance of radionuclides: underlying mechanisms and biotechnological applications. *Microb. Biotechnol.* 14, 810–828. doi: 10.1111/1751-7915.13718

Luo, Z. B., Wu, C., Zhang, C., Li, H., Lipka, U., and Polle, A. (2014). The role of ectomycorrhizas in heavy metal stress tolerance of host plants. *Environ. Exp. Bot.* 108, 47–62. doi: 10.1016/j.envexpbot.2013.10.018

Ma, Y., He, J., Ma, C., Luo, J., Li, H., Liu, T., et al. (2014). Ectomycorrhizas with Paxillus involutus enhance cadmium uptake and tolerance in Populus× canescens. *Plant Cell Environ.* 37, 627–642. doi: 10.1111/pce.12183

Malcová, R., Gryndler, M., and Vosátka, M. (2002). Magnesium ions alleviate the negative effect of manganese on Glomus claroideum BEG23. *Mycorrhiza* 12, 125–129. doi: 10.1007/s00572-002-0161-1

Malekzadeh, R., and Shahpiri, A. (2017). Independent metal-thiolate cluster formation in C-terminal Cys-rich region of a rice type 1 metallothionein isoform. *Int. J. Biol. Macromol.* 96, 436–441. doi: 10.1016/j.ijbiomac.2016.12.047

Marschner, H. (1995). *Mineral nutrition of higher plants 2nd edition*. Amsterdam: Elsevier.

Marschner, P., Godbold, D. L., and Jentschke, G. (1996). Dynamics of lead accumulation in mycorrhizal and non-mycorrhizal Norway spruce (Picea abies (L.) Karst.). *Plant Soil* 178, 239–245. doi: 10.1007/BF00011589

Martin, F., Kohler, A., Murat, C., Veneault-Fourrey, C., and Hibbett, D. S. (2016). Unearthing the roots of ectomycorrhizal symbioses. *Nat. Rev. Microbiol.* 14:760. doi: 10.1038/nrmicro.2016.149

Meharg, A. A. (2003). The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycol. Res.* 107, 1253–1265. doi: 10.1017/S0953756203008608

Mohammadhasani, F., Ahmadimoghadam, A., Asrar, Z., and Mohammadi, S. Z. (2017). Effect of Zn toxicity on the level of lipid peroxidation and oxidative enzymes activity in Badami cultivar of pistachio (Pistacia vera L.) colonized by ectomycorrhizal fungus. *Indian J. Plant. Physiol.* 22, 206–212. doi: 10.1007/s40502-017-0300-5

Mrnka, L., Kuchár, M., Cieslarová, Z., Matčjka, P., Száková, J., Tlustoš, P., et al. (2012). Effects of endo-and ectomycorrhizal fungi on physiological parameters and heavy metals accumulation of two species from the family Salicaceae. *Water Air Soil Pollut.* 223, 399–410. doi: 10.1007/s11270-011-0868-8

Nagajyoti, P. C., Lee, K. D., and Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: a review. *Environ. Chem. Lett.* 8, 199–216. doi: 10.1007/s10311-010-0297-8

Nilsen, A. R., Teasdale, S. E., Guy, P. L., Summerfield, T. C., and Orlovich, D. A. (2020). Fungal diversity in canopy soil of silver beech, Nothofagus menziesii (Nothofagaceae). *PLoS One* 15:e0227860. doi: 10.1371/journal.pone.0227860

Ogo, S., Yamanaka, T., Akama, K., Nagakura, J., and Yamaji, K. (2018). Influence of ectomycorrhizal colonization on cesium uptake by Pinus densiflora seedlings. *Mycobiology* 46, 388–395. doi: 10.1080/12298093.2018.1538074

Rai, G. K., Bhat, B. A., Mushtaq, M., Tariq, L., Rai, P. K., Basu, U., et al. (2021). Insights into decontamination of soils by phytoremediation: a detailed account on heavy metal toxicity and mitigation strategies. *Physiol. Plant* 173, 287–304. doi: 10.1111/ppl.13433

Rastgoo, L., and Alemzadeh, A. (2011). Biochemical responses of Gouan ('Aeluropus littoralis') to heavy metals stress. *Aust. J. Crop Sci.* 5:375. doi: 10.3316/informit.281334528451448

Ray, P., and Adholeya, A. (2009). Correlation between organic acid exudation and metal uptake by ectomycorrhizal fungi grown on pond ash in vitro. *Biometals* 22:275. doi: 10.1007/s10534-008-9163-6

Reddy, M. S., Kour, M., Aggarwal, S., Ahuja, S., Marmeisse, R., and Fraissinet Tachet, L. (2016). Metal induction of a Pisolithus albus metallothionein and its potential involvement in heavy metal tolerance during mycorrhizal symbiosis. *Environ. Microbiol.* 18, 2446–2454. doi: 10.1111/1462-2920.13149

Reddy, M. S., Prasanna, L., Marmeisse, R., and Fraissinet-Tachet, L. (2014). Differential expression of metallothioneins in response to heavy metals and their involvement in metal tolerance in the symbiotic basidiomycete Laccaria bicolor. *Microbiology* 160, 2235–2242. doi: 10.1099/mic.0.080218-0

Rog, I., Rosenstock, N. P., Körner, C., and Klein, T. (2020). Share the wealth: Trees with greater ectomycorrhizal species overlap share more carbon. *Mol. Ecol.* 29, 2321–2333. doi: 10.1111/mec.15351

Romero-Puertas, M. C., Terron-Camero, L. C., Pelaez-Vico, M. A., Olmedilla, A., and Sandalio, L. M. (2019). Reactive oxygen and nitrogen species as key indicators of plant responses to Cd stress. *Environ. Exp. Bot.* 161, 107–119. doi: 10.1016/j.envexpbot.2018.10.012

Rono, J. K., Le Wang, L., Wu, X. C., Cao, H. W., Zhao, Y. N., Khan, I. U., et al. (2021). Identification of a new function of metallothionein-like gene OsMT1e for cadmium detoxification and potential phytoremediation. *Chemosphere* 265:129136. doi: 10.1016/j.chemosphere.2020.129136

Rosas-Moreno, J., Pittman, J. K., and Robinson, C. H. (2021). Specific arbuscular mycorrhizal fungal–plant interactions determine radionuclide and metal transfer into Plantago lanceolata. *Plants People Planet* 3, 667–678. doi: 10.1002/ppp3.10185

Rosling, A. (2009). Trees, mycorrhiza and minerals–field relevance of in vitro experiments. *Geomicrobiol. J.* 26, 389–401. doi: 10.1080/01490450902929324

Rucińska-Sobkowiak, R. (2016). Water relations in plants subjected to heavy metal stresses. *Acta Physiol.* 38, 1–13. doi: 10.1007/s11738-016-2277-5

Saini, S., Kaur, N., and Pati, P. K. (2021). Phytohormones: Key players in the modulation of heavy metal stress tolerance in plants. *Ecotoxicol. Environ. Saf.* 223:112578. doi: 10.1016/j.ecoenv.2021.112578

Saitta, A., Anslan, S., Bahram, M., Brocca, L., and Tedersoo, L. (2018). Tree species identity and diversity drive fungal richness and community composition along an elevational gradient in a Mediterranean ecosystem. *Mycorrhiza* 28, 39–47. doi: 10.1007/s00572-017-0806-8

Schat, H., Llugany, M., and Bernhard, R. (2020). *Metal-specific patterns of tolerance, uptake, and transport of heavy metals in hyperaccumulating and nonhyperaccumulating metallophytes in Phytoremediation of contaminated soil and water*. Boca Raton: CRC Press, 171–188.

Shabani, L., and Sabzalian, M. R. (2016). Arbuscular mycorrhiza affects nickel translocation and expression of ABC transporter and metallothionein genes in Festuca arundinacea. *Mycorrhiza* 26, 67–76. doi: 10.1007/s00572-015-0647-2

Sharma, R. K., Agrawal, M., and Marshall, F. (2007). Heavy metal contamination of soil and vegetables in suburban areas of Varanasi. *India Ecotoxicol. Environ. Saf.* 66, 258–266. doi: 10.1016/j.ecoenv.2005.11.007

Sharma, S. S., and Dietz, K. J. (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot.* 57, 711–726. doi: 10.1093/jxb/erj073

Sheehan, D., Meade, G., and Foley, V. M. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* 360, 1–16. doi: 10.1042/bj3600001

Shi, L., Deng, X., Yang, Y., Jia, Q., Wang, C., Shen, Z., et al. (2019). A Cr (VI)-tolerant strain, Pisolithus sp1, with a high accumulation capacity of Cr in mycelium and highly efficient assisting Pinus thunbergii for phytoremediation. *Chemosphere* 224, 862–872. doi: 10.1016/j.chemosphere.2019.03.015

Shi, L., Dong, P., Song, W., Li, C., Lu, H., Wen, Z., et al. (2020). Comparative transcriptomic analysis reveals novel insights into the response to Cr (VI) exposure in Cr (VI) tolerant ectomycorrhizal fungi Pisolithus sp. 1 LS-2017. *Ecotoxicol. Environ. Saf.* 188:109935. doi: 10.1016/j.ecoenv.2019.109935

Shi, L., Xue, J., Liu, B., Dong, P., Wen, Z., Shen, Z., et al. (2018). Hydrogen ions and organic acids secreted by ectomycorrhizal fungi, Pisolithus sp1, are involved in the efficient removal of hexavalent chromium from waste water. *Ecotoxicol. Environ. Saf.* 161, 430–436. doi: 10.1016/j.ecoenv.2018.06.004

Shikha, K., Anuja, S., Radhika, A., and Reddy, M. S. (2019). Metallothioneins mediated intracellular copper homeostasis in Ectomycorrhizal fungus Suillus indicus. *Kavaka* 53, 48–54. doi: 10.36460/Kavaka/53/2019/48-54

Smith, S. E., and Read, D. J. (2010). *Mycorrhizal symbiosis*. Cambridge: Academic Press.

Sousa, N. R., Ramos, M. A., Marques, A. P., and Castro, P. M. (2012). The effect of ectomycorrhizal fungi forming symbiosis with Pinus pinaster seedlings exposed to cadmium. *Sci. Total Environ.* 414, 63–67. doi: 10.1016/j.scitotenv.2011.10.053

Srivastava, V., Sarkar, A., Singh, S., Singh, P., de Araujo, A. S., and Singh, R. P. (2017). Agroecological responses of heavy metal pollution with special emphasis on soil health and plant performances. *Front. Environ. Sci.* 5:64. doi: 10.3389/fenvs.2017.00064

Steidinger, B. S., Bhatnagar, J. M., Vilgalys, R., Taylor, J. W., Qin, C., Zhu, K., et al. (2020). Ectomycorrhizal fungal diversity predicted to substantially decline due to climate changes in North American Pinaceae forests. *J. Biogeogr.* 47, 772–782. doi: 10.1111/jbi.13802

Sun, J., Wang, R., Liu, Z., Ding, Y., and Li, T. (2013). Non-invasive microelectrode cadmium flux measurements reveal the spatial characteristics and real-time kinetics of cadmium transport in hyperaccumulator and nonhyperaccumulator ecotypes of Sedum alfredii. *J. Plant Physiol.* 170, 355–359. doi: 10.1016/j.jplph.2012.10.014

Sun, W., Yang, B., Zhu, Y., Wang, H., Qin, G., and Yang, H. (2021). Ectomycorrhizal fungi enhance the tolerance of phytotoxicity and cadmium accumulation in oak (Quercus acutissima Carruth.) seedlings: modulation of growth properties and the antioxidant defense responses. *Environ. Sci. Pollut. Res. Int.* 2021, 1–12. doi: 10.1007/s11356-021-16169-3

Suzuki, M., Tsukamoto, T., Inoue, H., Watanabe, S., Matsuhashi, S., Takahashi, M., et al. (2008). Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Mol. Biol.* 66, 609–617. doi: 10.1007/s11103-008-9292-x

Szuba, A., Karliński, L., Krzesłowska, M., and Hazubska-Przybył, T. (2017). Inoculation with a Pb-tolerant strain of Paxillus involutus improves growth and Pb tolerance of Populus× canescens under in vitro conditions. *Plant Soil* 412, 253–266. doi: 10.1007/s11104-016-3062-3

Szuba, A., Marczak, L., and Kozłowski, R. (2020). Role of the proteome in providing phenotypic stability in control and ectomycorrhizal poplar plants exposed to chronic mild Pb stress. *Environ. Pollut.* 264:114585. doi: 10.1016/j.envpol.2020.114585

Tang, Y., Shi, L., Zhong, K., Shen, Z., and Chen, Y. (2019). Ectomycorrhizal fungi may not act as a barrier inhibiting host plant absorption of heavy metals. *Chemosphere* 215, 115–123. doi: 10.1016/j.chemosphere.2018.09.143

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., et al. (2014). Global diversity and geography of soil fungi. *Science* 346:1256688. doi: 10.1126/science.1256688

Teotia, P., Kumar, M., Prasad, R., Kumar, V., Tuteja, N., and Varma, A. (2017). *Mobilization of micronutrients by mycorrhizal fungi in Mycorrhiza-Function, Diversity, State of the Art*. Cham: Springer. 9–26. doi: 10.1007/978-3-319-53064-2_2

Thompson, S. G., Ghiorso, A., Rasmussen, J. O., and Seaborg, G. T. (1949). Alpha-decay in isotopes of atomic number less than 83. *Phys. Rev.* 76:1406. doi: 10.1103/PhysRev.76.1406

Vaclavikova, M., Gallios, G. P., Hredzak, S., and Jakabsky, S. (2008). Removal of arsenic from water streams: an overview of available techniques. *Clean Technol. Environ. Policy* 10, 89–95. doi: 10.1007/s10098-007-0098-3

Van der Heijden, M. G., Martin, F. M., Selosse, M. A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

Veach, A. M., Stokes, C. E., Knoepp, J., Jumpponen, A., and Baird, R. (2018). Fungal communities and functional guilds shift along an elevational gradient in the southern Appalachian Mountains. *Microb. Ecol.* 76, 156–168. doi: 10.1007/s00248-017-1116-6

Wang, X., Agathokleous, E., Qu, L., Fujita, S., Watanabe, M., Tamai, Y., et al. (2018). Effects of simulated nitrogen deposition on ectomycorrhizae community structure in hybrid larch and its parents grown in volcanic ash soil: The role of phosphorous. *Sci. Total Environ.* 618, 905–915. doi: 10.1016/j.scitotenv.2017.08.283

Wani, R. A., Ganai, B. A. M., Shah, A., and Uqab, B. (2017). Heavy metal uptake potential of aquatic plants through phytoremediation technique–a review. *J. Bioremediat. Biodegrad.* 8:2. doi: 10.4172/2155-6199.1000404

Wei, S., Song, Y., and Jia, L. (2020). Influence of the slope aspect on the ectomycorrhizal fungal community of Quercus variabilis Blume in the middle part of the Taihang Mountains, North China. *J. Res.* 2020, 1–16. doi: 10.1007/s11676-019-01083-9

Wen, Z., Shi, L., Tang, Y., Shen, Z., Xia, Y., and Chen, Y. (2017). Effects of Pisolithus tinctorius and Cenococcum geophilum inoculation on pine in copper-contaminated soil to enhance phytoremediation. *Int. J. Phytoremed.* 19, 387–394. doi: 10.1080/15226514.2016.1244155

Wu, Q. S., Zou, Y. N., and Abd-Allah, E. F. (2014). *Mycorrhizal association and ROS in plants in Oxidative damage to plants*. Cambridge: Academic Press, 453–475. doi: 10.1016/B978-0-12-799963-0.00015-0

Wu, T. (2011). Can ectomycorrhizal fungi circumvent the nitrogen mineralization for plant nutrition in temperate forest ecosystems? *Soil Biol. Biochem.* 43, 1109–1117. doi: 10.1016/j.soilbio.2011.02.003

Xue, T., Li, X., Zhu, W., Wu, C., Yang, G., and Zheng, C. (2009). Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *J. Exp. Bot.* 60, 339–349. doi: 10.1093/jxb/ern291

Yu, P., Sun, Y., Huang, Z., Zhu, F., Sun, Y., and Jiang, L. (2020). The effects of ectomycorrhizal fungi on heavy metals' transport in Pinus massoniana and bacteria community in rhizosphere soil in mine tailing area. *J. Hazard Mater.* 381:121203. doi: 10.1016/j.jhazmat.2019.121203

Yu, X. Z., Lin, Y. J., and Zhang, Q. (2019). Metallothioneins enhance chromium detoxification through scavenging ROS and stimulating metal chelation in Oryza sativa. *Chemosphere* 220, 300–313. doi: 10.1016/j.chemosphere.2018.12.119

Zeng, F., Zhou, W., Qiu, B., Ali, S., Wu, F., and Zhang, G. (2011). Subcellular distribution and chemical forms of chromium in rice plants suffering from different levels of chromium toxicity. *J. Plant Nutr. Soil Sci.* 174, 249–256. doi: 10.1002/jpln.200900309

Zhang, C., Wang, X., Ashraf, U., Qiu, B., and Ali, S. (2017). Transfer of lead (Pb) in the soil-plant-mealybug-ladybird beetle food chain, a comparison between two host plants. *Ecotoxicol. Environ. Saf.* 143, 289–295. doi: 10.1016/j.ecoenv.2017.05.032

Zhang, J., Taniguchi, T., Xu, M., Du, S., Liu, G. B., and Yamanaka, N. (2014). Ectomycorrhizal fungal communities of Quercus liaotungensis along different successional stands on the Loess Plateau. *China J. Res.* 19, 395–403. doi: 10.1007/s10310-013-0433-y

Zhang, K., Tappero, R., Ruytinx, J., Branco, S., and Liao, H. L. (2021). Disentangling the role of ectomycorrhizal fungi in plant nutrient acquisition along a Zn gradient using X-ray imaging. *Sci. Total Environ.* 801:149481. doi: 10.1016/j.scitotenv.2021.149481

Zhang, Q., Gong, M., Liu, K., Chen, Y., Yuan, J., and Chang, Q. (2020). Rhizoglomus intraradices improves plant growth, root morphology and Phytohormone balance of *Robinia pseudoacacia* in arsenic-contaminated soils. *Front. Microbiol.* 11:1428. doi: 10.3389/fmicb.2020.01428

Zhi, J., Liu, X., Yin, P., Yang, R., Liu, J., and Xu, J. (2020). Overexpression of the metallothionein gene PaMT3-1 from Phytolacca americana enhances plant tolerance to cadmium. *Plant Cell Tissue Organ. Cult.* 143, 211–218. doi: 10.1007/s11240-020-01914-2

Zhu, T., Li, L., Duan, Q., Liu, X., and Chen, M. (2021). Progress in our understanding of plant responses to the stress of heavy metal cadmium. *Plant Signal. Behav.* 16:1836884. doi: 10.1080/15592324.2020.1836884

Zhu, W., Zhao, D. X., Miao, Q., Xue, T. T., Li, X. Z., and Zheng, C. C. (2009). Arabidopsis thaliana metallothionein, AtMT2a, mediates ROS balance during oxidative stress. *J. Plant Biol.* 52:585. doi: 10.1007/s12374-009-9076-0

Zimmer, D., Baum, C., Leinweber, P., Hrynkiewicz, K., and Meissner, R. (2009). Associated bacteria increase the phytoextraction of cadmium and zinc from a metal-contaminated soil by mycorrhizal willows. *Int. J. Phytoremed.* 11, 200–213. doi: 10.1080/15226510802378483

Zong, K., Huang, J., Nara, K., Chen, Y., Shen, Z., and Lian, C. (2015). Inoculation of ectomycorrhizal fungi contributes to the survival of tree seedlings in a copper mine tailing. *J. Res.* 20, 493–500. doi: 10.1007/s10310-015-0506-1

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ORIGINAL RESEARCH
published: 08 April 2022
doi: 10.3389/fmicb.2022.843010

Nutrients Regulate the Effects of Arbuscular Mycorrhizal Fungi on the Growth and Reproduction of Cherry Tomato

*Lei Wang, Xin Chen, Yeqin Du, Di Zhang and Zhanhui Tang**

State Environmental Protection Key Laboratory of Wetland Ecology and Vegetation Restoration, School of Environment, Northeast Normal University, Changchun, China

Arbuscular mycorrhizal fungi (AMF) colonize the rhizosphere of plants and form a symbiotic association with plants. Mycorrhizal symbionts have diversified ecological roles and functions which are affected by soil conditions. Understanding the effects of different AMF inoculation on plants under varied nutritional conditions is of great significance for further understanding the effects of the external environment regulating mycorrhizal symbiosis on plant phenotypic traits. In this study, the effects of four AMF inoculation treatments on the growth and reproductive performance of cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) were investigated under three nutrient levels by pot experiment. It was found that the growth-promoting effect of AMF on cherry tomato decreased with nutrient reduction, and the effects of the same AMF inoculation treatment on cherry tomato were different at different nutrient levels. Nutrient levels and AMF had interactive effects on flower characteristics, fruit yield, resource allocation, and seed germination of the cherry tomato. In addition, AMF could promote sexual reproductive investment. Nutrient levels and AMF also affected the accumulation of nitrogen and phosphorus in cherry tomato, and there were significant differences among different AMF inoculation treatments. The results indicated that nutrient differences could affect the symbiosis between AMF and plants, and confirmed that there were differences in the effects of the four AMF inoculation treatments on the growth and reproductive traits of plants. The differences in growth and reproduction characteristics of cherry tomato between different AMF inoculation treatments at different nutrient levels indicated that the effects of AMF mycorrhizal on the traits of cherry tomato were regulated by nutrients.

Keywords: arbuscular mycorrhizal fungi, nutrient differences, growth and reproductive strategy, nutrient accumulation, cherry tomato

OPEN ACCESS

Edited by:
Alok Kumar Srivastava, National Bureau of Agriculturally Important Microorganisms (ICAR), India

Reviewed by:
Raffaella Balestrini, Institute for Sustainable Plant Protection (CNR), Italy
Puffy Soundy, Tshwane University of Technology, South Africa
Simone Cantamessa, Council for Agricultural and Economics Research (CREA), Italy

***Correspondence:**
Zhanhui Tang
tangzh789@nenu.edu.cn

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *24 December 2021*
Accepted: *09 March 2022*
Published: *08 April 2022*

Citation:
Wang L, Chen X, Du Y, Zhang D and Tang Z (2022) Nutrients Regulate the Effects of Arbuscular Mycorrhizal Fungi on the Growth and Reproduction of Cherry Tomato. Front. Microbiol. 13:843010. doi: 10.3389/fmicb.2022.843010

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are ancient and widely distributed symbiotic fungi of plant roots. In terrestrial ecosystems, more than 80% of plants can form mycorrhizal symbiosis with AMF (Berruti et al., 2016; Genre et al., 2020). AMF plays an important role in the process of nutrient transfer between soil and plants. The common mycorrhizal networks (CMNs) formed by root peripheral hyphae is the main channel for nutrient exchange between aboveground and belowground in the ecosystem and plays an important regulatory role in the nutrient cycle of

the ecosystem (Bever et al., 2010; Cheng et al., 2012; Johnson et al., 2013; Yang et al., 2021). AMF provides host plants with water and soil mineral nutrient resources (especially phosphate) in exchange for photosynthates and lipids needed for growth and reproduction (Jeffries et al., 2003; Jiang et al., 2017; Wang et al., 2017; He et al., 2019). This mutually beneficial symbiotic relationship of AMF and host plants can not only satisfy their growth needs but also improve the photosynthetic efficiency and promote the growth and development, which improve productivity and the biomass accumulation of host plants (Van Geel et al., 2016; Keymer and Gutjahr, 2018; Jajoo and Mathur, 2021). Mycorrhizal symbionts can also alleviate abiotic stresses such as drought and nutrient deficiency to the host plant (Hoeksema et al., 2010; Doubková et al., 2013; Nouri et al., 2014; Augé et al., 2015). The effects of AMF symbionts on plant growth vary with environmental conditions and may show promoting, inhibiting, or neutral effects on plant growth (Facelli et al., 2010). It is noteworthy that AMF can also affect the reproductive process of host plants to a certain extent; however, the role of AMF in regulating the flowering and reproduction of host plants is often ignored in most studies (Adeyemi et al., 2021; Carmen et al., 2021; Kazadi et al., 2021; Khaekhum et al., 2021; Chen et al., 2022). The positive effects of AMF on plants are widely recognized, but the effects of the AMF symbiosis with plant roots on growth and reproductive performance are extremely complex under different cultivation conditions which require further investigation.

The growth and reproduction traits of plants are plastic to the changes of environment, especially the changes of soil nutrients (Kattia et al., 2015). Therefore, the plants will change their growth and reproduction strategies in response to the changes in nutrients. As an important node between aboveground and belowground ecosystems, AMF is sensitive to soil nutrient changes (Averill et al., 2018; Pan et al., 2020). Host plants need to consume a large amount of energy to maintain the growth and reproduction of AMF, although AMF can provide host plants with nutrients needed for growth and development when plants are in an environment containing highly available nutrients (Parniske, 2008; Smith and Smith, 2011). Based on the ecological economics theory of trade partnership, it is predicted that the host plant may have a tradeoff strategy of reducing the degree of mycorrhizal symbiosis and thus inhibiting the growth promotion effect of AMF, which may lead to the transformation of mycorrhizal function from mutualism to commensalism or even parasitism (Floss et al., 2013; Ma et al., 2020; Pan et al., 2020). Host plants may reduce their dependence on AMF and thus reduce their photosynthate allocation to AMF when nutrients are abundant (Jiang et al., 2018; Verlinden et al., 2018). When plants grow in nutrient-deficient environments, abiotic stress usually stimulates the colonization of AMF in roots and affects mycorrhizal structures (e.g., arbuscular abundance and vesicles) (Martínez-García et al., 2012). The external environment of nutrient deficiency is conducive to the formation of mycorrhizal, promotes the symbiosis between AMF and plants, and improves the ability of plants to adapt to the adverse environment (Cosme and Wurst, 2013; Fusconi, 2014; Pozo et al., 2015). AMF can effectively alleviate phosphorus deficiency stress faced by host plants when they are in a phosphorus-deficient environment (Smith et al., 2004; Yang et al., 2012). Interestingly, the establishment of a symbiotic relationship between AMF and plants seems to be regulated only by soil nitrogen and phosphorus content, while other mineral elements in soil do not affect mycorrhizal formation (Landis et al., 2004; Nouri et al., 2014). Although soil nutrient levels and AMF can affect plant growth and reproduction, it is not clear how direct or indirect nutrient exchange between plants and AMF affects plant adaptation strategies under different nutrient levels, and whether there are differences in the effects of different AMF inoculation treatments under different nutrient levels.

The effect of mycorrhizal symbionts on plants may be affected by abiotic environmental factors, such as soil nutrient levels. Dwarf cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) was selected as the plant material of this study because of its advantages of a short shoot, less space occupation, and short growth and reproduction cycle lasing 3–4 months (Rahim et al., 2019). We explored the effects of different nutrient levels and different AMF symbiosis and their interactions on the growth and reproductive strategies of cherry tomato, as well as the accumulation of nitrogen and phosphorus in cherry tomato. We hypothesized that (a) cherry tomato may become less dependent on AMF at a high nutrient level. However, with the decrease of nutrient level, the growth-promoting effect of AMF may be enhanced, and the promoting effect of different AMF inoculation treatments should be different; (b) AMF inoculation will change the reproductive strategy of cherry tomato at different nutrient levels. Especially at low nutrient levels, AMF may promote the absorption of nutrients and increase their investment in the reproduction of cherry tomato, and enhance the germination of offspring seeds; (c) different AMF treatments promoting/inhibiting the uptake and accumulation of nitrogen and phosphorus in cherry tomato under different nutrients. To test these hypotheses, we inoculated cherry tomatoes with four AMF treatments under three nutrient levels in the greenhouse, aiming to explore the response of growth and reproduction strategies of cherry tomato and nutrient accumulation to AMF treatments under different nutrient levels.

MATERIALS AND METHODS

Seed Disinfection and Germination

The seeds of dwarf cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) with full grains and consistent size were selected and soaked in 0.5% potassium permanganate solution for 5 min, 10% H_2O_2 for 10 min, and 5% $NaClO_3$ for 1 min, respectively, for surface disinfection. The disinfected seeds were washed with pure water and then placed in petri dishes with moist filter paper. The petri dishes with seeds were placed in a constant temperature light incubator at 25°C (20% illumination and 60% humidity) for germination.

Preparation of Soil Substrate

Using a mixture of peat soil and sand as soil substrate (**Table 1**), the following 3 nutrition treatments were set: high nutrition level [High, 1:1 (volume), peat soil: sand], medium nutrition level

(Medium, 1:2, peat soil: sand), and low nutrition level (Low, 1:3, peat soil: sand). The prepared soil matrix was autoclaved at 126°C for 120 min, and then put into a flowerpot (inner diameter 16.5 cm, height 12.5 cm), wiped, and sterilized with 95% alcohol. A 2 L of soil matrix was added to each flowerpot.

Arbuscular Mycorrhizal Fungi Inoculation

Four AMF inoculation treatments were set up as follows: *Funnelliformis mosseae* (Fm), *Rhizophagus intraradices* (Ri), *Glomus versiforme* (Gv), and an equal mixture of the three fungi (Ma) (AMF strain information and propagation method in **Supplementary Material**). The dosage of the above treatments was 40 g (containing 1,000 spores), respectively. The control treatment (CK) without AMF inoculation was set at the same time. After wetting the soil with pure water, four fungus agent were added into each pot. To ensure the same microbial conditions except for AMF among different inoculation treatments, an equal number of bactericidal inoculums and 40 ml of water filtrate of non-sterilized microbial inoculums (filtered by a filter membrane with an aperture of 20 μm) were applied in the non-inoculation treatment (CK). One germinated seed was put on the fungus agent to ensure that the seeds can fully contact with the fungus agent after taking root, and then cover the germinated seeds and the fungus agent with a small amount of soil substrate. Finally, sufficient amount of pure water was added to make the whole soil substrate wet. There were 15 (3 nutrient levels × 5 inoculations including CK) treatment combinations in total, and each treatment combination was repeated 10 times for a total of 150 pots in this study.

Plant Cultivation

All the pots with different treatments were placed in a greenhouse for cultivation with a photoperiod of 12 h/12 h (light/dark), an optical quantum flux density of 550–600 $\mu mol/m^2s$, temperature of 30/25°C (day/night), and humidity of 40–50%. The plants were checked and watered every day to keep the soil moist. The positions of pots were randomly changed every week and the growth of the plants was monitored and recorded regularly until the cherry tomatoes were mature.

Measurement

The flowering time was recorded after the first cherry tomato flower opened. Five plants were randomly selected from each treatment and the flower longevity was recorded. Blooming flowers with a petal opening angle of 180° were sampled to measure flower diameter and single flower weight (n = 5). The pollen number in single flowers was counted under an optical microscope after the crushed anthers were made into a suspension using 20% sodium hexametaphosphate solution (n = 5). Pollen viability of cherry tomato was determined by triphenyl tetrazole chloride method (Sutyemez, 2011) (n = 5). The number of flowers per plant was recorded every day until the plant was harvested (n = 10).

Fruit number and fresh weight were measured after harvest. Mycorrhizal colonization was measured by trill benzene blue staining (Brundrett et al., 1984). The stems, leaves, roots, and fruits of cherry tomato were placed in a drying oven at 80°C for 48 h. The biomass (the dry weight) of the stems, leaves, roots, and fruits were weighed, respectively, to calculate the root–shoot ratio and reproductive allocation (n = 10). The seeds were picked out after fresh cherry tomato fruits were crushed in water and record the number of seeds in each fruit (n = 5). Seed weight was weighed after drying in natural ventilation. These seeds were germinated after disinfection referring to the method in 2.1, and the seed germination percentage was calculated. The total contents of nitrogen and phosphorus in the aboveground and belowground parts of cherry tomato are determined by element analyzer (model: EA3100 Elemental Analyzer, Italy) after the plants were dried, crushed, and sieved (100 mesh) (n = 5).

Data Statistics and Analysis

Before analysis, all data are checked to see whether they conform to normal distribution using the 1-Simple Kolmogorov–Smirnov Z test and variance homogeneity using the Bartlett test. All data conform to normal distribution and variance homogeneity. The effects of nutrients and AMF on the growth and reproduction of cherry tomato were tested by two-factor ANOVA. Multiple comparisons between treatments were performed using the least significant difference (LSD) test for differences at 0.05 significance level. All statistical analyses were performed using SPSS (25.0) (SPSS Inc., Chicago, IL, United States).

RESULTS

Mycorrhizal Infection

Arbuscules were formed in the root system of cherry tomato after AMF inoculation (**Figures 1A,B**), while no endophytic mycelia were found in the root system of cherry tomato without AMF inoculation. The root mycorrhizal infection of cherry tomato showed an extremely significant difference among different AMF inoculation treatments (P = 0.001). Soil nutrient level and the interaction between nutrients and AMF inoculation did not significantly affect the root infection rate (**Table 2**). Under the same nutrient levels, the infection rate of different AMF was also different. Overall, the infection rates of Gv and Ma were significantly higher than Fm and Ri at medium and low nutrient levels, and the infection rates of Gv were higher than Fm at a high nutrient level (**Figure 1C**).

TABLE 1 | Basic characteristics of experimental soil substrate.

Properties	Value (Mean ± SE)	
	Peat soil	Sand
Density (kg/m^3)	508.933 ± 12.639	1,462.400 ± 7.965
pH	4.905 ± 0.025	0
Organic matter, OM (%)	14.647 ± 0.165	0
Total nitrogen, TN (mg/kg)	4,796.343 ± 37.616	0
Available nitrogen, AN (mg/kg)	314.720 ± 17.638	0
Total phosphorus, TP (mg/kg)	6,231.953 ± 113.720	0
Available phosphorus, AP (mg/kg)	186.208 ± 2.407	0
Available potassium, AK (mg/kg)	4,169.227 ± 115.888	0

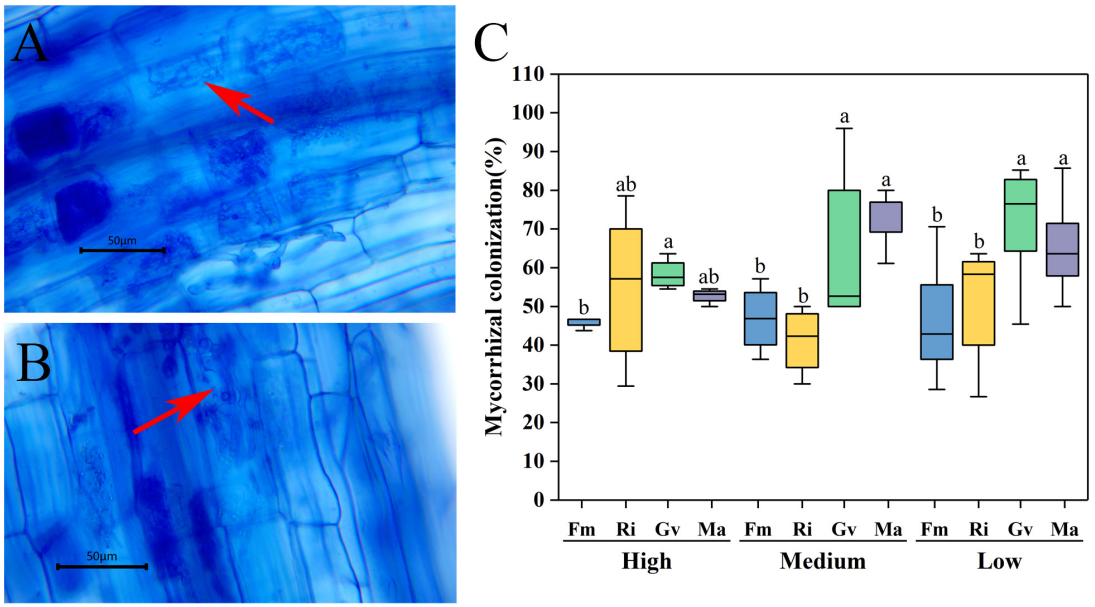


FIGURE 1 | Mycorrhizal infection of cherry tomato root inoculated with arbuscular mycorrhizal fungi (AMF) at different nutrient levels. **(A,B)** Arbuscular structure of root (arrow); **(C)** Mycorrhizal colonization of cherry tomato under different nutrient levels. Different letters indicate that there were significant differences among different AMF inoculation treatments at the same nutrient level ($P < 0.05$), the same as below.

TABLE 2 | Effects of nutrient levels, AMF inoculation treatments and their interaction on root infection rate, growth and reproductive traits of cherry tomato.

Indicators	Nutrient levels (*df* = 2)		AMF (*df* = 4)		Nutrient levels *AMF (*df* = 8)	
	F	*P*	*F*	*P*	*F*	*P*
Mycorrhizal colonization rate	0.71	0.497	6.55	**0.001**	1.16	0.343
Aboveground biomass	176.82	**<0.001**	4.65	**0.002**	1.71	0.102
Belowground biomass	24.03	**<0.001**	3.16	**0.016**	0.94	0.490
Total biomass	165.16	**<0.001**	4.46	**0.002**	2.14	**0.036**
Root-shoot ratio	2.93	0.057	0.37	**0.012**	0.99	0.446
Flowering time	2.55	0.082	5.89	**<0.001**	5.15	**<0.001**
Flower longevity	1.07	0.353	2.32	0.071	1.41	0.218
Flower diameter	3.50	**0.033**	0.69	0.602	2.36	**0.022**
Flower weight	21.89	**<0.001**	1.55	0.193	2.77	**0.008**
Pollen number	0.91	0.408	0.55	0.701	0.75	0.646
Pollen viability	1.98	0.149	3.89	**0.008**	0.38	0.928
Flower number	14.01	**<0.001**	5.33	**0.001**	4.62	**<0.001**
Fruit number	3.81	**0.025**	2.88	**0.026**	3.81	**0.025**
Fruit biomass	22.20	**<0.001**	10.94	**<0.001**	2.33	**0.027**
Reproductive allocation	3.60	**0.032**	7.78	**<0.001**	3.14	**0.004**
Seeds in a fruit	4.76	**0.012**	2.74	**0.036**	1.31	0.254
1000-grain seed weight	7.63	**0.002**	1.15	0.351	1.45	0.216
Germination percentage	10.07	**<0.001**	5.00	**0.003**	3.80	**0.003**
Aboveground nitrogen accumulation	566.15	**<0.001**	21.95	**<0.001**	6.07	**<0.001**
Belowground nitrogen accumulation	324.87	**<0.001**	36.99	**<0.001**	7.76	**<0.001**
Total nitrogen accumulation	833.42	**<0.001**	37.44	**<0.001**	8.06	**<0.001**
Aboveground phosphorus accumulation	1360.17	**<0.001**	42.47	**<0.001**	2.41	**0.038**
Belowground phosphorus accumulation	188.94	**<0.001**	50.06	**<0.001**	21.07	**<0.001**
Total phosphorus accumulation	1615.47	**<0.001**	72.93	**<0.001**	9.80	**<0.001**

The P values with significant differences were bolded.
** means the interaction between nutrient levels and AMF.*

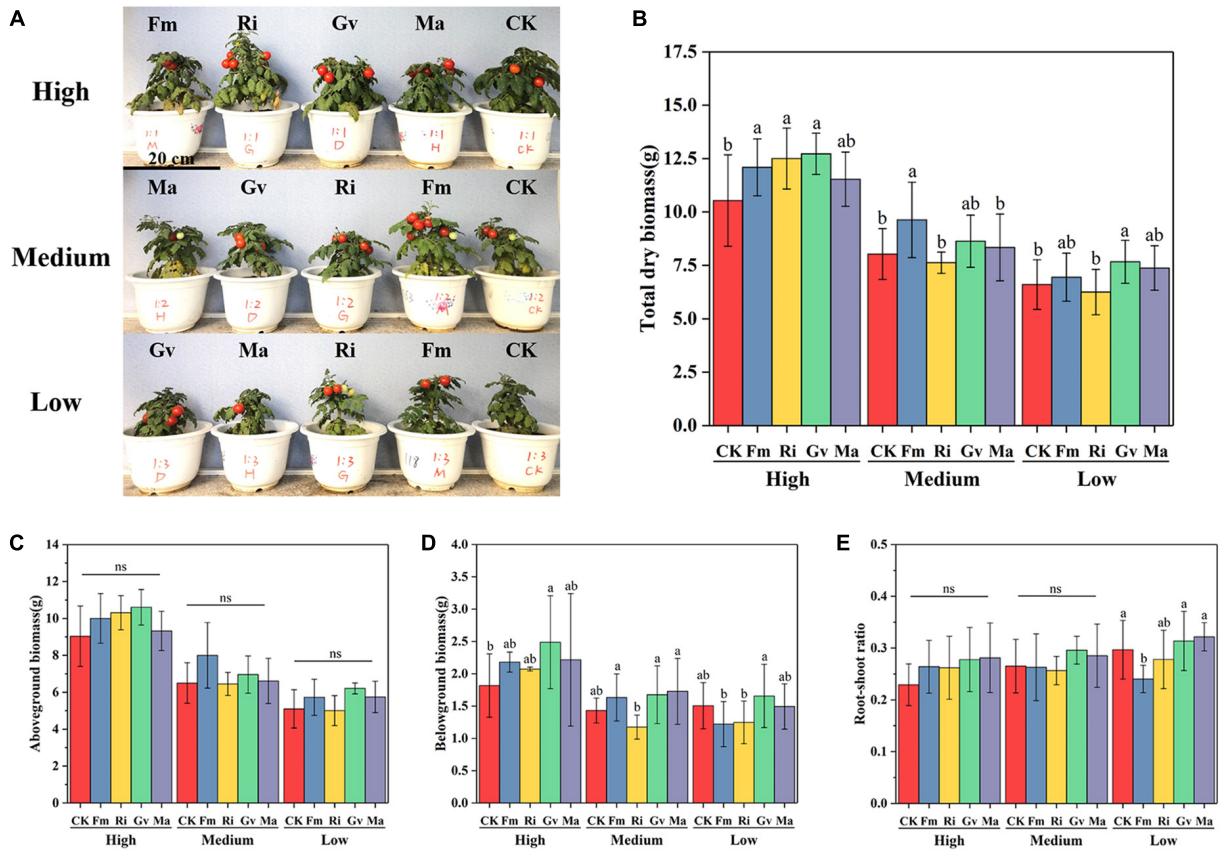


FIGURE 2 | Growth **(A)**, total biomass **(B)**, aboveground biomass **(C)**, belowground biomass **(D)**, and root shoot ratio **(E)** of cherry tomato inoculated with different AMF treatments under different nutrient levels. ns denotes no significant difference between AMF inoculation treatments at the same nutrient level ($P > 0.05$), the same as below.

Growth and Biomass Allocation

The aboveground and belowground biomass of cherry tomato was significantly affected by soil nutrient level and AMF inoculation treatment. The total biomass of cherry tomato was significantly affected by soil nutrient level, AMF, and their interactions. However, the root–shoot ratio was only significantly affected by AMF inoculation treatment (**Table 2**). The growth performance of cherry tomatoes under different AMF treatments were different under the three nutrient levels (**Figure 2A**). Aboveground biomass and root–shoot ratio did not change significantly after the four AMF inoculation treatments were applied. But the three single AMF treatments significantly increased the total biomass of cherry tomato, and Gv inoculation significantly increased the belowground biomass at a high nutrient level (**Figures 2B–E**). Fm inoculation could significantly increase the accumulation of total biomass, and the total biomass was significantly higher than that of Ri and Ma inoculation at the medium nutrient level (**Figure 2B**). AMF inoculation at medium nutrient levels did not significantly affect aboveground and belowground biomass and root–shoot ratio (**Figures 2C–E**). It is noteworthy that Ri inoculation significantly reduced belowground biomass compared to Fm, Gv, and Ma treatments (**Figure 2D**). When the nutrient level was lowest, aboveground and belowground biomass and root–shoot ratio did not change significantly after inoculation with four AMF treatments, while inoculation with Gv significantly promoted total biomass accumulation (**Figures 2B–E**).

Reproductive Characters

AMF inoculation and the interaction between AMF inoculation and nutrient level can significantly affect the flowering time of cherry tomato (**Table 2**). The flowering time of cherry tomato was significantly advanced by inoculating Fm and Ri at a high nutrient level, significantly advanced by inoculating Ri and Gv at a medium nutrient level and significantly promoted by Gv and Ma at a low nutrient level (**Figure 3A**). Nutrient levels, AMF inoculation, and their interactions had no significant effect on single flower longevity (**Table 2**). The single flower longevity of cherry tomato decreased after Fm inoculation at high nutrient levels (**Figure 3B**). The fresh weight and diameter of a single flower of cherry tomato were significantly different under different nutrient levels and the interaction between nutrient levels and AMF (**Table 2**). Inoculating Fm significantly reduced the flower diameter at high nutrient levels. At the medium nutrient level, inoculating Fm significantly increased the flower diameter and flower weight, while inoculating Ri significantly reduced the fresh flower weight of cherry tomato. When the nutrient level is at the lowest level, AMF inoculation treatment

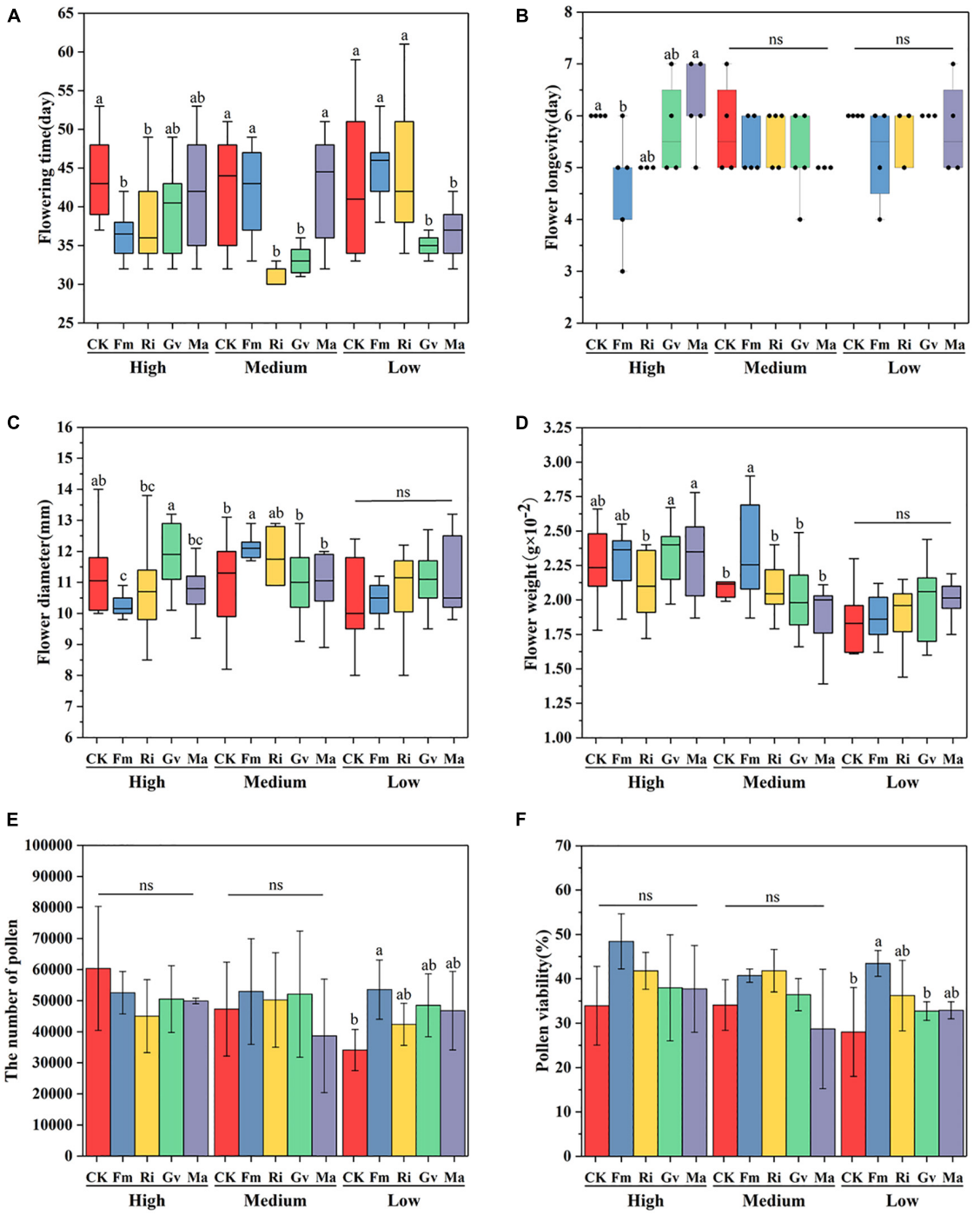


FIGURE 3 | Flowering time **(A)**, single flower longevity **(B)**, single flower diameter **(C)**, single flower weight **(D)**, pollen quantity **(E)**, and pollen viability **(F)** of cherry tomato inoculated with AMF under different nutrient levels.

has no significant effect on flower diameter and flower weight (**Figures 3C,D**). AMF inoculation did not affect the pollen number and pollen viability of cherry tomato at high and medium nutrient levels, whereas Fm inoculation significantly increased the pollen number and pollen viability at low nutrient levels (**Figures 3E,F**).

Soil nutrient level, AMF, and their interaction had significant effects on flower number, fruit number, fruit biomass, and reproductive allocation of cherry tomato (**Table 2**). Ri and Gv inoculation significantly increased the flower number of cherry tomato under high nutrient levels. There were no significant differences in the fruit number of cherry tomato among the four

AMF inoculation treatments under high nutrient levels. When the cherry tomato was grown at a medium nutrient level, Fm inoculation significantly increased its flower number, and Fm and Ri inoculation also significantly increased the fruit number. Fm inoculation treatment increased the number of flowers and fruits, but Fm, Ri, and Ma inoculation treatment only increased the flower number of cherry tomatoes at the lowest nutrient level (**Figures 4A,B**). Fm inoculation could increase the fruit biomass of cherry tomato at all three nutrient levels, but Ri inoculation only significantly increased the fruit biomass at the medium nutrient level. Gv inoculation could promote the increase of fruit biomass at medium and low nutrient levels, while Ma inoculation increased the fruit biomass of cherry tomato at high and low nutrient levels (**Figure 4C**). Fm and Ri inoculation at the medium and lower nutrient levels significantly increased the reproductive allocation, while Ma inoculation promoted resources allocation to the reproductive organs under a high nutrient level (**Figure 4D**).

Soil nutrient level and AMF inoculation had significant effects on seed number per fruit of cherry tomato, while 1,000-grain weight of seeds was only affected by soil nutrient level (**Table 2**). Fm and Ma inoculation significantly increased the number of seeds per fruit but had no effects on the seed 1,000-grain weight at a high nutrient level. The four AMF inoculation treatments had no significant effects on the seed number per fruit, while Ri inoculation significantly increased the seed weight at the medium nutrient level. Ma inoculation tended to increase the seed number per fruit but did not affect seed weight at low nutrient levels (**Figures 5A,B**). Seed germination percentage was significantly affected by soil nutrient levels, AMF inoculation, and their interactions (**Table 2**). Seed germination percentage could be improved by inoculating Fm at a high nutrient level and by inoculating Gv and Ma under low nutrient levels (**Figure 5C**).

Accumulation of Nitrogen and Phosphorus

The accumulation of nitrogen and phosphorus in cherry tomato was significantly affected by soil nutrient level, AMF inoculation, and their interaction (**Table 2**). Fm, Gv, and Ma inoculation promoted the aboveground nitrogen accumulation of cherry tomato. The four AMF inoculation treatments all increased the belowground and total nitrogen accumulation of cherry tomato under high nutrient levels. Four AMF inoculation treatments significantly increased the aboveground and total nitrogen accumulation of cherry tomato under medium nutrient level, while Fm, Gv, and Ma inoculation treatments increased the belowground nitrogen accumulation of cherry tomato. When cherry tomato was grown under low nutrient level, inoculation of Gv and Ma significantly increased the aboveground nitrogen accumulation, belowground nitrogen accumulation, and total nitrogen accumulation of cherry tomato (**Figures 6A,C**). In addition, Fm and Gv inoculation at the three nutrient levels all significantly increased the aboveground phosphorus accumulation of cherry tomato. The effects of different AMF inoculation on belowground phosphorus accumulation were different under different nutrient conditions. Fm and Gv inoculation under high nutrient levels can increase the belowground phosphorus accumulation. Gv and Ma inoculation at a medium nutrient level and Gv inoculation at a low nutrient level significantly promoted the belowground phosphorus accumulation and total phosphorus accumulation. Ri inoculation at medium and low nutrient levels decreased the belowground phosphorus accumulation of cherry tomato. Fm and Ma inoculation also significantly reduced the belowground phosphorus accumulation under low nutrient levels, and inoculation of Ri and Ma under low nutrient levels was not conducive to total phosphorus accumulation in cherry tomatoes (**Figures 6B,D**).

DISCUSSION

Effects of Nutrient Conditions on Arbuscular Mycorrhizal Fungi Infection and Growth Promotion

Although AMF colonization on cherry tomato roots was high (41.15–71.30%), our study did not find that AMF inoculation at a high nutrient level reduced the dependence of cherry tomato on AMF. It was found that the dependence of cherry tomato on AMF was stronger at a higher nutrient level. With the decrease of nutrient level, the promoting effect of AMF decreased to a certain extent (**Figure 2B**), which indicated that the high nutrient level in this experiment was not enough to inhibit the colonization of AMF and its contribution to cherry tomato. Our study results showed that the four AMF inoculation treatments showed significant differences in affecting the growth and reproduction of cherry tomato. On the one hand, the accumulation of plant biomass depends on the symbiotic effect of AMF, and on the other hand, it is also affected by soil nutrients. There seems to be a positive correlation between infection rate and biomass (Ma et al., 2007). Different AM fungi in soil with different nutrient levels may have different mechanisms in promoting plant growth and development. Currently, it is generally accepted that AMF can regulate the balance of plant hormones and thus participate in promoting plant growth, especially play an important role in the regulation of auxin (IAA), gibberellin (GA), cytokinin, ethylene, and other plant hormones and signaling substances (Pozo et al., 2015; Shi et al., 2015). Furthermore, a study has found that AMF can improve the activity of photosynthesis key enzyme (ribulose bisphosphate carboxylase, RuBisCO), and thus increase the net photosynthetic rate of leaf, which facilitated the accumulation of photosynthates (Pozo et al., 2015). However, the effect of enhancing photosynthesis is also restricted by AMF species, which indicated that AMF have different functions under different nutrient conditions.

Effects of Arbuscular Mycorrhizal Fungi on Reproductive Characters of Cherry Tomato Under Different Nutrient Conditions

Our study confirmed that the inoculation of AMF could advance the flowering of cherry tomatoes, but this advanced effect is

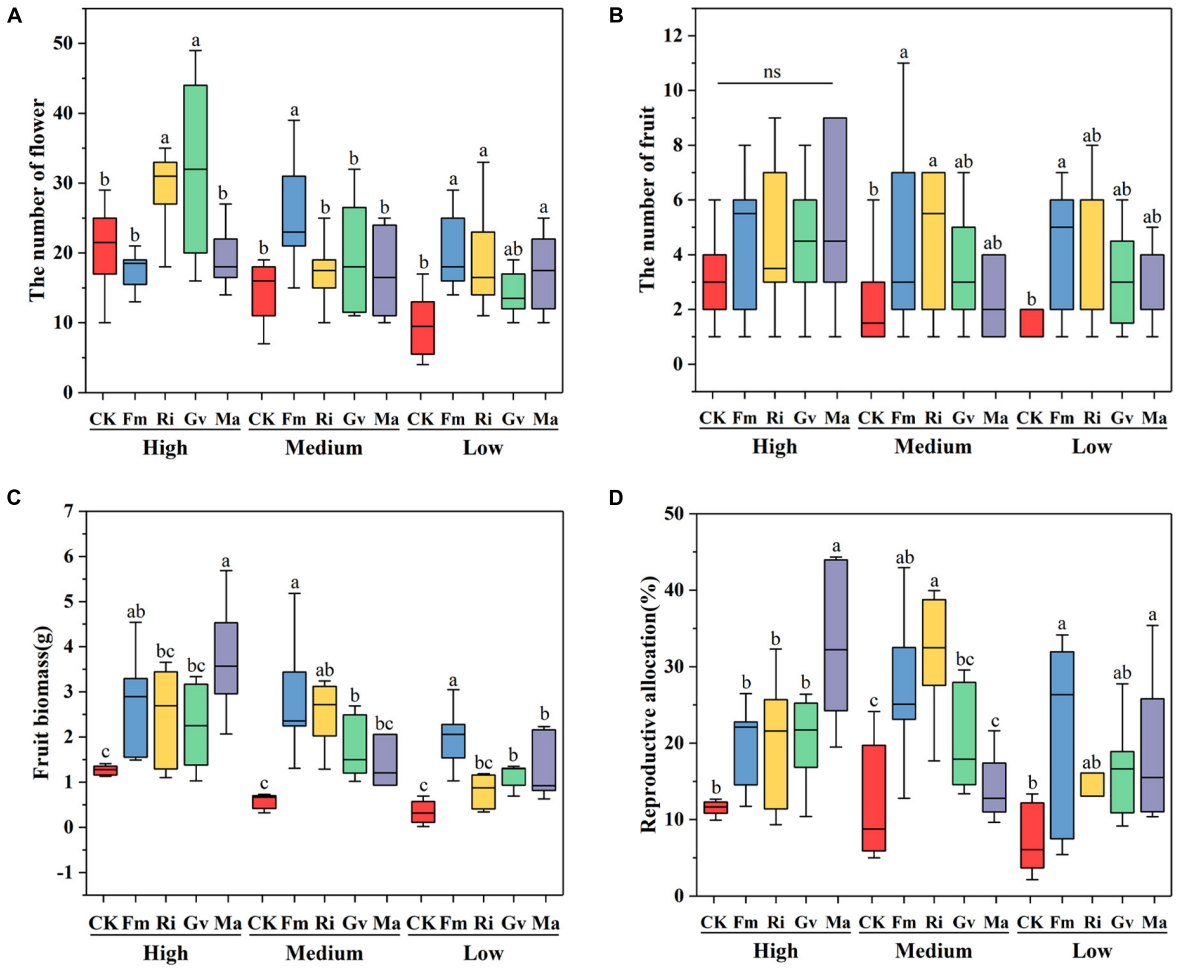


FIGURE 4 | Effects of AMF inoculation on flower number **(A)**, fruit number **(B)**, fruit biomass **(C)**, and reproductive allocation **(D)** of cherry tomato under different nutrient levels.

limited by AMF species. The earlier flowering of plants is mainly promoted by a large amount of phosphorus provided to the host plant after AMF inoculation (Oehl et al., 2011; Kazadi et al., 2021). However, this is not the only reason. According to the C/N ratio theory, the formation of flower organs in plants is controlled by the ratio of sugar content to nitrogen content (C/N) in plants (Kraus and Kraybili, 1918). Because photosynthesis is also affected by nitrogen content (Evans, 1983), the nitrogen supply provided by AMF after symbiosis with plants can also promote the flowering of plants, which is consistent with the results of nitrogen accumulation. On the other hand, flower bud differentiation is also affected by hormone levels and effective assimilate interactions in plants. Some AMF can promote the uptake of potassium in plants, and increase the synthesis of auxin (IAA) and gibberellin (GA) in plants, thus inducing flower bud formation and flowering earlier (Asmelash et al., 2016; Liu et al., 2018). Flower diameter and weight seem to be affected only under medium nutrient levels inoculated by *F. mosseae*, while under the low nutrient level, inoculation with *F. mosseae* significantly promoted pollen viability and pollen number of cherry tomato. This effect may be because AMF provides more phosphorus and potassium to plants and thus increases plant investment in floral organs under the low nutrient level. It is well known that the formation of flower and pollen require phosphorus and potassium. AMF may contribute to flower production by providing both phosphorus and potassium to plants during flowering (Conversa et al., 2015; Pereyra et al., 2019). Increasing pollen number and viability under low nutrient levels may also be a reproductive strategy for plants to symbiosis with AMF when nutrients are scarce, which may be more conducive to the continuation of plant offspring under nutrient deficiency conditions.

We also found that inoculation of AMF has a positive effect on the flower and fruit set of cherry tomato. Especially at low nutrient levels, the promotion effect of AMF inoculation on flower numbers was more obvious. The result of this positive effect was that cherry tomatoes may have more fruit and increased the biomass of the fruit, which was also proved in our study (**Figure 4**). For most flowering plants, the number of flowers is proportional to the biomass due to resource restriction.

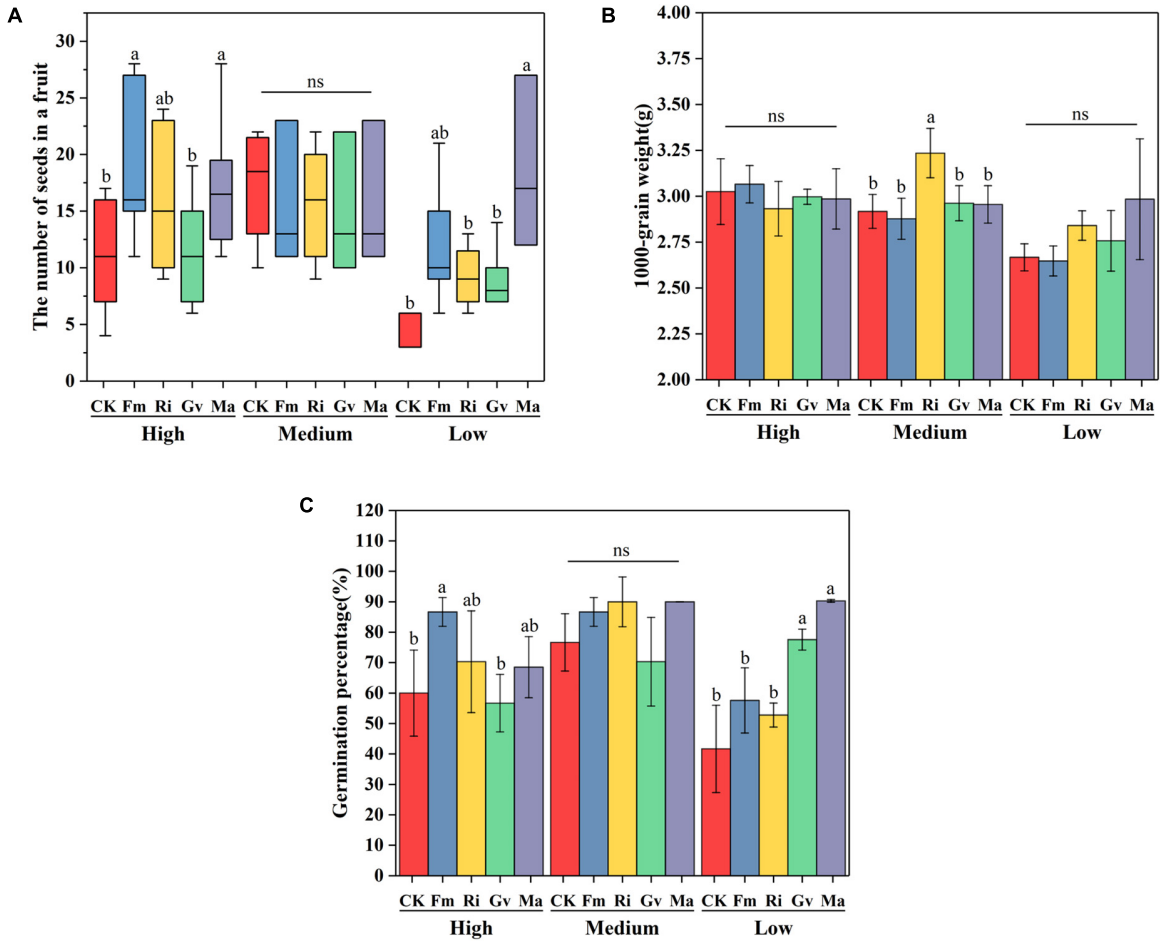


FIGURE 5 | Effects of AMF inoculation on seed number in a fruit **(A)**, seed 1,000-grain weight **(B)**, and seed germination percentage **(C)** of cherry tomato under different nutrient levels.

Phosphorus is one of the most important limiting factors. The symbiosis of AMF can ameliorate the restriction of resources, especially phosphorus, on plant growth. When nutrients are relatively scarce, AMF inoculation can increase the number of flowers of tomato (David and Roger, 1990; Püschel et al., 2014). Moreover, the increase in flower number may also be caused by the increased concentration of photosynthetic products and plant hormones regulated by AMF (Bona et al., 2017). In particular, the increase of the content of gibberellin (GA) in plants can make plants produce more flowers (Bona et al., 2015). We found that the number of cherry tomato fruits did not seem to be directly affected by the number of flowers, although the number of flowers was a major factor affecting the number of cherry tomato fruits. In this study, the increase in flower numbers of the cherry tomato did not directly increase fruit numbers under high nutrient conditions. In addition, the effects of different AMF inoculation on the number of cherry tomato flowers and fruits were also different under different nutrient conditions. The fruit develops from the ovary or together with other parts of the flower. The growth of the ovary relies mainly on a large number of hormones and nutrients. The phosphate and the production of regulating hormones provided by AMF after symbiosis can promote the growth and development of the ovary (Zhang et al., 2014; Hart et al., 2015; Battini et al., 2016; Bona et al., 2017). Nevertheless, the effects of different nutrient levels on cherry tomato reproduction under different AMF inoculation treatments were different. This suggests that changes in fruit yield may be an adaptive strategy for cherry tomato to tradeoff between AMF infection and soil nutrient resource restriction.

Effects of Arbuscular Mycorrhizal Fungi on the Reproductive Allocation of Cherry Tomato Under Different Nutrient Conditions

We found that inoculation of single AMF species did not seem to promote the reproductive investment of cherry tomato at a high nutrient level. But the reproductive allocation of cherry tomato increased after being inoculated with the mixed AMF species. It suggests that communities containing multiple AMF species may jointly promote the reproductive investment of cherry tomato under natural conditions. Our results also confirmed that the

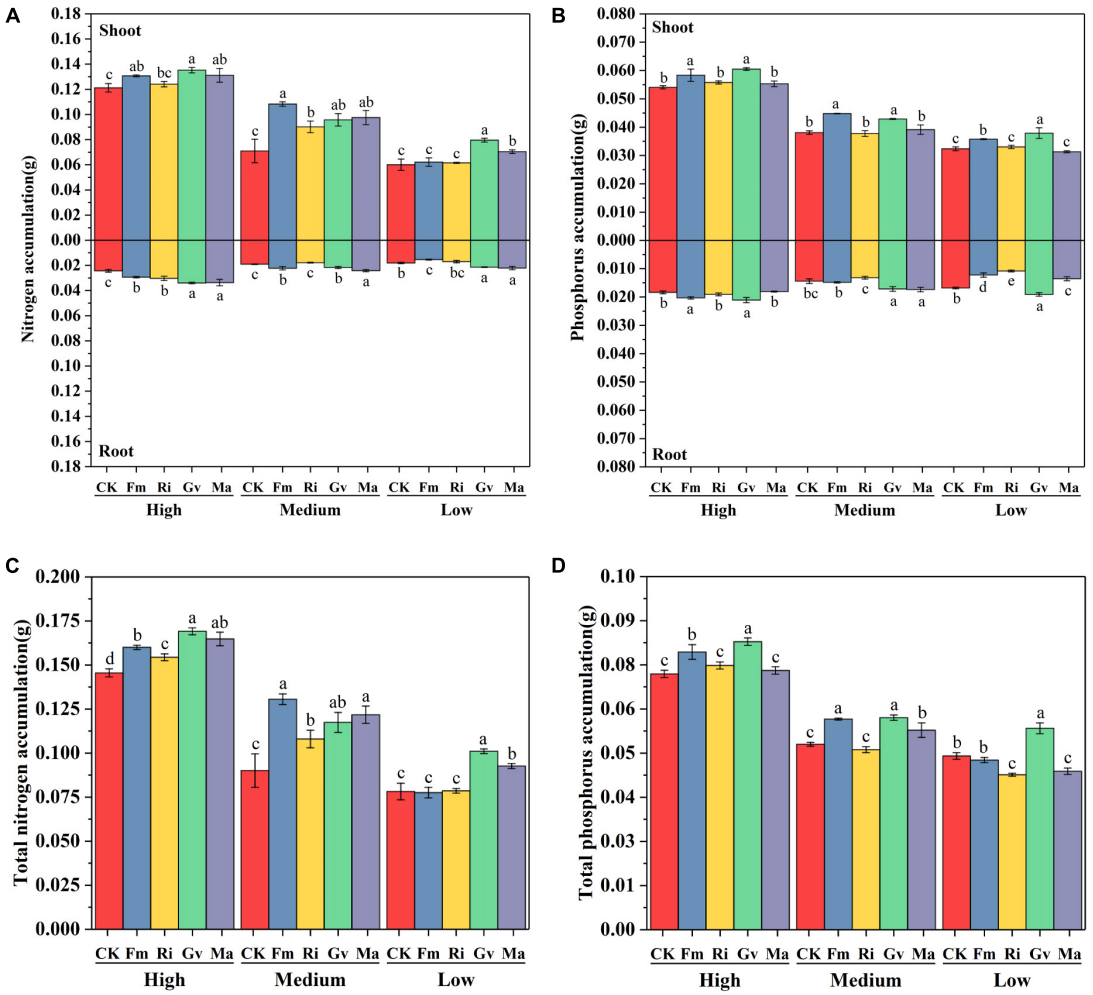


FIGURE 6 | Effects of AMF inoculation on nitrogen accumulation **(A)**, phosphorus accumulation **(B)**, total nitrogen accumulation **(C)**, and total phosphorus accumulation **(D)** of aboveground and belowground cherry tomato under different nutrient levels.

mixed AMF species inoculation treatment (Ma) also increased the reproductive allocation at the low nutrient level. The results indicated that the extracellular mycelia formed after the infection of cherry tomato with multiple AMF greatly increased the decomposition and utilization efficiency of unavailable nutrients in the soil, thus increasing the uptake of nitrogen and phosphorus by cherry tomato and increasing the resource investment in reproduction (Barrett et al., 2011; Schnepf et al., 2011; Zhang et al., 2014). For single AMF inoculation, the effect of *F. mosseae* was the most prominent, which significantly increased the reproductive allocation of cherry tomato at medium and low nutrient levels. This also fully indicated that when the growth and reproduction of cherry tomato were stressed by nutrients, the functions of different AMF inoculation treatments were different. *F. mosseae* had a stronger ability to relieve such stress, which can promote the increase of reproductive allocation and improve the reproductive success of cherry tomato.

In our study, we found that mixed AMF inoculations (Ma) increased seed number in two extreme soil environments (high and low nutrient), which also appears to be phosphorus-driven (Bona et al., 2017). However, it is very interesting to note that although inoculation with *R. intraradices* at the medium nutrient level did not affect the cherry tomato seed number, it did affect the weight of the seeds. This suggests that AMF inoculation may increase the reproductive allocation of the host plant by putting more resources into offspring seed (such as increasing seed diameter and nutrient content) (Liu et al., 2018). The germination percentage of cherry tomato seeds increased after AMF inoculation at a low nutrient level, because the seed germination process was affected by endogenous hormone levels and organic matter accumulation (Li et al., 2012; Han and Yang, 2015). The improvement of seed germination percentage can prove that AMF can promote plant resource investment in seeds, which indicates that AMF may improve the ability of plants to sexually reproduce in an adverse environment. Therefore, the effects of the same/different AMF inoculation under different nutrient conditions are also different, which may be driven by the context-dependency of AMF–plant symbiosis (such as

phosphorus content or phosphorus–nitrogen ratio) or influenced by the functional differences of AMF under heterogeneous soil conditions (Johnson et al., 2015).

Effects of Arbuscular Mycorrhizal Fungi on Nitrogen and Phosphorus Accumulation of Cherry Tomato Under Different Nutrient Conditions

Our study found that AMF enhanced total nitrogen accumulation of cherry tomato at high and medium nutrient levels, while at a low nutrient level, promotion effects of AMF inoculation excluding *F. mosseae* on total nitrogen accumulation seemed to disappear. Cherry tomato inoculated with *F. mosseae* reduced root nitrogen accumulation at a low nutrient level. Different AMF species showed a great difference in influencing phosphorus accumulation. We found that *F. mosseae* and *G. versiforme* had a significant promotion effect on the accumulation of phosphorus in the aboveground of cherry tomato. When cherry tomato was inoculated with AMF under low nutrient level, some AMF inhibited the absorption and accumulation of phosphorus in the root system and decreased the total accumulation of phosphorus. The allocation pattern of nutrients in the aboveground and belowground of plants is a concrete embodiment of the survival strategy of maximizing the utilization of plant resources. The infection of AMF on cherry tomato changed nutrient allocation patterns in aboveground and belowground parts. AMF extracellular mycelium can accelerate the mineralization of organic phosphorus and the dissociation of hard-to-melt phosphorus in the soil, making it easier for plants to absorb. AMF extracellular mycelium also can promote the absorption of ammonia nitrogen and nitrate nitrogen by plants (Leigh et al., 2009; Schnepf et al., 2011). The effect of AMF on plant nutrient absorption was affected by soil nutrient differences and showed different promoting effects. When nutrient conditions were good, plants could absorb nutrients together through roots and mycelia, while in a nutrient-deficient environment, the presence of AMF increased the belowground energy consumption of plants, resulting in a decrease in the ability of plants to accumulate nutrients. Our results indicate that functional differences of AMF lead to variations in the nutrient accumulation in cherry tomato, and such variations are also affected by nutrient levels.

CONCLUSION

This study showed that cherry tomato growth and reproduction were closely related to soil nutrient content and AM symbiosis. The function of AM symbiosis also varies with soil nutrient levels. We found that AM symbiosis had direct promoting effects on the growth and nutrient accumulation of cherry tomato, and confirmed that AM symbiosis promoted flower and seed setting of cherry tomato. In addition, the phenotypic response of the growth and reproduction of cherry tomato to mycorrhizal symbiosis under different nutrient conditions was revealed, and the promoting effects of different AM fungi on cherry tomato growth were found to be different under heterogeneous conditions. It proved that different nutrient levels in soil determined the difference of cherry tomato's benefits from mycorrhizal symbiosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LW: experiment, data curation, methodology, visualization, writing-original draft, writing-review, and editing. XC, YD, and DZ: experiment and data curation. ZT: funding acquisition, supervision, resources, review, and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the National Natural Science Foundation of China (grant no. 31470446).

ACKNOWLEDGMENTS

We are grateful to the reviewers who helped improve the article with constructive comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.843010/full#supplementary-material

REFERENCES

Adeyemi, N. O., Atayese, M. O., Sakariyawo, O. S., Azeez, J. O., Olubode, A. A., Ridwan, M., et al. (2021). Influence of different arbuscular mycorrhizal fungi isolates in enhancing growth, phosphorus uptake and grain yield of soybean in a phosphorus deficient soil under field conditions. *Commun. Soil Sci. Plant Anal.* 52, 1171–1183. doi: 10.1080/00103624.2021.1879117

Asmelash, F., Bekele, T., and Birhane, E. (2016). The potential role of arbuscular mycorrhizal fungi in the restoration of degraded lands. *Front. Microbiol.* 7:1095. doi: 10.3389/fmicb.2016.01095

Augé, R. M., Toler, H. D., and Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25, 13–24. doi: 10.1007/s00572-014-0585-4

Averill, C., Dietze, M. C., and Bhatnagar, J. M. (2018). Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. *Glob. Change Biol.* 24, 4544–4553. doi: 10.1111/gcb.14368

Barrett, G., Campbell, C. D., Fitter, A. H., and Hodge, A. (2011). The arbuscular mycorrhizal fungus *Glomus hoi* can capture and transfer nitrogen from organic patches to its associated host plant at low temperature. *Appl. Soil Ecol.* 48, 102–105. doi: 10.1016/j.apsoil.2011.02.002

Battini, F., Cristani, C., Giovannetti, M., and Agnolucci, M. (2016). Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont *Rhizophagus intraradices*. *Microbiol. Res.* 183, 68–79. doi: 10.1016/j.micres.2015.11.012

Berruti, A., Lumin, E., Balestrini, R., and Bianciotto, V. (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. *Front. Microbiol.* 6:1559. doi: 10.3389/fmicb.2015.01559

Bever, J. D., Dickie, I. A., Facelli, E., Facelli, J. M., Klironomos, J., Moora, M., et al. (2010). Rooting theories of plant community ecology in microbial interactions. *Trends Ecol. Evol.* 25, 468–478. doi: 10.1016/j.tree.2010.05.004

Bona, E., Cantamessa, S., Massa, N., Manassero, P., Marsano, F., Copetta, A., et al. (2017). Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study. *Mycorrhiza* 27, 1–11. doi: 10.1007/s00572-016-0727-y

Bona, E., Lingua, G., Manassero, P., Cantamessa, S., Marsano, F., Todeschini, V., et al. (2015). AM fungi and PGP pseudomonads increase flowering, fruit production, and vitamin content in strawberry grown at low nitrogen and phosphorus levels. *Mycorrhiza* 25, 181–193. doi: 10.1007/s00572-014-0599-y

Brundrett, M. C., Piché, Y., and Peterson, R. L. (1984). A new method for observing the morphology of vesicular-arbuscular mycorrhizae. *Can. J. Bot.* 62, 2128–2134. doi: 10.1139/b84-290

Carmen, B., Amélia, C., Paulo, E. L., and Cinta, C. (2021). On-farm reduced irrigation and fertilizer doses, and arbuscular mycorrhizal fungal inoculation improve water productivity in tomato production. *Sci. Hortic.* 288:110337. doi: 10.1016/j.scienta.2021.110337

Chen, K., Kleijn, D., Scheper, J., and Fijen, T. P. M. (2022). Additive and synergistic effects of arbuscular mycorrhizal fungi, insect pollination and nutrient availability in a perennial fruit crop. *Agric. Ecosyst. Environ.* 325:107742. doi: 10.1016/j.agee.2021.107742

Cheng, L., Booker, F. L., Tu, C., Burkey, K. O., Zhou, L., Shew, H. D., et al. (2012). Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. *Science* 337, 1084–1087. doi: 10.1126/science.1224304

Conversa, G., Bonasia, A., Lazzizera, C., and Elia, A. (2015). Influence of biochar, mycorrhizal inoculation, and fertilizer rate on growth and flowering of *Pelargonium* (*Pelargonium zonale* L.) plants. *Front. Plant Sci.* 6:429. doi: 10.3389/fpls.2015.00429

Cosme, M., and Wurst, S. (2013). Interactions between arbuscular mycorrhizal fungi, rhizobacteria, soil phosphorus and plant cytokinin deficiency change the root morphology, yield and quality of tobacco. *Soil Biol. Biochem.* 57, 436–443. doi: 10.1016/j.soilbio.2012.09.024

David, R. B., and Roger, T. K. (1990). Regulation of reproduction in wild and cultivated *Lycopersicon esculentum* Mill. by vesicular-arbuscular mycorrhizal infection. *Oecologia* 84, 74–81. doi: 10.1007/BF00665598

Doubková, P., Vlasáková, E., and Sudová, R. (2013). Arbuscular mycorrhizal symbiosis alleviates drought stress imposed on *Knautia arvensis* plants in serpentine soil. *Plant Soil* 370, 149–161. doi: 10.1007/s11104-013-1610-7

Evans, J. R. (1983). Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Physiol.* 72, 297–302. doi: 10.1104/pp.72.2.297

Facelli, E., Smith, S. E., Facelli, J. M., Christophersen, H. M., and Smith, F. A. (2010). Underground friends or enemies: model plants help to unravel direct and indirect effects of arbuscular mycorrhizal fungi on plant competition. *New Phytol.* 185, 1050–1061. doi: 10.1111/j.1469-8137.2009.03162.x

Floss, D. S., Levy, J. G., Lévesque-Tremblay, V., Pumplin, N., and Harrison, M. J. (2013). DELLA proteins regulate arbuscule formation in arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 110, E5025–E5034. doi: 10.1073/pnas.1308973110

Fusconi, A. (2014). Regulation of root morphogenesis in arbuscular mycorrhizae: What role do fungal exudates, phosphate, sugars and hormones play in lateral root formation? *Ann. Bot.* 113, 19–33. doi: 10.1093/aob/mct258

Genre, A., Lanfranco, L., Perotto, S., and Bonfante, P. (2020). Unique and common traits in mycorrhizal symbioses. *Nat. Rev. Microbiol.* 18, 649–660. doi: 10.1038/s41579-020-0402-3

Han, C., and Yang, P. (2015). Studies on the molecular mechanisms of seed germination. *Proteomics* 15, 1671–1679. doi: 10.1002/pmic.201400375

Hart, M., Ehret, D. L., Krumbein, A., Leung, C., Murch, S., Turi, C., et al. (2015). Inoculation with arbuscular mycorrhizal fungi improves the nutritional value of tomatoes. *Mycorrhiza* 25, 359–376. doi: 10.1007/s00572-014-0617-0

He, J., Zhang, C., Dai, H., Liu, H., and Wang, E. (2019). A LysM receptor heteromer mediates perception of arbuscular mycorrhizal symbiotic signal in rice. *Mol. Plant.* 12, 1561–1576. doi: 10.1016/j.molp.2019.10.015

Hoeksema, J. D., Chaudhary, V. B., Gehring, C. A., Johnson, N. C., Karst, J., Koide, R. T., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* 13, 394–407. doi: 10.1111/j.1461-0248.2009.01430.x

Jajoo, A., and Mathur, S. (2021). Role of arbuscular mycorrhizal fungi as an underground savior for protecting plants from abiotic stresses. *Physiol. Mol. Biol. Plants* 27, 2589–2603. doi: 10.1007/s12298-021-01091-2

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, J. M. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertil. Soils* 37, 1–16. doi: 10.1007/s00374-002-0546-5

Jiang, S., Liu, Y., Luo, J., Qin, M., Johnson, N. C., Opik, M., et al. (2018). Dynamics of arbuscular mycorrhizal fungal community structure and functioning along a nitrogen enrichment gradient in an alpine meadow ecosystem. *New Phytol.* 220, 1222–1235. doi: 10.1111/nph.15112

Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., et al. (2017). Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science* 356, 1172–1175. doi: 10.1126/science.aam9970

Johnson, N. C., Angelard, C., Sanders, I. R., and Kiers, E. T. (2013). Predicting community and ecosystem outcomes of mycorrhizal responses to global change. *Ecol. Lett.* 16, 140–153. doi: 10.1111/ele.12085

Johnson, N. C., Wilson, G. W. T., Wilson, J. A., Miller, R. M., and Bowker, M. A. (2015). Mycorrhizal phenotypes and the law of the minimum. *New Phytol.* 205, 1473–1484. doi: 10.1111/nph.13172

Kattia, P. L., Brian, B., Samuel, S., and Jane, M. (2015). The ubiquity of phenotypic plasticity in plants: a synthesis. *Ecol. Evol.* 5, 3389–3400. doi: 10.1002/ece3.1603

Kazadi, A. T., Lwalaba, J. L., Ansey, B. K., Muzulukwau, J. M., Katabe, G. M., Karul, M. I., et al. (2021). Effect of phosphorus and Arbuscular Mycorrhizal Fungi (AMF) inoculation on growth and productivity of maize (*Zea mays* L.) in a tropical ferralsol. *Gesunde Pflanz* 74, 159–165. doi: 10.1007/s10343-021-00598-8

Keymer, A., and Gutjahr, C. (2018). Cross-kingdom lipid transfer in arbuscular mycorrhiza symbiosis and beyond. *Curr. Opin. Plant Biol.* 44, 137–144. doi: 10.1016/j.pbi.2018.04.005

Khaekhum, S., Ekprasert, J., Suebrasri, T., Seemakram, W., Monglcolthanaruk, W., Riddech, N., et al. (2021). Co-inoculation of an endophytic and arbuscular mycorrhizal fungus improve growth and yield of *Helianthus tuberosus* L. under field condition. *J. Fungi* 7:976. doi: 10.3390/jof7110976

Kraus, E. J., and Kraybili, H. R. (1918). *Vegetation and Reproduction with Special Reference to the Tomato*. Corvallis, OR: Oregon Agricultural College Experiment Station, 149.

Landis, F. C., Gargas, A., and Givnish, T. J. (2004). Relationships among arbuscular mycorrhizal fungi, vascular plants and environmental conditions in oak savannas. *New Phytol.* 164, 493–504. doi: 10.1111/j.1469-8137.2004.01202.x

Leigh, J., Hodge, A., and Fitter, A. H. (2009). Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytol.* 181, 199–207. doi: 10.1111/j.1469-8137.2008.02630.x

Li, S., Gu, H., Mao, Y., Yin, T., and Gao, H. (2012). Effects of tallow tree seed coat on seed germination. *J. For. Res.* 23, 229–233. doi: 10.1007/s11676-011-0217-1

Liu, S., Guo, H., Xu, J., Song, Z., Song, S., Tang, J., et al. (2018). Arbuscular mycorrhizal fungi differ in affecting the flowering of a host plant under two soil phosphorus conditions. *J. Plant Ecol.* 11, 623–631. doi: 10.1093/jpe/rtx038

Ma, N., Yokoyama, K., and Marumoto, T. (2007). Effect of peat on mycorrhizal colonization and effectiveness of the arbuscular mycorrhizal fungus *Gigaspora margarita*. *Soil Sci. Plant Nutr.* 53, 744–752. doi: 10.1111/j.1747-0765.2007.00204.x

Ma, X., Geng, Q., Zhang, H., Bian, C., Chen, H., Jiang, D., et al. (2020). Global negative effects of nutrient enrichment on arbuscular mycorrhizal fungi, plant diversity and ecosystem multifunctionality. *New Phytol.* 229, 2957–2969. doi: 10.1111/nph.17077

Martínez-García, L. B., de Dios, M. J., and Pugnaire, F. I. (2012). Impacts of changing rainfall patterns on mycorrhizal status of a shrub from arid environments. *Eur. J. Soil Biol.* 50, 64–67. doi: 10.1016/j.ejsobi.2011.12.005

Nouri, E., Breuillin-Sessoms, F., Feller, U., and Reinhardt, D. (2014). Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in *Petunia hybrida*. *PLoS One* 9:e90841. doi: 10.1371/journal.pone.0090841

Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K., and Gladstone, A. D. S. (2011). Advances in Glomeromycota taxonomy and classification. *IMA Fungus* 2, 191–199. doi: 10.5598/imafungus.2011.02.02.10

Pan, S., Wang, Y., Qiu, Y., Chen, D., Zhang, L., Ye, C., et al. (2020). Nitrogen-induced acidification, not N-nutrient, dominates suppressive N effects on arbuscular mycorrhizal fungi. *Glob. Change Biol.* 26, 6568–6580. doi: 10.1111/gcb.15311

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbiosis. *Nat. Rev. Microbiol.* 6, 763–775. doi: 10.1038/nrmicro1987

Pereyra, M., Grilli, G., and Galetto, L. (2019). Root-associated fungi increase male fitness, while high simulated herbivory decreases indirect defenses in *Croton lachnostachyus* plants. *Plant Ecol.* 220, 29–39. doi: 10.1007/s11258-018-0900-9

Pozo, M. J., López-Ráez, J. A., Azcón-Aguilar, C., and García-Garrido, J. M. (2015). Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. *New Phytol.* 205, 1431–1436. doi: 10.1111/nph.13252

Püschel, D., Rydlová, J., and Vosátka, M. (2014). Can mycorrhizal inoculation stimulate the growth and flowering of peat-grown ornamental plants under standard or reduced watering? *Appl. Soil Ecol.* 80, 93–99. doi: 10.1016/j.apsoil.2014.04.001

Rahim, M. A., Rabbi, A. Z., Afrin, K. S., Jung, H. J., Kim, H. T., Park, J. I., et al. (2019). Differential expression pattern of lignin biosynthetic genes in dwarf cherry tomato (*Solanum lycopersicum* var. *cerasiforme*). *Plant Breed. Biotech.* 7, 229–236. doi: 10.9787/PBB.2019.7.3.229

Schnepf, A., Jones, D., and Roose, T. (2011). Modelling nutrient uptake by individual hyphae of arbuscular mycorrhizal fungi: temporal and spatial scales for an experimental design. *Bull. Math. Biol.* 73, 2175–2200. doi: 10.1007/s11538-010-9617-1

Shi, Z., Mickan, B., Feng, G., and Chen, Y. (2015). Arbuscular mycorrhizal fungi improved plant growth and nutrient acquisition of desert ephemeral *Plantago minuta* under variable soil water conditions. *J. Arid Land* 7, 414–420. doi: 10.1007/s40333-014-0046-0

Smith, F. A., and Smith, S. E. (2011). What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? *Plant Soil* 348, 63–79. doi: 10.1007/s11104-011-0865-0

Smith, S. E., Smith, A. F., and Jakobsen, I. (2004). Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytol.* 162, 511–524. doi: 10.1111/j.1469-8137.2004.01039.x

Sutyemez, M. (2011). Pollen quality and pollen production in some almond culvitars under Kaharamanmaras (Turkey) ecological conditions. *Afr. J. Agric. Res.* 6, 12357–12373. doi: 10.5897/AJAR11.504

Van Geel, M., De, B. M., Lievens, B., and Honnay, O. (2016). Crop-specific and single-species mycorrhizal inoculation is the best approach to improve crop growth in controlled environments. *Agron. Sustain. Dev.* 36:37. doi: 10.1007/s13593-016-0373-y

Verlinden, M. S., Ven, A., Verbruggen, E., Janssens, I. A., Wallander, H., and Vicca, S. (2018). Favorable effect of mycorrhizae on biomass production efficiency exceeds their carbon cost in a fertilization experiment. *Ecology* 99, 2525–2534. doi: 10.1002/ecy.2502

Wang, W., Shi, J., Xie, Q., Jiang, Y., Yu, N., and Wang, E. (2017). Nutrient exchange and regulation in arbuscular mycorrhizal symbiosis. *Mol. Plant* 10, 1147–1158. doi: 10.1016/j.molp.2017.07.012

Yang, L., Zou, Y., Tian, Z., Wu, Q., and Kua, K. (2021). Effects of beneficial endophytic fungal inoculants on plant growth and nutrient absorption of trifoliate orange seedlings. *Sci. Hortic.* 277:109815. doi: 10.1016/j.scienta.2020.109815

Yang, S., Grønlund, M., Jakobsen, I., Grotemeyer, M. S., Rentsch, D., Miyao, A., et al. (2012). Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the *PHOSPHATE TRANSPORTER1* gene family. *Plant Cell* 24, 4236–4251. doi: 10.1105/tpc.112.104901

Zhang, L., Fan, J., Ding, X., He, X., Zhang, F., and Feng, G. (2014). Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biol. Biochem.* 74, 177–183. doi: 10.1016/j.soilbio.2014.03.004

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ORIGINAL RESEARCH
published: 25 April 2022
doi: 10.3389/fmicb.2022.855890

Interactive Effects of *Epichloë* Endophytes and Arbuscular Mycorrhizal Fungi on Saline-Alkali Stress Tolerance in Tall Fescue

Hui Liu, Huimin Tang, Xiaozhen Ni, Yajie Zhang and Yingchao Wang*

College of Life Sciences, Dezhou University, Dezhou, China

Epichloë endophytes and arbuscular mycorrhizal fungi (AMFs) are two important symbiotic microorganisms of tall fescue (*Lolium arundinaceum*). Our research explores the combined effects of endophytes and AMF on saline-alkali stress. The finding revealed that a significant interaction between *Epichloë* endophytes and AMF, and saline-alkali stress occurred in the growth and physiological parameters of tall fescue. Endophyte infection significantly enhanced tall fescue resistance to saline-alkali stress by increasing shoot and root biomass and nutrient uptake (organic carbon, total nitrogen, and total phosphorus concentration), and accumulating K^+ while decreasing Na^+ concentration. Furthermore, the beneficial effect of endophytes was enhanced by the beneficial AMF, *Claroideoglomus etunicatum* (CE) but was reduced by the detrimental AMF, *Funneliformis mosseae* (FM). Our findings highlight the importance of interactions among multiple microorganisms for plant performance under saline-alkali stress.

Keywords: endophyte, arbuscular mycorrhizal fungi, interaction, tall fescue, saline-alkali stress

OPEN ACCESS

Edited by:
George Newcombe, University of Idaho, United States

Reviewed by:
Kamlesh Kumar Meena, National Institute of Abiotic Stress Management (ICAR), India
Ajit Varma, Amity University, India

****Correspondence:***
Hui Liu
zhangxiushuang522@163.com

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *16 January 2022*
Accepted: *10 March 2022*
Published: *25 April 2022*

Citation:
Liu H, Tang H, Ni X, Zhang Y and Wang Y (2022) Interactive Effects of Epichloë Endophytes and Arbuscular Mycorrhizal Fungi on Saline-Alkali Stress Tolerance in Tall Fescue. Front. Microbiol. 13:855890. doi: 10.3389/fmicb.2022.855890

INTRODUCTION

Epichloë endophytes [Clavicipitaceae, Hypocreales, and Ascomycota] are endophytic fungi that can infect and coexist with above-ground portions of host plants and have been shown to impact their tolerance to certain biotic and abiotic stresses such as drought, low nitrogen, salt, cold, heat, heavy metals, insects, nematodes, and diseases by either enhancing the fitness and productivity of host plants or producing a range of alkaloids and other secondary metabolites (Schardl et al., 2004; Wang et al., 2021). In recent years, there has been a lot of research interest in the salt tolerance of hosts bearing *Epichloë* endophytes (Rodriguez et al., 2008; Reza and Mirlohi, 2010). Some studies found that under salt stress, endophyte infection enhanced growth, activity of enzymes of nitrogen metabolism, nitrogen use efficiency, and photosynthetic ability (Wang et al., 2019), and that *Hordeum brevisubulatum* infected with *Epichloë bromicola* had greater growth, including improved antioxidant potential, increased nutrient absorption, and osmotic and ionic adjustment (Song et al., 2015; Chen T. X. et al., 2018). Endophytes, on the other hand, may also decrease the salt tolerance of their hosts. For example, Ren et al. (2006) demonstrated that endophyte infection significantly decreased tiller number under high salt stress. Another study indicated that

endophytes did not influence the biomass production of *Festuca rubra* under soil salinity conditions (Zabalgogeazcoa et al., 2006).

Plants can also deal with soil environmental stress by interacting with arbuscular mycorrhizal fungi (AMFs), which are members of the Glomeromycota phylum and form mutualistic relationships with more than 80% of terrestrial plant roots belowground and are found in saline and alkaline soils (Aliasgharzadeh et al., 2001; Wilde et al., 2009). Numerous studies have indicated that AMFs play an important role in improving host plant saline-alkaline stress tolerance by improving nutrient and water uptake, maintaining ion balance, increasing photosynthetic efficiency, and inducing the antioxidant defense system (Estrada et al., 2013; Abd-Allaa et al., 2019; Ben-Laouane et al., 2020; Moreira et al., 2020; Yang et al., 2020). In addition, Qiu et al. (2020) found that outcomes of salt stress tolerance of plants varied with AMF species identity. In addition, Parihar et al. (2020) indicated that multi-species AMF inoculation was superior to single AMF inoculation in improving plant production under salt stress conditions.

In grass species, dual infection of a host plant with leaf endophytes and below-ground AMFs is common. Interactions between grass endophytes and AMFs are complex, and studies focused on the two have been inconclusive based on relationships among the plant, AMFs, and grass endophytes. AMF colonization rates could be inhibited (Müller, 2003; Omacini et al., 2006; Mack and Rudgers, 2008) or enhanced (Novas et al., 2005, 2009; Arrieta et al., 2015) by leaf endophytes, and results are dependent on the combination of the AMFs and endophytes as well as soil environmental conditions. Our previous study has discovered that interactions between endophytes and AMFs, as well as their combined effects on growth of host plants depended on both the identity and richness of AMFs (Liu et al., 2019). In addition, Liu et al. (2011) demonstrated that the competition between AMFs and endophytes was determined by resource supply and host carbohydrate content. According to Zhou et al. (2016), tripartite interactions among endophytes, AMFs, and *Achnatherum sibiricum* were influenced by nutrient supply.

Many studies have been conducted to investigate the individual effects of *Epichloë* endophytes and AMFs on the growth of host plants and of the impact of saline-alkaline stress (Song et al., 2015; Chen T. X. et al., 2018; Wang et al., 2019; Moreira et al., 2020; Yang et al., 2020). Research on interactions of the two microorganisms infecting the same host plant is limited, especially regarding saline-alkali stress environments. This study was designed to evaluate the role of *Epichloë* endophytes and AMF species, as well as their interaction effects on the growth of tall fescue (*Lolium arundinaceum*) under saline-alkali stress conditions. As for AMFs, colonization with two AMFs, *Funneliformis mosseae* (FM) and *Claroideoglomus etunicatum* (CE), alone and in combination, as well as non-inoculated (M-), was considered. We analyzed the growth characteristics and physiological parameters of tall fescue. Specifically, we addressed the following questions: (1) does endophyte infection ameliorate saline-alkali stress performance of the host, and (2) when endophyte and AMF infections are both present, is the effect of endophyte infection on the host's growth and saline-alkali tolerance influenced by the AMF?

MATERIALS AND METHODS

Plants and Fungi

Endophyte-infected (E+) tall fescue seeds were naturally infected with *Epichloë coenophialum* (Morgan-Jones and Gams, 1982; Leuchtmann et al., 2014), and uninfected (E–) seeds were acquired by eliminating the endophyte by long-term storage of E+seeds at room temperature. This procedure reduces the viability of the endophyte but not the seeds (Clay and Holah, 1990). The seeds used in this experiment were several generations removed from the storage treatment and came from freely cross-pollinated field-grown parents. The E+ and E– seeds were provided by Professor Anzhi Ren at Nankai University. The E+ and E– seeds were sown in 160 pots (80 pots for E+ and 80 pots for E– plants), with 20 seeds per pot, in sterilized vermiculite. After germination, the seedlings were thinned to 15 per pot (21 cm in diameter × 16 cm in height). The endophyte status of the plants was checked both immediately before and after the experiment by microscopic examination of leaf sheaths stained with aniline blue, as described by Latch and Christensen (1985).

FM and CE were used in this study, and these two fungi were prepared by trap culture in pots using *Sorghum bicolor* as the host plant under controlled greenhouse conditions for 3 months. The soil was then dried, and the roots were cut into < 1-cm pieces, which were subsequently homogenously mixed. The amount of inoculum for single species inoculation (FM or CE) was 100 g per pot; for the FM + CE treatment, the amount was 50 g for each AMF per pot. The M- treatment received a 100-g autoclaved inoculum and 50 ml of a non-autoclaved inoculum filtrate (passed through a 10-μm sieve) to correct for possible differences in the microbial community between the AMF and M- treatments.

Experimental Design

The experiment was conducted from October 15, 2020 to February 15, 2021 based on a fully crossed 2 × 4 × 4 factorial design. The first factor, endophyte infection status, contained two levels: E+ and E–. The second factor, AMF inoculum, contained four levels: M-, inoculation with FM, inoculation with CE, and a combination inoculum of FM + CE. The third factor, saline-alkali treatment, contained four intensities: 0, 200, 400, and 600 mM. Four salts, NaCl, Na_2SO_4, $NaHCO_3$, andNa_2CO_3, were mixed in 9:1:1:9 M ratios to simulate a range of mixed saline-alkali stress conditions. There were 32 treatment combinations in total, and each was repeated five times. During the first 6 weeks, tall fescue seedlings were grown free of saline-alkali to ensure functional mycorrhiza and to avoid the effects of saline-alkali stress on fungi development (Feng et al., 2002). The seedlings were treated with modified 1/2 strength Hoagland nutrient solution supplemented with additional 0–, 200–, 400–, and 600-mM, as well as with 50 mM saline-alkali on the first and second days to avoid saline-alkali shock. Final concentrations of saline-alkali in each treatment were applied from the third day onward. Every 3 days, the treatment solution was replaced to maintain consistent stress conditions, and the position of each pot was changed randomly.

Harvest and Measurements

After 8 weeks of saline-alkali treatment, the shoots and roots were separated and harvested. Each pot's roots were washed and divided into two sub-samples. AMF colonization was measured using a sample of approximately 2 g that was cleared in 10% KOH and stained in 1% trypan blue (Phillips and Hayman, 1970). AMF colonization rate was recorded using the cross-hair eyepiece method under a dissecting microscope at 40 × magnification (McGonigle et al., 1990). The remainder of the sample was used to calculate biomass and other parameters. The biomass of the plants was determined after roots and shoots were oven dried at 80°C for 24 h. The plants were ground and digested at a high temperature in a PerkinElmer microwave using an HNO_3:HCl mixture (9:1). Phosphorus concentration was measured using acid-dissolved molybdenum, antimony, and scandium colorimetry (Chen et al., 2017). The concentrations of Na^+ and K^+ were assessed using ICP-OES in an Optima 7000 DV spectrophotometer (PerkinElmer, United States). Plant organic carbon (C) concentration in the shoots and roots was measured using the K_2CrO_7-H_2SO_4 oxidation method (Tanveer et al., 2014). Total nitrogen (N) was determined using a dry combustion method and an elemental analyzer (Vario EL/micro cube, Elementar, Hanau, Germany; Zhou et al., 2016).

Statistical Analyses

The effects of *Epichloë* endophytes, AMFs, and saline-alkali stress on AMF colonization rate, plant biomass, nutrient concentration parameters (C, N, and P), and cations (Na^+ and K^+) were analyzed by three-factor ANOVA with SPSS 20.0 (SPSS Inc., Chicago, IL, United States). When a significant effect was detected, differences in means between different treatments were determined by Duncan's multiple range tests at a probability of 0.05. A redundancy analysis (RDA) was performed using *Epichloë* endophytes, AMF treatments, and saline-alkali stress (S) as explanatory variables, with growth (biomass) and physiological (C, N, P, Na^+, and K^+) parameters as response variables. After a Monte Carlo permutation test with 499 permutations, statistical significance was determined by stepwise forward selection.

RESULTS

Arbuscular Mycorrhizal Fungi Colonization Rate

AMF structures were not observed in the roots of M- treatments, whereas plants inoculated with AMF showed 9–63.3% root colonization rate. AMF colonization rate was significantly influenced by the *Epichloë* endophyte × saline-alkali stress interaction (**Table 1**). In the control treatment (0 mM), there was no difference in AMF colonization rate between E– and E+ plants, either with inoculation with FM or CE, or the mixture of FM and CE (FM + CE); however, higher colonization rate was found in E+ than in E– plants when inoculated with CE and the mixture of FM and CE (FM + CE) in the 200–, 400–, and 600-mM saline-alkali stress treatments, and no significant difference was observed between the E+ and E– plants when inoculated with FM (**Figure 1**).

Growth Parameters

The endophytes, AMFs, and saline-alkali stress all had a significant interactive effect on shoot and root biomass (**Table 1**). In the control treatment (0 mM), E+ had comparable shoot and root biomass to the E– plants regardless of AMF inoculation. Under saline-alkali conditions, with the M- treatment, the shoot and root biomass of the E+ plants was greater than that of the E– plants in the 200- and 400-mM saline-alkali stress treatments. When inoculated with AMF alone, FM had a detrimental effect on shoot and root biomass, while CE was found to be beneficial to host growth in the 200- and 400-mM stress treatments. The combination of FM and CE (FM + CE) had a similar effect on host growth as the CE. The growth advantage of E+ relative to the E– plants was reduced by FM but increased by CE and those simultaneously containing the FM and CE mixture (FM + CE) in the 200- and 400-mM saline-alkali treatments. No obvious difference was observed between the E+ and E– plants in the 600-mM saline-alkali treatment regardless of AMF inoculation (**Figures 2A,B**).

Carbon, Nitrogen, and Phosphorus Concentrations

C concentration in the shoots and roots showed no significant difference between the E+ and E– plants in the control treatment (0 mM). However, in the M- treatment, C concentration under stress (200, 400, and 600 mM) conditions was higher in E+ than in the E– plants in both the shoots and roots. FM inoculation significantly decreased C concentration in the shoots and roots of both the E+ and E– plants, and there was no difference between the E+ and E– plants when inoculated with FM either in the 200–, 400–, or 600-mM saline-alkali treatment. Unlike FM, the E+ plants had a higher C concentration in the shoots and roots than the E– plants when inoculated with CE, and a synergistic effect between CE and the endophytes was observed, with C concentration in the shoots and roots of host plants simultaneously infected by CE and endophytes being significantly greater than that of plants infected with either CE or the endophytes separately. The combination of FM and CE (FM + CE) had an effect on C concentration similar to that of CE (**Table 2** and **Figures 3A,B**).

Saline-alkali stress significantly decreased the N and P concentration in the shoots and roots, and the decrease was significantly influenced by *Epichloë* endophyte × AMF (**Table 2**). In shoots and roots, the E+ plants had approximately 37 and 31% higher N concentrations, and 41 and 33% P concentrations, respectively, than the E– plants. The beneficial effects of endophytes were inhibited by the detrimental AMF, FM, and promoted by the beneficial AMF, CE, and the mixture of FM and CE (FM + CE) in the stress treatments (**Figures 4** and **5**).

Na^+ and K^+ Concentration

The results showed that as the stress concentrations increased, the Na^+ concentration in the shoots and roots of tall fescue

TABLE 1 | Three-way ANOVA for the effects of endophytes (E), arbuscular mycorrhizal fungi (AMFs, M), and saline-alkali stress (S) on AMF colonization rate, biomass, Na^+ and K^+ in shoots and roots of tall fescue.

	AMF colonization rate	Biomass		Na^+		K^+	
		Shoot	Root	Shoot	Root	Shoot	Root
Endophytes (E)	37.768***	30.789***	141.524***	77.542***	1667.281***	98.574***	37.151***
AMF (M)	20.784***	109.944***	217.096***	126.138***	1201.875***	219.637***	58.935***
Saline-alkali (S)	9.075***	595.509***	1080.803***	2348.554***	17194.196***	1467.438***	232.668***
E × M	10.995***	8.433***	11.416***	37.741***	205.126***	19.442***	3.913***
E × S	5.149**	11.877***	43.068***	9.015***	134.329***	13.780***	1.742^{ns}
M × S	0.271^{ns}	12.315***	50.434***	21.773***	195.896***	23.651***	1.877^{ns}
E × M × S	1.051^{ns}	2.677*	3.668**	6.323***	52.708***	1.293^{ns}	1.086^{ns}

*The numeric data in the table is F-value. *, **, ***, and ns represent significance at the P < 5, 1, and 0.1% levels and non-significance, respectively.*

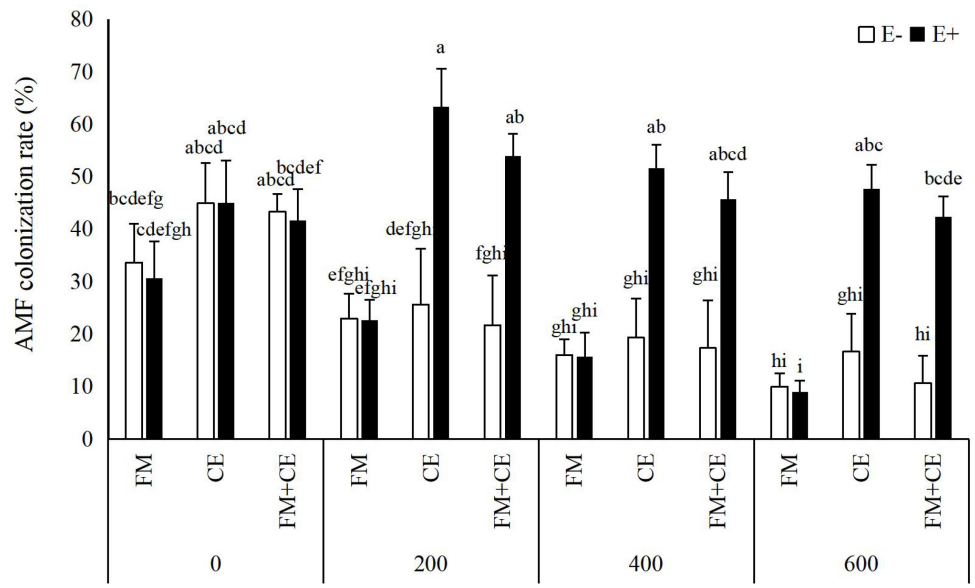


FIGURE 1 | Arbuscular mycorrhizal fungi (AMF) colonization rate of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress. Values are means ± SE. Different letters denote means that are significantly different ($P < 0.05$).

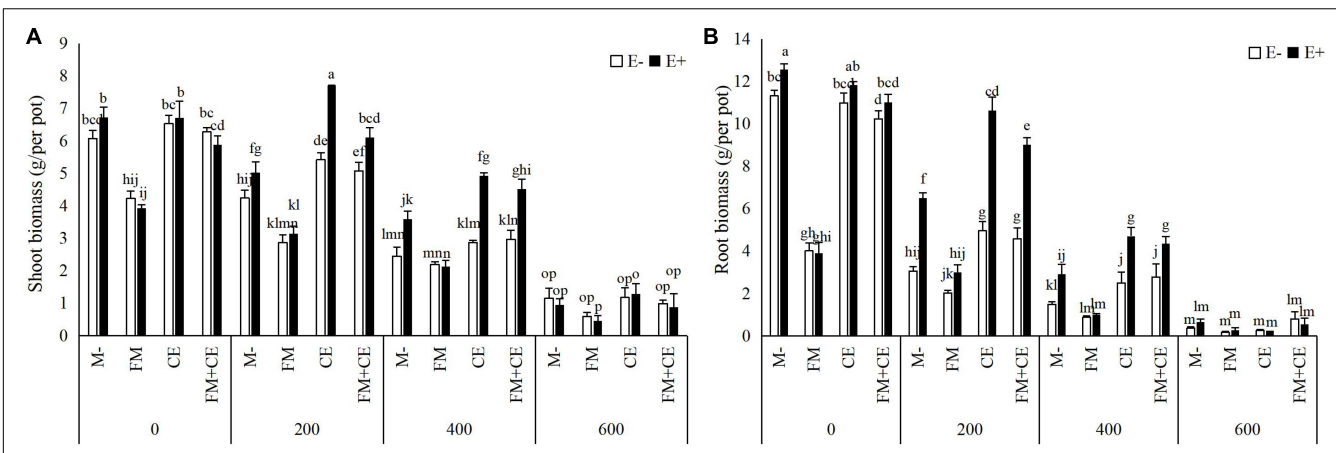


FIGURE 2 | Biomass in shoots and roots of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress. **(A)** shoot biomass, **(B)** root biomass. Values are means ± SE. Different letters denote means that are significantly different ($P < 0.05$).

TABLE 2 | Three-way ANOVA for the effects of endophytes (E), AMFs (M), and saline-alkali stress (S) on C, N, and P concentrations in shoots and roots of tall fescue.

	C concentration		N concentration		P concentration	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
Endophytes (E)	203.822***	546.680***	290.541***	178.755***	818.466***	84.781***
AMF (M)	362.740***	1458.130***	425.737***	565.720***	1780.575***	336.368***
Saline-alkali (S)	488.028***	536.472***	272.580***	283.243***	15.725***	73.061***
E × M	41.748***	42.618***	16.169***	20.557***	111.896***	10.765***
E × S	31.284***	62.494***	28.369***	28.032***	46.050***	2.383^{ns}
M × S	24.002***	14.596***	18.059***	56.267***	142.904***	4.155***
E × M × S	7.830***	7.876***	11.952***	5.721***	11.976***	2.306*

*The numeric data in the table is F-value. *, **, *** and ns represent significance at the P < 5, 1, and 0.1% levels and non-significance, respectively.*

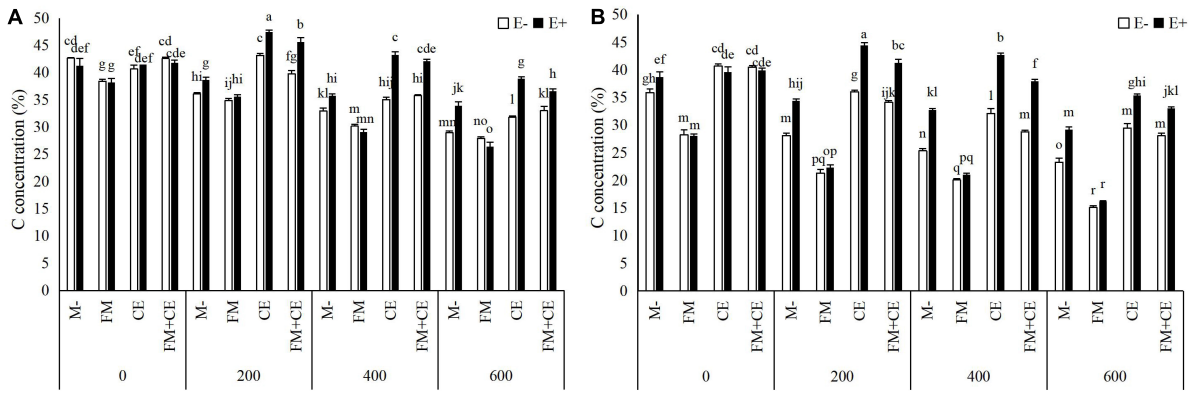


FIGURE 3 | Carbon (C) concentration in shoots and roots of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress. **(A)** shoot C concentration, **(B)** root C concentration. Values are means ± SE. Different letters denote means that are significantly different ($P < 0.05$).

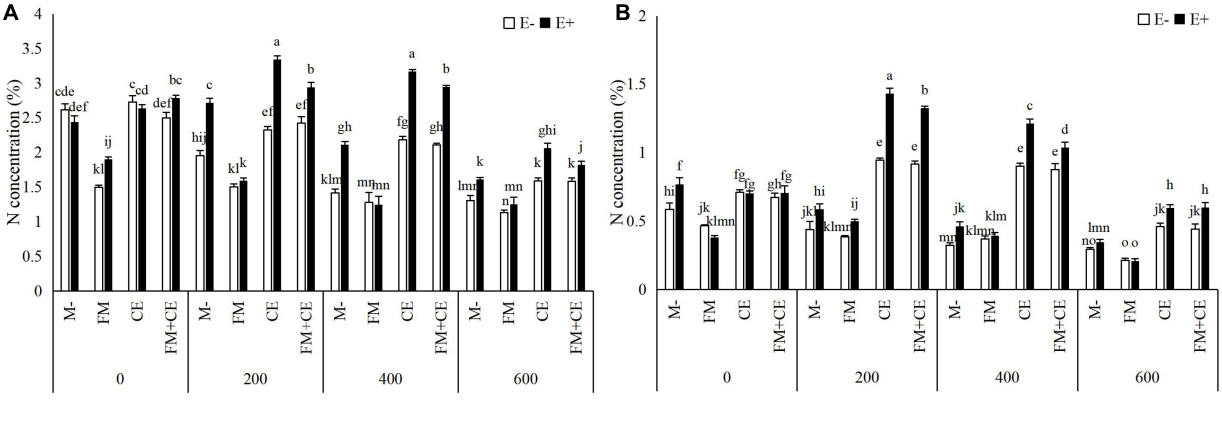


FIGURE 4 | Nitrogen (N) concentration in shoots and roots of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress. **(A)** shoot N concentration, **(B)** root N concentration. Values are means ± SE. Different letters denote means that are significantly different ($P < 0.05$).

increased. However, *Epichloë* endophyte presence alleviated this change, with the E+ plants having significantly lower Na^+ concentrations in the shoots and roots than the E– plants in the M- treatment. FM inoculation increased Na^+ concentration when compared to the M- treatment, especially in the roots. CE inoculation decreased Na^+ concentration when compared to the M- treatment. The mixture of FM and CE (FM + CE) had an effect similar to that of CE. In addition, there was a significant interaction between the endophytes and AMF species identity on Na^+ concentration, with the E+ plants having higher shoot and root Na^+ concentrations than the E– plants when inoculated with FM but lower shoot and root Na^+ concentrations when

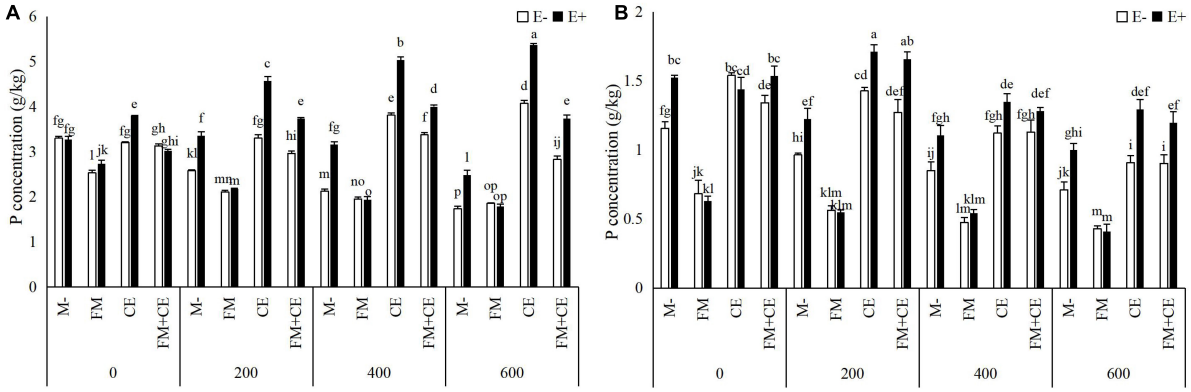


FIGURE 5 | Phosphorus (P) concentration in shoots and roots of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress. **(A)** shoot P concentration, **(B)** root P concentration. Values are means ± SE. Different letters denote means that are significantly different ($P < 0.05$).

TABLE 3 | Na^+ and K^+ concentrations in shoots and roots of tall fescue with (E+) and without (E–) endophytes and colonized with (AMF) and without (M-) AMF under saline-alkali stress (mean ± SE, $n = 3$).

		Na^+				K^+			
		Shoot		Root		Shoot		Root	
		E–	E+	E–	E+	E–	E+	E–	E+
0	M-	0.19 ± 0.01m	0.20 ± 0.01m	0.22 ± 0.01r	0.19 ± 0.01st	3.37 ± 0.11ab	3.39 ± 0.10ab	1.73 ± 0.08cd	1.79 ± 0.03bc
	FM	0.16 ± 0.01m	0.17 ± 0.01m	0.18 ± 0.01st	0.18 ± 0.01t	3.21 ± 0.01bcd	3.11 ± 0.00de	1.48 ± 0.05efgh	1.57 ± 0.06defg
	CE	0.22 ± 0.01m	0.17 ± 0.00m	0.20 ± 0.01rs	0.15 ± 0.01u	3.31 ± 0.02abc	3.42 ± 0.07a	2.09 ± 0.03a	2.03 ± 0.04a
	FM + CE	0.14 ± 0.02m	0.15 ± 0.02m	0.15 ± 0.01u	0.15 ± 0.01u	3.27 ± 0.05abcd	3.16 ± 0.09cd	1.98 ± 0.07ab	2.09 ± 0.06a
200	M-	0.95 ± 0.05ij	0.83 ± 0.01jk	0.75 ± 0.01j	0.53 ± 0.00n	2.04 ± 0.05hij	2.49 ± 0.02f	1.31 ± 0.15hij	1.57 ± 0.12defg
	FM	1.08 ± 0.04hi	1.18 ± 0.04gh	0.82 ± 0.01hi	0.85 ± 0.01g	2.05 ± 0.01hij	1.96 ± 0.07ij	1.26 ± 0.06hijkl	1.26 ± 0.05hijkl
	CE	1.00 ± 0.07i	0.63 ± 0.08l	0.56 ± 0.00n	0.40 ± 0.01q	2.49 ± 0.09f	2.95 ± 0.02e	1.55 ± 0.07def	1.81 ± 0.05bc
	FM + CE	1.07 ± 0.04hi	0.75 ± 0.02k	0.62 ± 0.01l	0.42 ± 0.00pq	2.23 ± 0.06gh	2.57 ± 0.04f	1.42 ± 0.05fgh	1.65 ± 0.07cde
400	M-	1.34 ± 0.06f	0.99 ± 0.09i	0.92 ± 0.01e	0.62 ± 0.01l	1.51 ± 0.03kl	1.89 ± 0.03j	1.17 ± 0.10ijklm	1.31 ± 0.05hij
	FM	1.26 ± 0.00fg	1.64 ± 0.05d	0.83 ± 0.00ghi	0.81 ± 0.00i	1.34 ± 0.12lm	1.29 ± 0.05m	1.11 ± 0.04jklmn	1.07 ± 0.05klmno
	CE	1.07 ± 0.03hi	0.77 ± 0.03k	0.60 ± 0.00m	0.44 ± 0.00p	2.15 ± 0.04h	2.55 ± 0.04f	1.37 ± 0.10ghi	1.63 ± 0.07cdef
	FM + CE	1.14 ± 0.05gh	0.85 ± 0.03jk	0.70 ± 0.01k	0.51 ± 0.00o	2.12 ± 0.06hi	2.39 ± 0.04fg	1.14 ± 0.10jklm	1.42 ± 0.09fgh
600	M-	2.01 ± 0.02b	1.75 ± 0.02cd	1.19 ± 0.03a	1.15 ± 0.01b	1.10 ± 0.06n	1.54 ± 0.07k	0.89 ± 0.01op	1.07 ± 0.09lmno
	FM	2.31 ± 0.03a	2.36 ± 0.10a	1.09 ± 0.01c	1.10 ± 0.01c	0.81 ± 0.03o	0.82 ± 0.05o	0.82 ± 0.01p	0.79 ± 0.01p
	CE	1.78 ± 0.02c	1.46 ± 0.03e	1.05 ± 0.01d	0.84 ± 0.01gh	1.52 ± 0.06kl	1.95 ± 0.02ij	0.99 ± 0.01mnop	1.29 ± 0.01hijk
	FM + CE	1.95 ± 0.05b	1.68 ± 0.05cd	1.17 ± 0.01b	0.89 ± 0.01f	1.46 ± 0.04klm	1.86 ± 0.09j	0.92 ± 0.04nop	1.16 ± 0.05ijklm

inoculated with CE and that containing both FM and CE mixture (FM + CE; **Tables 1** and **3**).

A decrease in K^+ concentration was observed in the shoots and roots as the stress concentrations increased. However, the presence of the endophytes alleviated the decrease, particularly in shoot K^+ concentration. In the M- treatment, the E+ plants had significantly higher K^+ concentrations in the shoots in the 200–, 400–, and 600-mM saline-alkali treatments, as well as a higher K^+ concentration in the roots in the 200-mM saline-alkali treatments. FM inoculation had no significant effect on K^+ concentration in shoots and roots of the E– plants but significantly decreased K^+ concentration in shoots and roots of THE E+ plants, resulting in disappearance of the E+ advantage over the E– plants. Under all stress conditions, a synergistic effect occurred between CE and the endophytes, and K^+ concentration in the shoots and roots of host plants simultaneously infected by CE and the endophytes was significantly greater than that of plants infected with either CE or the endophytes separately (**Tables 1** and **3**).

Redundancy Analysis

Three factors, *Epichloë* endophytes, AMFs, and saline-alkali stress (S), as well as growth and physiological parameters of host plants, biomass, nutrients (C, N, and P), and cation (Na^+ and K^+) concentration, were used for RDA to investigate the contributions of both the endophytes and the AMFs to the growth of tall fescue under saline-alkali stress conditions. Axis 1 of the

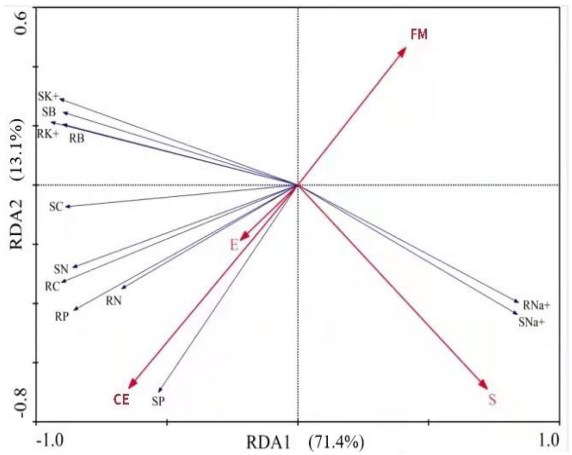


FIGURE 6 | Redundancy analysis (RDA) of *Epichloë* endophytes (E), AMFs (FM, *Funneliformis mosseae* and CE, *Claroideoglomus etunicatum*) and saline-alkali stress (S) on growth and physiological parameters of tall fescue under saline-alkali stress. Shoot biomass (SB), root biomass (RB), shoot C (SC), root C (RC), shoot N (SN), root N (RN), shoot P (SP), root P (RP), shoot Na^+ (SNa^+), root Na^+ (RNa^+), shoot K^+ (SK^+), root K^+ (RK^+).

RDA plot explained 71.4% of the total variance by being positively correlated with endophytes and CE and negatively correlated with S and FM. Axis 2 explained 13.1% of the total variance. The endophytes, FM, CE, and S explained 4, 15, 36, and 44% of the total variance, respectively (**Figure 6**).

DISCUSSION

Many studies have reported the *Epichloë* endophytes' ability to increase host resistance to abiotic stresses (Wang J. F. et al., 2020; Wang Z. F. et al., 2020; Chen et al., 2021; Liu et al., 2021; Wang et al., 2021). Among abiotic stress conditions, we demonstrated that *Epichloë*-infected tall fescue outperformed uninfected plants in saline-alkali soil. The ability of *Epichloë* to improve resistance to NaCl stress, a single physiological stress, has been demonstrated in many studies (Chen S. et al., 2018; Chen T. X. et al., 2018, 2019; Wang et al., 2019; Chen et al., 2021); however, very little is known about how *Epichloë* infection impacts mixed saline-alkali stress. The results of our study, first, revealed that endophyte presence resulted in higher biomass of shoots and roots under saline-alkali stress (200 and 400 mM) relative to that of the E– plants, which was consistent with previous research results (Chen T. X. et al., 2018, 2019; Wang et al., 2019). Second, *Epichloë* endophyte infection significantly decreased the concentration of Na^+ and increased the concentration of K^+ under saline-alkali conditions, which was consistent with the findings of Song et al. (2015). The reduction of Na^+ due to endophytes can alleviate the damage of plant cells by decreasing the inhibition of enzymes, disrupting K^+ acquisition, and inhibiting K^+-dependent metabolic processes on the one hand, and reducing oxidative stress on the other (Chen T. X. et al., 2018; Chen T. X. et al., 2019; Chen J. B. et al., 2019). Under saline-alkali stress, K^+ accumulation by endophytes is important for stomatal conductance and maintenance of normal plant activities (Chen J. B. et al., 2019). *Epichloë* endophytes regulate the balance of Na^+ and K^+ in plants, thus maintaining normal metabolic processes in cells and improving the adaptation of plants to saline-alkali environments (Song et al., 2015; Chen S. et al., 2018; Wang et al., 2021). Third, the E+ plants had higher concentrations of C, N, and P in the shoots and roots than the E– plants under saline-alkali stress conditions, which can improve plant metabolism by promoting protein synthesis and increasing the concentration of compatible osmolytes, as well as helping to maintain cell membrane integrity and reducing electrolyte leakage (Song et al., 2015; Chen T. X. et al., 2018; Wang et al., 2021).

Both *Epichloë* and AMF metabolize carbohydrates from host plants. Previous studies have produced inconsistent results when *Epichloë* and AMF simultaneously interact with a host, reporting that AMF root colonization rate was inhibited (Omacini et al., 2006; Liu et al., 2011) or promoted (Novas et al., 2005; Vignale et al., 2016) by *Epichloë* endophyte infection. In our study, we demonstrated that *Epichloë* and saline-alkali stress had significant interactive effects on AMF colonization rate. Under the control treatment, *Epichloë* endophyte infection had no significant effects on AMF colonization rate regardless of AMF species. Under the saline-alkali stress treatment, the presence of *Epichloë* endophytes significantly decreased the colonization of FM but increased the colonization rate of CE and the mixture of FM and CE (FM + CE). These discrepancies of *Epichloë* endophytes in AMF colonization may be attributed to AMF species (Larimer et al., 2012; Zhou et al., 2016) and environmental conditions (Li et al., 2019). Larimer et al. (2012) showed that *Epichloë elymi* significantly promoted the colonization rate of FM but inhibited the colonization rate of *Claroideoglomus claroideum* (CC). Zhou et al. (2016) found that *Epichloë* endophyte infection significantly reduced the colonization rate of CE but did not affect the colonization rate of FM. Li et al. (2019) demonstrated that the effect of *Epichloë* endophyte infection on the colonization rate of CE was influenced by soil water content. Endophyte infection significantly reduced the colonization rate of CE at 70% soil water content but had no effect on that of CE at either 50 or 30% soil water content.

When tripartite interactions among *Epichloë* endophytes, AMFs, and host plants were considered, some studies found that the effects of *Epichloë* endophyte infection on host plants were influenced by AMFs (Zhou et al., 2016; Liu et al., 2017, 2019; Li et al., 2019). For example, Zhou et al. (2016) reported that the effects of *Epichloë* endophyte infection on the shoot biomass of *A. sibiricum* changed from neutral with the M- treatment to positive with the FM inoculation treatment under sufficient N and P conditions. Li et al. (2019) indicated that there was no significant effect of *Epichloë* endophyte infection on the total P content of *Lolium perenne* with the M- treatment but significantly increased total P content with AMF inoculation treatment under 70% soil water content conditions. Liu et al. (2019) discovered that the outcomes of tripartite interactions among *Epichloë* endophytes, AMFs, and host plants varied with AMF identity. The beneficial effect of endophyte infection

on plant shoot biomass decreased in response to FM but increased in response to *Rhizophagus intraradices* (RI). No obvious difference was observed between the E+ and E–plants when inoculated either with CE or CC. Some studies, however, reported no interaction between *Epichloë* endophytes and AMFs in host plants (Omacini et al., 2006; Mack and Rudgers, 2008; Larimer et al., 2012). In our study, we found that the interaction between *Epichloë* endophytes and AMFs has a significant influence on the saline-alkali resistance of tall fescue, and that this was dependent on the species of AMFs. For the E– plants, host plant resistance to saline-alkali stress was decreased by FM but increased by CE. FM inoculation significantly decreased plant biomass (shoot and root biomass), nutrient concentrations (C, N, and P), and K^+ concentration while increasing Na^+ concentration. Contrary to FM, CE inoculation significantly increased these response variables. However, there was an antagonistic interaction between the *Epichloë* endophytes and the detrimental AMF, FM, but a synergistic interaction between the *Epichloë* endophytes and the beneficial AMF, CE, in plant biomass (shoot and root biomass), nutrient concentrations (C, N, and P), and ion concentration (Na^+ and K^+) for the E+ plants. Thus, the differences between the E+ and E– plants under saline-alkali stress conditions were reduced by FM but increased by CE.

According to the RDA analysis, the contribution of endophytes was less than that of AMFs and further suggested that the AMF species played an important role in *Epichloë* endophyte-host-AMF tripartite interactions. This result was similar to the findings of Zhou et al. (2016) who found that the contribution of endophytes to *A. sibiricum* was less than that of AMFs under conditions of nutrient stress. AMFs were present in the roots of host plants, and they directly absorbed N and P *via* external AMF hyphae in soil (Kong et al., 2019), whereas *Epichloë* endophytes lived in the above-ground tissues of the host plants (Wang J. F. et al., 2020) and indirectly affected nutrient absorption by changing the root's morphological and physiological characteristics (Chen et al., 2020, 2021). This may be the reason that could explain why *Epichloë* endophytes have lower contribution than AMFs under saline-alkali stress conditions.

CONCLUSION

In conclusion, a significant interaction among the *Epichloë* endophytes, AMFs, and saline-alkali stress occurred in tall fescue growth and physiological parameters. Endophyte infection significantly enhanced tall fescue resistance to saline-alkali stress by increasing biomass, nutrient uptake, and accumulation of K^+ while decreasing Na^+ concentration; this beneficial effect of the endophytes was enhanced by the beneficial AMF, CE, but reduced by the detrimental AMF, FM. Our study reinforces the currently limited finding that *Epichloë* endophytes and AMFs interact in complex ways to influence the growth of their shared host grasses, especially under stress conditions. Further research should be conducted to investigate the ecological implications of the combined effects of *Epichloë* endophytes and AMFs under field conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HL designed the research and revised and polished the manuscript. HT, XN, YZ, and YW performed the experiments. HL and HT analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (32001103) and Dezhou University Science Research Foundation (2019xjrc317).

REFERENCES

Abd-Allaa, M. H., Nafadya, N. A., Bashandya, S. R., and Hassan, A. A. (2019). Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. *Rhizosphere* 10:100148. doi: 10.1016/j.rhisph.2019.100148

Aliasgharzadeh, N., Rastin, S. N., Towfighi, H., and Alizadeh, A. (2001). Occurrence of arbuscular mycorrhizal fungi in saline soils of the Tabriz Plain of Iran in relation to some physical and chemical properties of soil. *Mycorrhiza* 11, 119–122. doi: 10.1007/s005720100113

Arrieta, A., Iannones, L. J., Scervino, J., Vignale, M., and Novas, M. (2015). Afoliar endophyte increases the diversity of phosphorus-solubilizing rhizospheric fungi and mycorrhizal colonization in the wild grass *Bromus auleticus*. *Fungal Ecol.* 17, 146–154. doi: 10.1016/j.funeco.2015.07.001

Ben-Laouane, R., Baslam, M., Ait-El-Mokhtar, M., Anli, M., Boutasknit, A., Ait-Rahou, Y., et al. (2020). Potential of native arbuscular mycorrhizal fungi, rhizobia, and/or green compost as Alfalfa (*Medicago sativa*) enhancers under salinity. *Microorganisms* 8:1695. doi: 10.3390/microorganisms8111695

Chen, J. B., Zong, J. Q., Li, D. D., Chen, Y., Wang, Y., Guo, H. L., et al. (2019). Growth response and ion homeostasis in two bermudagrass (*Cynodon dactylon*) cultivars differing in salinity tolerance under salinity stress. *Soil Sci. Plant Nutr.* 4, 419–429. doi: 10.1080/00380768.2019.1631125

Chen, T. X., Li, C. J., White, J. F., and Nan, Z. B. (2019). Effect of the fungal endophyte *Epichloë bromicola* on polyamines in wild barley (*Hordeum brevisubulatum*) under salt stress. *Plant Soil* 436, 29–48. doi: 10.1007/s11104-018-03913-x

Chen, S., Chen, T., Yao, X., and Lv, H. U. I. (2018). Physicochemical properties of an asexual *Epichloe* endophyte modified wild barely in the presence of salt stress. *Pak. J. Bot.* 50, 2105–2111.

Chen, T. X., Johnson, R., Chen, S. H., Lv, H., Zhou, J., and Li, C. J. (2018). Infection by the fungal endophyte *Epichloë bromicola* enhances the tolerance of wild barley (*Hordeum brevisubulatum*) to salt and alkali stresses. *Plant Soil* 428, 353–370. doi: 10.1007/s11104-018-3643-4

Chen, T. X., White, J. F., and Li, C. J. (2021). Fungal endophyte *Epichloë bromicola* infection regulates anatomical changes to account for salt stress tolerance in wild barley (*Hordeum brevisubulatum*). *Plant Soil* 461, 533–546. doi: 10.1007/s11104-021-04828-w

Chen, Y. L., Deng, Y., Ding, J. Z., Hu, H. W., Xu, T. L., Li, F., et al. (2017). Distinct microbial communities in the active and permafrost layers on the Tibetan Plateau. *Mol. Ecol.* 26, 6608–6620. doi: 10.1111/mec.14396

Chen, Z. J., Jin, Y. Y., Yao, X., Chen, T. X., Wei, X. K., Li, C. J., et al. (2020). Fungal endophyte improves survival of *Lolium perenne* in low fertility soils by increasing root Growth, metabolic activity and absorption of nutrients. *Plant Soil* 452, 185–206. doi: 10.1007/s11104-020-04556-7

Clay, K., and Holah, J. (1990). Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285, 1742–1745. doi: 10.1126/science.285.5434.1742

Estrada, B., Aroca, R., Maathuis, F. J. M., Barea, J. M., and Ruiz-Lozano, J. M. (2013). Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize tolerance to salinity through improved ion homeostasis. *Plant Cell Environ.* 36, 1771–1782. doi: 10.1111/pce.12082

Feng, G., Zhang, F., Li, X., Tian, C., Tang, C., and Rengel, Z. (2002). Improved tolerance of maize plants to salt stress by arbuscular mycorrhiza is related to higher accumulation of soluble sugars in roots. *Mycorrhiza* 12, 185–190. doi: 10.1007/s00572-002-0170-0

Kong, L., Gong, X. W., Zhang, X. L., Zhang, W. Z., Sun, J., and Chen, B. L. (2019). Effects of arbuscular mycorrhizal fungi on photosynthesis, ion balance of tomato plants under saline-alkali soil condition. *J. Plant Nutr.* 43, 682–698. doi: 10.1080/01904167.2019.1701029

Larimer, A. L., Bever, J. D., and Clay, K. (2012). Consequences of simultaneous interactions of fungal endophytes and arbuscular mycorrhizal fungi with a shared host grass. *Oikos* 121, 2090–2096. doi: 10.1111/j.1600-0706.2012.20153.x

Latch, G. C. M., and Christensen, M. J. (1985). Artificial infection of grasses with endophytes. *Ann. Appl. Biol.* 107, 17–24. doi: 10.1111/j.1744-7348.1985.tb01543.x

Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106, 202–215. doi: 10.3852/106.2.202

Li, F., Deng, J., Nzabanita, C., Li, Y. Z., and Duan, T. Y. (2019). Growth and physiological responses of perennial ryegrass to an AMF and an *Epichloë* endophyte under different soil water contents. *Symbiosis* 79, 151–161. doi: 10.1007/s13199-019-00633-3

Liu, H., Chen, W., Wu, M., Wu, R. H., Zhou, Y., Gao, Y. B., et al. (2017). Arbuscular mycorrhizal fungus inoculation reduces the droughtresistance advantage of endophyte-infected versus endophyte-free *Leymus chinensis*. *Mycorrhiza* 27, 791–799. doi: 10.1007/s00572-017-0794-8

Liu, H., Wu, M., Liu, J. M., Qu, Y. B., Gao, Y. B., and Ren, A. Z. (2019). Tripartite interactions between endophyte, arbuscular mycorrhizal fungi and *Leymus chinensis*. *Microb. Ecol.* 79, 98–109. doi: 10.1007/s00248-019-01394-8

Liu, Q., Parsons, A. J., Xue, H., Fraser, K., Ryan, G. D., Newman, J. A., et al. (2011). Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal *Glomus* spp. fungi in *Lolium perenne* depends on resource supply and host carbohydrate content. *Funct. Ecol.* 25, 910–920. doi: 10.1111/j.1365-2435.2011.01853.x

Liu, Y. L., Hou, W. P., Jin, J., Christensen, M. J., Gu, L. J., Cheng, C., et al. (2021). *Epichloe gansuensis* increases the tolerance of *Achnatherum inebrians* to low-P stress by modulating amino acids metabolism and phosphorus utilization efficiency. *J. Fungi* 7:390. doi: 10.3390/jof7050390

Mack, K. M. L., and Rudgers, J. A. (2008). Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes. *Oikos* 117, 310–320. doi: 10.1111/j.2007.0030-1299.15973.x

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., and Swan, J. A. (1990). Anew method which gives an objective measure of colonization ofroots by vesicular-arbuscular mycorrhizal fungi. *New Phytol.* 115, 495–501. doi: 10.1111/j.1469-8137.1990.tb00476.x

Moreira, H., Pereira, S. I. A., Vega, A., Castro, P. M. L., and Marques, A. P. G. C. (2020). Synergistic effects of arbuscular mycorrhizal fungi and plant growth-promoting bacteria benefit maize growth under increasing soil salinity. *J. Environ. Manage.* 257:109982. doi: 10.1016/j.jenvman.2019.109982

Morgan-Jones, G., and Gams, W. (1982). Notes on Hyphomycetes. XLI. an endophyte of *Festuca arundinacea* and the anamorph of *Epichloë typhina*, new taxa in one of two new sections of Acremonium. *Mycotaxon* 50, 113–117.

Müller, J. (2003). Artificial infection by endophytes affects growth and mycorrhizal colonisation of *Lolium perenne*. *Funct. Plant Biol.* 30, 419–424. doi: 10.1071/FP02189

Novas, M. V., Cabral, D., and Godeas, A. M. (2005). Interaction between grass endophytes and mycorrhizas in *Bromus setifolius* from Patagonia, Argentina. *Symbiosis* 40, 23–30.

Novas, M. V., Iannone, L. J., Godeas, A. M., and Cabral, D. (2009). Positive association between mycorrhiza and foliar endophytes in *Poa bonariensis*, a native grass. *Mycol. Prog.* 8, 75–81. doi: 10.1007/s11557-008-0579-8

Omacini, M., Eggers, T., Bonkowski, M., Gange, A. C., and Jones, T. H. (2006). Leaf endophytes affect mycorrhizal status and growth of co-infected and neighbouring plants. *Funct. Ecol.* 20, 226–232. doi: 10.1111/j.1365-2435.2006.01099.x

Parihar, M., Rakshit, A., Rana, K., Meena, R. P., and Joshi, D. C. (2020). A consortium of arbuscular mycorrizal fungi improves nutrient uptake, biochemical response, nodulation and growth of the pea (*Pisum sativum* L.) under salt stress. *Rhizosphere* 15:100235. doi: 10.1016/j.rhisph.2020.100235

Phillips, J. M., and Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55, 158–161. doi: 10.1016/s0007-1536(70)80110-3

Qiu, Y. J., Zhang, N. L., Zhang, L. L., Zhang, X. L., Wu, A. P., Huang, J. Y., et al. (2020). Mediation of arbuscular mycorrhizal fungi on growth and biochemical parameters of *Ligustrum vicaryi* in response to salinity. *Physiol. Mol. Plant Pathol.* 112:101522. doi: 10.1016/j.pmpp.2020.101522

Ren, A. Z., Gao, Y. B., Zhang, J., and Zhang, J. (2006). Effect of endophyte infection on salt resistance of ryegrass. *Acta Ecol. Sin.* 26, 1750–1757.

Reza, S. M., and Mirlohi, A. (2010). *Neotyphodium* endophytes trigger salt resistance in tall and meadow fescues. *J. Plant Nutr. Soil Sci.* 173, 952–957. doi: 10.1002/jpln.200900345

Rodriguez, R. J., Henson, J., Van, V. E., Hoy, M., Wright, L., Beckwith, F., et al. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *ISME J.* 2, 404–416. doi: 10.1038/ismej.2007.106

Schardl, C. L., Leuchtmann, A., and Spiering, M. J. (2004). Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* 55, 315–340. doi: 10.1146/annurev.arplant.55.031903.141735

Song, M., Chai, Q., Li, X., Yao, X., Li, C., Christensen, M. J., et al. (2015). An asexual *Epichloë endophyte* modifies the nutrient stoichiometry of wild barley (*Hordeum brevisubulatum*) under salt stress. *Plant Soil* 387, 153–165. doi: 10.1007/s11104-014-2289-0

Tanveer, S. K., Zhang, J. L., Lu, X. L., Wen, X. X., Wei, W., Yang, L., et al. (2014). Effect of corn residue mulch and N fertilizer application on nitrous oxide (N_2O) emission and wheat crop productivity under rain-fed condition of Loess Plateau China. *Int. J. Agric. Biol.* 16, 505–512.

Vignale, M. V., Iannone, L. J., PinCEt, A. D., De Battista, J. P., and Novas, M. V. (2016). Effect of epichloid endophytes and soil fertilization onarbuscular mycorrhizal colonization of a wild grass. *Plant Soil* 405, 279–287. doi: 10.1007/s11104-015-2522-5

Wang, J. F., Hou, W. P., Christensen, M. J., Li, X. Z., Xia, C., Li, C. J., et al. (2020). Role of *Epichloe*endophytes in improving host grass resistance ability and soil properties. *J. Agric. Food Chem.* 68, 6944–6955. doi: 10.1021/acs.jafc.0c01396

Wang, Z. F., Li, C. J., and White, J. (2020). Effects of *Epichloë* endophyte infection on growth, physiological properties and seed germination of wild barley under saline conditions. *J. Agro. Crop Sci.* 206, 43–51. doi: 10.1111/jac.12366

Wang, J. F., Hou, W. P., Christensen, M. J., Xia, C., Chen, T., Zhang, Z. X., et al. (2021). The fungal endophyte *Epichloë gansuensis* increases NaCltolerance in *Achnatherum inebrians* through enhancing the activity of plasma membrane H+-ATPase and glucose-6-phosphate dehydrogenase. *Sci. China Life Sci.* 63, 452–465. doi: 10.1007/s11427-020-1674-y

Wang, J. F., Tian, P., Christensen, M. J., Zhang, X. X., Li, C. J., and Nan, Z. B. (2019). Effect of *Epichloë gansuensis* endophyte on the activity of enzymes of nitrogen metabolism, nitrogen use efficiency and photosynthetic ability of *Achnatherum inebrians* under various NaCl concentrations. *Plant Soil* 435, 57–68. doi: 10.1007/s11104-018-3868-2

Wilde, P., Manal, A., Stodden, M., Sieverding, E., Hildebrandt, U., and Bothe, H. (2009). Biodiversity of arbuscular mycorrhizal fungi in roots and soils of two salt marshes. *Environ. Microbiol.* 11, 1548–1561. doi: 10.1111/j.1462-2920.2009.01882.x

Yang, Y. R., Cao, Y. P., Li, Z. X., Zhukova, A., Yang, S. T., Wang, J. L., et al. (2020). Interactive effects of exogenous melatonin and *Rhizophagus intraradices* on saline-alkaline stress tolerance in *Leymus chinensis*. *Mycorrhiza* 30, 357–371. doi: 10.1007/s00572-020-00942-2

Zabalgogeazcoa, I., Romo, M., Keck, E., Vázquez de Aldana, B. R., García Ciudad, A., and García Criado, B. (2006). The infection of *Festuca rubra* subsp. pruinosa by *Epichloë festucae*. *Grass Forage Sci.* 61, 71–76. doi: 10.1111/j.1365-2494.2006.00509.x

Zhou, Y., Li, X., Qin, J. H., Liu, H., Chen, W., Niu, Y., et al. (2016). Effects of simultaneous infections of endophytic fungi andarbuscular mycorrhizal fungi on the growth of their shared hostgrass *Achnatherum sibiricum* under varying N and P supply. *Fungal Ecol.* 20, 56–65. doi: 10.1016/j.funeco.2015.11.004

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