TOWARDS A FUNCTIONAL CHARACTERIZATION OF PLANT BIOSTIMULANTS

EDITED BY: Paolo Carletti, Andrew Merchant, Andrés Calderín García and Carlos Alberto Silva

PUBLISHED IN: Frontiers in Plant Science



frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88966-794-9
DOI 10.3389/978-2-88966-794-9

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TOWARDS A FUNCTIONAL CHARACTERIZATION OF PLANT BIOSTIMULANTS

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Citation: Carletti, P., Merchant, A., García, A. C., Silva, C. A., eds. (2021). Towards a Functional Characterization of Plant Biostimulants. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-794-9

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frontiers in Plant Science

EDITORIAL
published: 13 April 2021
doi: 10.3389/fpls.2021.677772

Editorial: Towards a Functional Characterization of Plant Biostimulants

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Keywords: humic substances, microorganism, abiotic and biotic stress, protein hydrolysates, seaweed extracts, bacteria

Editorial on the Research Topic

Towards a Functional Characterization of Plant Biostimulants

The second United Nations Sustainable Development Goal aims to reach "zero hunger" by 2030. To achieve this, an increase in crop yield is expected while simultaneously increasing the efficiency of non-renewable resource use, protection of soil quality and improvement of agroecological biodiversity. It is therefore necessary and advantageous to learn from nature to develop environmentally friendly inputs and technologies (Perminova et al., 2019). Plant biostimulants are a promising tool to address this issue. A plant biostimulant is any substance, microorganism (or mixture thereof) capable of improving plant nutrition, leading to improved resistance to abiotic stress or food quality traits, independently of its nutrient content (du Jardin, 2015; Rouphael and Colla, 2020). Biostimulants are products that stimulate plant nutrition and growth, commonly acting at low doses. Biostimulants comprise a wide range of compound classes, commonly including (but not limited to) humic substances, amino acids, protein hydrolysates, carbohydrates, algae-derived products, and root growth promoting bacteria. As a result of this broad scope encompassing a wide range of chemical characteristics, characterization of novel compounds with biostimulant activities is frequently reported.

The functional characterization of biostimulants is fast emerging as a new frontier in plant, soil, and microbial research. The synergies, competition and interactions between organisms that take place upon the diversity of chemical and structural components and pools that constitute the phyto-soil system are complex. The magnitude of the challenge to characterize these will require the most advanced tools, technologies and expertise available often in a cross disciplinary context. Ultimately, harnessing the potential benefits of biostimulant function will require a range of approaches from both the fundamental and applied fields of research, in both lab and field conditions. The objective of this Research Topic was to bring together expertise and contributions from around the world to highlight progress and identify future challenges for biostimulant research.

In this special Research Topic of Frontiers in Plant Science, we highlight some of the latest developments in the detection and functional characterization of biostimulants to enhance plant growth and survival to ultimately improve the management of plant, soil and microbial systems. The potential use of biostimulants holds great promise to alleviate plant stress conditions, improve productivity and promote survival whilst in some cases reducing demand for scarce and essential inputs such as fertilizer. To date, the characterization of biostimulants and their complex network of interactions has encompassed

OPEN ACCESS

Edited and reviewed by:
Abraham J. Escobar-Gutiérrez, Institut National de Recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), France

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *08 March 2021*
Accepted: *16 March 2021*
Published: *13 April 2021*

Citation:
Carletti P, García AC, Silva CA and Merchant A (2021) Editorial: Towards a Functional Characterization of Plant Biostimulants. Front. Plant Sci. 12:677772. doi: 10.3389/fpls.2021.677772

a wide diversity of studies, the individual scope of which is determined by the chemical, structural or organismal focus of an observed effect. For example, a chemical elicitor may need to be isolated from a complex chemical matrix. Alternatively, a biological extract may elicit a stress alleviating effect that may also be crop-dependent. All such permutations fall under the development of our understanding of biostimulation and represent progress within this exciting avenue of research. The present Research Topic contains a wide scope of such examples.

Obtaining materials that demonstrate biostimulant activity has cast a wide net, partly to stay within accepted boundaries of sustainability, partly due to economics, but also in an effort to obtain novel chemical mixtures of biological origin. Several authors demonstrate biostimulant activity from a range of biological materials from marine environments such as seaweed extracts (Islam et al.) and coral microbiota (Ocampo-Alvarez et al.) through to terrestrial environments including arbuscular mycrorrhizal fungi (González-González et al.), multispecies microbial biostimulants (Nazari and Smith, Mickan et al.), humic-like substances isolated from lignin rich agro-industrial residues (Savy et al.), soil-derived humic substances (Jindo et al.), rhizospheric organic acids (Macias-Benitez et al.) and even hydrolysed animal protein (Casadesús et al.). Similarly, soil amendments that enhance the chemical and structural complexity of the soil environment, such as the addition of biochar (Tartaglia et al.) show considerable promise and are the subject of significant attention for improve plant growth, fruit yield and affecting gene expression. It is likely that the adoption of such materials by industry requires the identification of chemical entities within these complex matrices that elicit the biostimulant response. Characterizing the chemical composition of such mixtures requires both advanced tools and the metabolomics approach (Savy et al.; Lucini et al.) and in some cases novel biostatistical approaches (Savy et al.) to cope with such chemical and biochemical complexities. Ultimately, if consistent biostimulant effects can be observed across sufficient environmental variation, harnessing the benefits of crude materials such as those demonstrated here may suffice. However, further understanding of the function, mode of action and potential of these materials across a range of environments is, without doubt, a profitable venture.

At the other end of the spectrum, isolating and characterizing specific chemical elicitors of biostimulant activity represents a powerful step toward functional characterization. The isolation of specific chemical elicitors demonstrating biostimulant activity is uncommon. However, several promising candidates such as Omeprazole (Van Oosten et al.), as a stimulant of nitrogen use efficiency and Thuricin 17 (highlighted by both Nazari and Smith and Lyu et al.) as a promising candidate for "second generation" plant growth promoters and biostimulants (e.g., Nazari and Smith) are highlighted here.

The amelioration of stress conditions is a major subset of biostimulant research and highlights the sometimes-subtle distinction between increasing growth and enhancing survival. An array of plant stress conditions are studied in this Frontiers Research Topic reflecting the scope of renewed interest in this discipline. Biostimulant activity is shown to enhance tolerance to the effects of both biotic stress (e.g., *Phytophthora cinnamomi* infection, Islam et al.) to the effects of heat (Carmody et al.), saline conditions (Ocampo-Alvarez et al.), the combined effects of temperature and nutrient deficit (Casadesús et al.) and the combined effects of pathogenic bacteria, growth stage and nutrient deficiency on plant defense mechanisms for precautionary induced expression (Verly et al.). An equally important consideration for biostimulant use is the target tissues/organisms of application to develop a solid framework for targeted use in field conditions. Application of biostimulants and biofertilizers to the seed (Campobenedetto et al.; Dal Cortivo et al.) are shown to have lasting effects not only in growth promotion and tolerance to heat stress, but also in influencing populations of the resultant phytomicrobiome. This, in turn, undoubtedly has a range of downstream effects on the phtyomicrobiome (see for e.g., Dal Cortivo et al.) and modifications similar to latter developmental-stage interventions showing significant promise (see for e.g., Mickan et al.; Lyu et al.; Macias-Benitez et al.; Dal Cortivo et al.; Moradtalab et al.). In addition, small shifts in environmental conditions can have marked influences and confounded effects on biostimulant activity (see Allen and Allen; Lucini et al.) as well as the process of biostimulant extraction and/or processing (Carmody et al.). Only through the characterization of such vulnerabilities in the maintenance and enhancement of biostimulant activity will enable industrial application to prosper.

The property of biological complexity is both a blessing and a curse and highlighted among many studies both here (Lyu et al.; Macias-Benitez et al.; Mickan et al.; Moradtalab et al.) and in the wider literature. The characterization of complex systems must avoid over-simplification, and recognize the beneficial properties of complexity, often imparting multiple nodes of regulation in moderating growth. Interdependencies in chemical and biological components (see for e.g., Macias-Benitez et al.; Moradtalab et al.) are both a major challenge to the reliable application of biostimulants but may also represent a significant benefit in moderating the effects of biostimulant application to enhance survival.

Finally, the demonstration and characterization of sustained biostimulant activity under field conditions is vital to promote industrial-scale uptake for the plethora of potential applications in both production and conservation efforts. For this reason, field application such as that discussed by Lyu et al. and demonstrated by Jindo et al.; Dal Cortivo et al.; and Mickan et al. as well as potential for horticultural application (Casadesús et al.; González-González et al.) are an important advancement in biostimulant research and deserve considerable attention. It is clear that the functional characterization of biostimulant properties and mode of action directly confronts the challenge of understanding highly complex interactions amongst biological entities often among variable environments. Contributions to this field of research, be them incremental or revolutionary, are likely to lead to significant outcomes for industrial application, conservation, ecological intensification and the sustainability of food, forage, fiber, and biofuel cropping systems through the displacement of more resource intensive management tools and practices.

AUTHOR CONTRIBUTIONS

AM wrote the first draft. PC, AG, and CS added notes and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

PC was supported by University of Padova, grant DOR2178525/21. CNPq (307447-2019-7 grant) provided a scholarship for CS. AM was supported by the Australian Research Council Future Fellowship FT120100200. AG was supported by FAPERJ: 2012028010 grant and CNPq: 306867/2018-4 grant.

ACKNOWLEDGMENTS

The authors recognize the contributions and constructive dialogue from the contributing authors regarding their papers in this Research Topic. The authors also recognize The University of Sydney, Università di Padova, Universidade Federal Rural do Rio de Janeiro, and Universidade Federal de Lavras for their support.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 December 2019
doi: 10.3389/fpls.2019.01507

Omeprazole Treatment Enhances Nitrogen Use Efficiency Through Increased Nitrogen Uptake and Assimilation in Corn

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OPEN ACCESS

Edited by:
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University of Padua, Italy

Reviewed by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 02 July 2019
Accepted: 30 October 2019
Published: 04 December 2019

Citation:
Van Oosten MJ, Dell'Aversana E, Ruggiero A, Cirillo V, Gibon Y, Woodrow P, Maggio A and Carillo P (2019) Omeprazole Treatment Enhances Nitrogen Use Efficiency Through Increased Nitrogen Uptake and Assimilation in Corn. Front. Plant Sci. 10:1507. doi: 10.3389/fpls.2019.01507

Omeprazole is a selective proton pump inhibitor in humans that inhibits the H^+/K^+-ATPase of gastric parietal cells. Omeprazole has been recently shown to act as a plant growth regulator and enhancer of salt stress tolerance. Here, we report that omeprazole treatment in hydroponically grown maize improves nitrogen uptake and assimilation. The presence of micromolar concentrations of omeprazole in the nutrient solution alleviates the chlorosis and growth inhibition induced by low nitrogen availability. Nitrate uptake and assimilation is enhanced in omeprazole treated plants through changes in nitrate reductase activity, primary metabolism, and gene expression. Omeprazole enhances nitrate assimilation through an interaction with nitrate reductase, altering its activation state and affinity for nitrate as a substrate. Omeprazole and its targets represent a novel method for enhancing nitrogen use efficiency in plants.

Keywords: benzimidazole, nitrogen use efficiency, proton pump inhibitor, maize, nitrogen assimilation, nitrate reductase

HIGHLIGHT

Omeprazole enhances nitrogen uptake and assimilation in corn.

INTRODUCTION

In recent decades, increasing nitrogen use efficiency (NUE) has emerged as critical target for reducing the requirements for chemical fertilizers and lessen the potential environmental impacts of nitrogen fertilization in agriculture. The Green Revolution resulted in dramatic increases in crop productivity allowing modern agriculture to keep up with a tripling in the global population over the last seventy years (Godfray et al., 2010). However, higher yield and productivity were in part achieved through a sevenfold increase in nitrogen fertilizers use (Hirel et al., 2007; Lassaletta et al., 2014), which, in turn, had critical consequences on the environment. The chemical synthesis of ammonia, ammonium, urea, and nitrate for use in agriculture through the Haber-Bosch process produces 450 million tons of chemical fertilizer per year consuming up to 5% of the global natural gas production (Smith, 2002; Smill, 2004; Erisman et al., 2008; Chen et al., 2018). It is estimated

that 50% of the nitrogen atoms in humans today originate from nitrogen fixed from the Haber-Bosch process (Erisman et al., 2008). Moreover, for each metric ton of NH_3 produced 1.9 metric tons of CO_2 are released into the environment (Rafiqul et al., 2005). Overuse and poor management of chemical fertilizers, in particular in developing countries, have caused environmental degradation and pollution and reduction of the arable land (Keeney and Hatfield, 2001). Among fertilizers, nitrogen (N) represents the single highest input cost for many crops and, since its production is energy intensive, this cost is dependent on the price of energy (Rothstein, 2007). N containing compounds, which are typically used as chemical fertilizers, are generally mobile in the soil, and only 30%– 40% of the applied N is taken up by plants (Raun and Johnson, 1999), with the remainder lost for leaching, surface run-off, denitrification, volatilization, and microbial consumption. Increasing NUE by only 1% could globally save $2.3 billion annually (Raun and Johnson, 1999). Based on indications by Hirel et al. (2007), the Organization for Economic Co-operation and Development (OECD) described NUE as the ratio between the amount of N fertilizer exported from the field by the crops and the amount of N fertilizer applied. However, NUE depends not only on the plants' ability to uptake nitrate but also on nitrate assimilatory reduction and translocation efficiency (Reich et al., 2014). Until now, crops, like maize, have been selected almost exclusively under nonlimiting N conditions. In contrast, attempts to reduce the use of nitrogen fertilizers have faced the inability of crop plants to adapt to a low nitrogen availability leading to significant decrease in crop yield (Masclaux-Daubresse et al., 2010). Nitrogen is of pivotal importance in plant metabolism. Nitrate reductase (NR), the first enzyme in the nitrate assimilation pathway catalyzing the reduction of NO_3^- in NO_2^- is strictly dependent on nitrate availability at cellular level (Carillo et al., 2005; Annunziata et al., 2017). This enzyme is considered as the limiting step in the overall process of plant growth and productivity (Kaiser et al., 1999). Nitrate is required for full levels of NR gene expression, since signals from nitrogen metabolism play an important role in inducing the expression of NR gene Nia (Oaks, 1974).

It is therefore critical to better understand the physiological and molecular basis of N assimilation and use in plants to design new strategies to improve NUE.

Omeprazole (OP) is a member of the family of substituted benzimidazoles that act as proton pump inhibitors (PPIs) in mammals (Baumann and Baxendale, 2013). OP interacts with P-Type IIC ATPases and inhibit H^+/K^+ ATPases in the gut lumen, reducing the pacification of the gut (Shin et al., 2009). Plants do not appear to have Type IIC ATPases that move potassium or sodium across the membrane; instead they transport Na^+ and K^+ using the family of NHX-type antiporters (Axelsen and Palmgren, 1998; Hasegawa, 2013). Notwithstanding plants do not possess functional orthologues of H^+/K^+ ATPases, OP does influence the physiological processes of the plant (Sweadner and Donnet, 2001). OP has previously been shown to act at micromolar concentrations in tomato and basil as an enhancer of growth (Van Oosten et al., 2017a; Carillo et al., 2019d; Cirillo et al., 2019) and it was proven to enhance nitrogen and potassium uptake and their loading into the shoots in lettuce and basil (Carillo et al., 2019c; Carillo et al., 2019d). OP also increased plant tolerance to salt stress through several adaptive mechanisms (Van Oosten et al., 2017a; Van Oosten et al., 2017b; Rouphael et al., 2018; Carillo et al., 2019c; Carillo et al., 2019d). While the targets of OP that are responsible for these responses in terms of growth and stress tolerance are unclear, it was quite evident that OP affects plant metabolism and NUE on many levels. We hypothesized that OP could interact directly with mechanisms of nitrate uptake and assimilation and that a study examining the role of OP on plants under nitrogen stress would be informative. Therefore, we determined if plant metabolism under high (10 mM NO_3^-) or low (1 mM NO_3^-) inorganic N availability may change in response to OP treatment in order to understand the role played by OP in improving NUE. Here, we demonstrate that addition of micromolar concentrations of OP to the nutrient solution alleviates chlorosis and growth inhibition, induced by low nitrogen availability, through changes in NR activity, primary metabolism, and gene expression.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The p1619 line (Pioneer Hi-Bred International, Johnston Iowa USA) of maize (*Zea mays* L.) was used for the experiments. For hydroponic experiments, maize seeds were imbibed for 48 h in tap water with aeration and germinated on filter paper wetted for three days and transferred to hydroponics. Two modified Hoagland's solutions supplemented with Hidromix S micronutrients (Vlagro, Cieti Italy) (1g/L) were used: low nitrogen with 1mM NO_3^- in test-run experiments showed clear signs of nitrogen stress with reduced growth and chlorosis while high nitrogen with 10mM NO_3^- demonstrated excellent growth and no signs of nitrogen stress. Therefore, the selected concentrations (1 vs. 10 mM NO_3^-) allowed us to visually differentiate plants growing under optimal vs. suboptimal N availability without causing irreversible metabolic dysfunctions and cell death in the short term (Carillo et al., 2008). Three replicates containing six plants each were made for each nutrient regimen and OP treatment. The OP at final concentration of 1 μM was supplied to the nutrient solution to a set of replicates for OP treatment starting from 14 days after germination. The 1 μM OP was selected based on previous experiments in which this concentration was found optimal as growth enhancer (Van Oosten et al., 2017; Cirillo et al., 2019). Nutrient solutions with and without OP were changed every four days for the first 2 weeks and every 3 days for the final week of the experiment. Plants were grown in a climate-controlled greenhouse with 8 h of supplemental lighting (1,000 μmol/m²/s) and day/night temperature of 28°C/18°C as per Eddy and Hahn (2010).

Biometric Measurements

At the end of the experiment, 4 weeks DAST, SPAD values (Chlorophyll Meter SPAD-502Plus, Konica Minolta) were measured from 20 leaves of each treatment. Roots and shoots were separated and weighed for fresh weight and total leaf area

was calculated using ImageJ (Abramoff et al., 2004). Roots and shoots were then dried for five days at 65°C and dry weight was measured.

Net Uptake Assay and Kinetic Parameters

The net nitrate uptake rate (NNUR) was measured by a depletion method adapted from (Sorgonà et al., 2011). Maize seeds were imbibed for 48 h in tap water with aeration and germinated on filter paper wetted with one quarter strength Hoagland's solution with or without 1 μM OP and then transferred to 10 cm × 50 cm trays with washed sand. Sand was kept moist with watering and quarter strength Hoagland's solution with or without 1 μM OP. Three-week-old maize plants were washed three times and divided into 1-g pools and incubated in 10 ml of apoplastic equilibration solution containing 100 μM KH_2PO_4, 250 μM K_2SO_4, and 200 μM $MgSO_4$. Net nitrate uptake was measured for 1 h and for four biological replicates using 0, 100, and 500 μM KNO_3^- and 0, 1, 10, 50, and 100 μM OP.

Microsomal Membrane Isolation and ATPase Assays

Total microsomal membranes were isolated as per Yang and Murphy, 2003, using 5 g of separated root and shoot tissue. ATPase activity was measured with an ATPase/GTPase Activity Assay Kit (Sigma-Aldrich, Cat. No. MAK113). Four biological replicates of freshly prepared microsomes from roots and shoots were tested using 10 μl of the microsomal fraction in conjunction with 0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 50, 100, and 1,000 μM omeprazole. Sodium ortho-vanadate (1 mM), a strong suppressor of ATPase activity was added to the negative controls. ATPase activity was measured after a 30-min incubation time at 620 nm.

RNA Extraction and Quantitative RT-PCR

Roots and shoots were separated and snap frozen in liquid nitrogen at 4 weeks DAST. Total RNA and quantitative RT-PCR was performed as in Van Oosten et al. (2017a). Relative expression levels were calculated using molybdenum cofactor biosynthesis (GRMZM2G067176) as an internal standard (Best et al., 2016). All primers were designed to amplify a cDNA fragment of 120 bp (+/− 10 bp) with an annealing temperature of 55°C (+/− 1°C). All primers were determined to be within 5% efficiency. The ΔΔCt method was used for relative quantification. Three biological replicates were used to calculate the relative expression of each gene. Results were statistically analyzed using Student's T-Test for each treatment compared to high N controls. Primers used in this study are listed in **Supplementary Table 1**.

NR Assays in Maize Shoots

NR protein was extracted according to Scheible et al. (1997) from maize. NR activity was assayed by a method modified by Gibon et al. (2004). Leaf extracts (20 μl), as well as nitrite standards ranging from 0 to 10 nmol, were pipetted into microplate wells and incubated with 45 μl medium containing 50 mM Hepes/KOH pH 7.5, 1 mM sodium fluoride, 5 μM Na_2MoO_4, 10 μM flavin adenine dinucleotide, 0.5 mM DTT, 15 μM leupeptin, 10 mM potassium nitrate, and 5 mM EDTA (for maximal activity) or 10 mM $MgCl_2$ (for selective activity). The reaction was started by the addition of NADH to a final concentration of 0.8 mM. The reaction was stopped with 5 μl of 0.6 M zinc acetate. Then, 15 μl of 0.25 mM phenazine methosulfate (PMS) were added, and the microplates were incubated for 15 min at room temperature. Finally, 120 μl of 1% (w/v) sulphanilamide and 0.02% (w/v) N(1-naphtyl)ethylenediamine dihydrochloride in 3 N HCl were added. After 20 min, the OD was red at 540 nm.

OP-Dependent Modulation of NR Catalytic Properties

For testing the effects of OP on NR *in vitro*, velocities were determined spectrophotometrically using purified *Arabidopsis thaliana* NR (≥ 0.5 U mg^{-1} protein) obtained from Sigma-Aldrich. Enzyme activity was determined using the assay system described above in presence of OP (0, 1, 10, or 50 μM) in the assay solutions containing nitrate 0.08, 0.16, 0.4, 0.8, 1.5 mM. Global fitting analysis, maximum rate (V_{max}) and Michaelis constant (K_m) were calculated by nonlinear regression using the enzyme kinetics module (SigmaPlot 12.5; Systat Software GmbH, Erkrath, Germany).

Measurements of Other Enzymes of Central C and N Metabolism

Frozen leaf and root tissues were reduced to a homogenous powder and stored at −80°C until required for the enzyme assays. The extraction buffer consisted of 500 mM HEPES, pH 7.5, containing 100 mM $MgCl_2$, 10 mM EDTA, 10 mM EGTA, 2 mM leupeptin, 0.5 mM DTT, 0.1% (v/v) Triton, and 1% (w/v) polyvinylpolypyrrolidone. All extractions were performed at 4°C. A robot-based platform was used to measure the activity of enzymes involved in central C, and N metabolism. ADP glucose pyrophosphorylase (AGPase), glucokinase (GlcK), glucose-6P dehydrogenase (G6PDH), glutamate dehydrogenase (GLDH), phosphoenolpyruvate carboxylase (PEPC), and pyruvate kinase (PK) activities were assayed by using the protocols described by Gibon et al. (2004). Additional methods were used for the cytosolic and mitochondrial citrate synthase (C-CS and M-CS) (Nunes-Nesi et al., 2007), and NADP-, and NAD-isocitrate dehydrogenase (NADP- and NAD-ICDH) (Zhang et al., 2010).

Quantification of Ions and Metabolites

Ions and organic acids were assayed according to Ferchichi et al. (2018). Primary amino acids and proline were extracted and assayed according to Woodrow et al. (2017). Hydrogen peroxide (H_2O_2) amounts were evaluated according to (Baptista et al., 2007). Total proteins, starch, and soluble sugars were determined according to Carillo et al. (2012).

Statistical Analysis

Shoots from six and twenty plants for each treatment were used for biometric and SPAD measurements, respectively. The

other analyses were performed on three biological replicates for each treatment. Biometric measurements were statistically analyzed using the Student's t-test. The PCA analysis was assess using Minitab 16.8 statistical software (Ciarmiello et al., 2015). The score plot and loading matrix were determined based on the first and second principal components (PCs). A heatmap was generated using the https://biit.cs.ut.ee/CLUSTVIS/online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage. Morpho-physiological parameters and mineral composition data were visualized using a false color scale, with red indicating an increase and blue a decrease of values (Carillo et al., 2019a).

RESULTS

Plant Growth

Plants grown in hydroponics with 1 (low N) or 10 mM NO_3^- (high N) with and without 1 μM omeprazole (OP) are shown in **Figure 1**. In nonstress conditions (high N), OP treatment did not significantly increase growth in terms of fresh weight (**Figures 1** and **2A–D**) or leaf area (**Figure 2F**). OP alone induced a slight but significant ($p < 0.05$) increase of the SPAD-index, which has been significantly correlated with chlorophyll concentration, according to absorbance/transmittance measurements (Liu et al., 2017) (**Figure 2E**).

In nitrogen stress conditions (low N), leaf fresh weight, dry weight, and area were decreased by 50%, 47%, and 61%, respectively (**Figures 2A**, **B**, **F**) and 31% in roots (**Figures 2A**, **C**). Plants where visibly chlorotic (**Figure 1**) and SPAD-index values were 81% lower compared to high N controls ($p < 0.001$) (**Figure 2E**). Also root fresh and dry biomass were similarly affected with a decrease of 31% and 36%, respectively (**Figures 2B**, **D**). The presence of OP in the growth media had a significant effect in low N conditions by reversing the reduced growth effects of N stress. Growth in terms of fresh weight of shoots and roots was increased by 58% and 71%, respectively in OP treated plants compared to control plants (**Figures 2A**, **C**). Biomass accumulation was similarly affected with shoot biomass increasing by 61% and root biomass by 68% compared to controls without OP. Leaf area in OP treated plants in low N was 61% larger than control plants (**Figure 2F**). Leaves of OP treated plants in low N were also visibly greener (**Figure 1**) and SPAD-chlorophyll values were fivefold higher than untreated low N plants (<0.001) (**Figure 2E**). Overall, OP treatment did not significantly affect growth in high N conditions, however in low N conditions, it almost completely alleviated the symptoms of N stress induced by 1 mM NO_3^- availability

Effect of OP on Nitrogen Uptake

In order to determine if OP directly or indirectly affects nitrate uptake, we performed a nitrate uptake assays in increasing concentrations of OP. Our previous findings indicated that high concentrations of OP were inhibitory to growth (Van Oosten et al., 2017). Optimal (1 μM) and higher concentrations of OP were used in a nitrate uptake experiment to determine

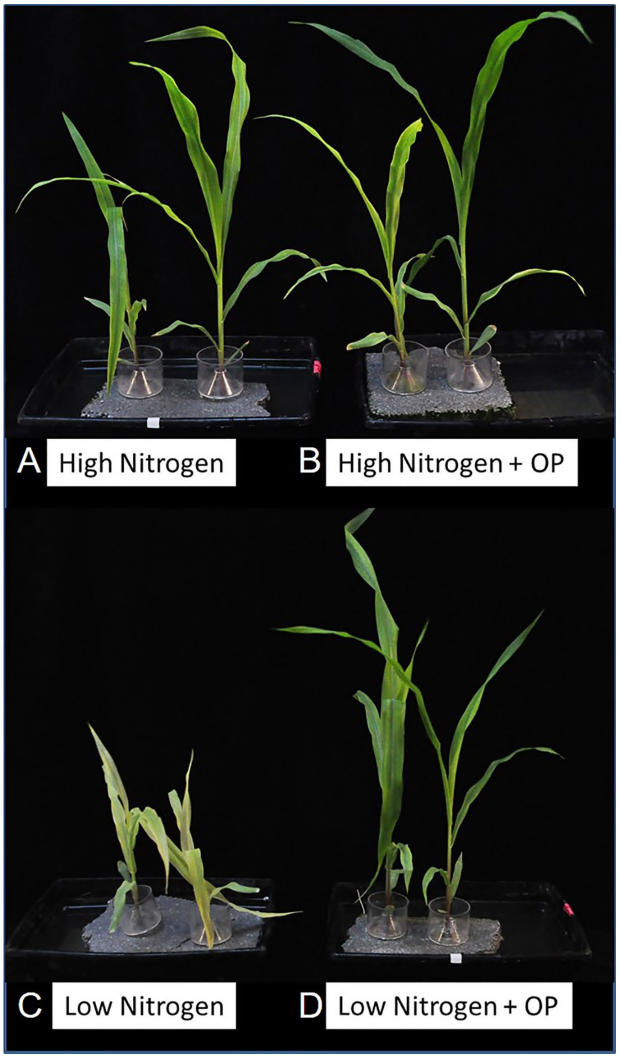


FIGURE 1 | Phenotype of hydroponically grown maize (*Zea mays* L.) plants in nitrogen stress conditions with 1 μM omeprazole (OP). **(A)** Control plants supplied with 10 mM NO_3^- (high N), **(B)** 1 μM OP treated high N plants, **(C)** Nitrogen stressed plants supplied with 1 mM NO_3^- (low N), **(D)** 1μM OP treated low N plants.

if high concentrations of OP were affecting nitrate uptake, and therefore, growth. Nitrate uptake of 3-week-old maize roots was assayed in the presence of 1, 10, 50, and 100 μM OP. Roots were incubated with 0, 100, and 500 μM nitrate in the presence and absence of OP. Nitrate uptake was evaluated using a method adapted from Sorgonà et al. (2011) to assess the ability of OP to affect both low-affinity and high-affinity uptake. The inducible low-affinity transport system (LATS) typically functions at concentrations higher than 250 μM NO_3^-, whereas the high-affinity transport system (HATS) functions in the range of 10–250 μM NO_3^- (Garnett et al., 2009). Nitrate uptake was affected by OP in a dose dependent manner. At low nitrate concentrations associated with HATS uptake (100 μM NO_3^-), OP increased NO_3^- uptake by 30% at 1 μM OP and by 27% at 10 μM OP. Higher OP concentrations had either a reduction effect

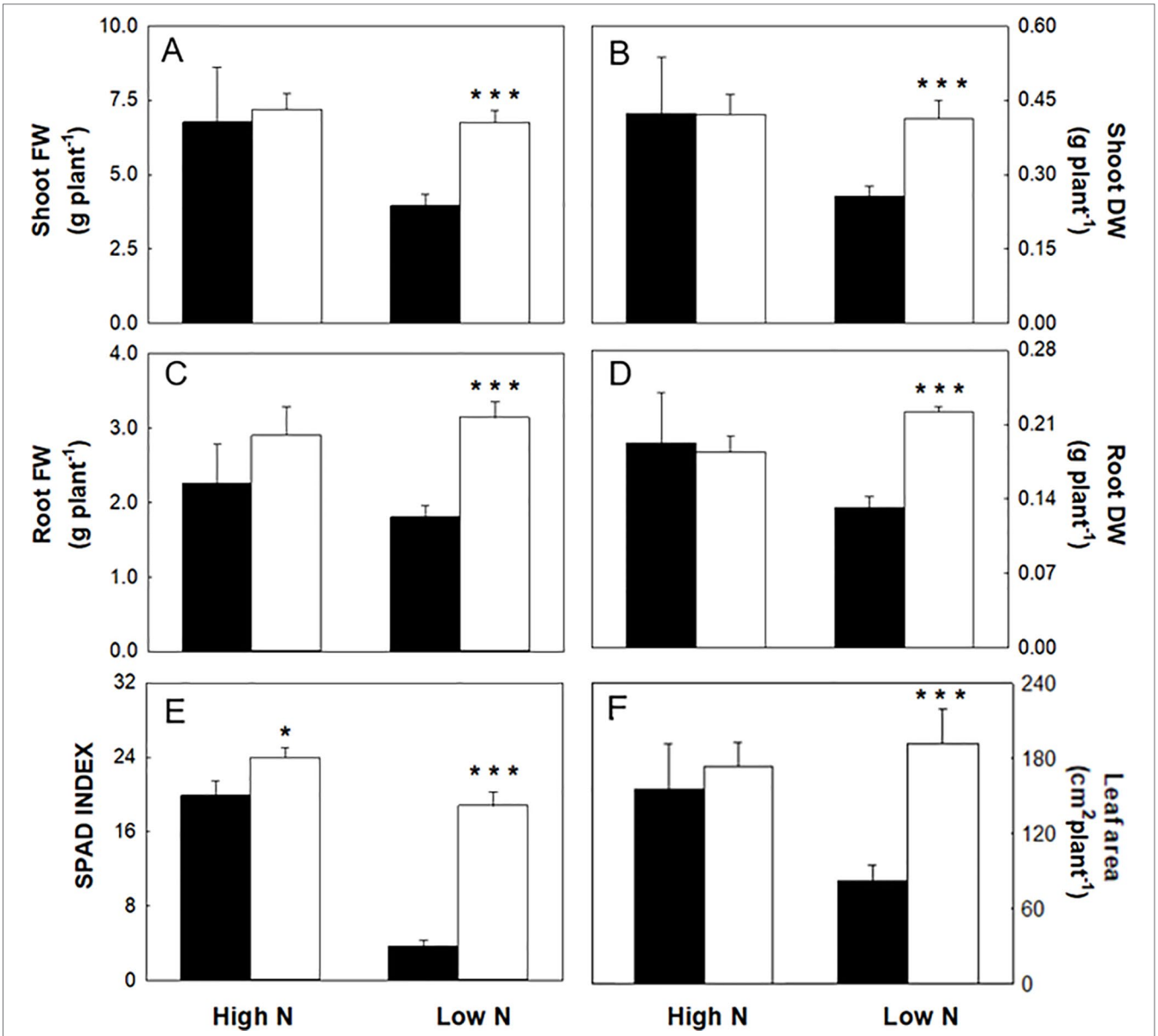


FIGURE 2 | Biometrics and SPAD index of maize plants hydroponically grown in control (10 mM NO_3^-, high N) and stress (1 mM NO_3^-, low N) nitrogen conditions with (black bars) and without 1 μM omeprazole (OP) (white bars). **(A)** FW of shoots, **(B)** DW of shoots, **(C)** FW of roots, **(D)** DW of roots, **(E)** SPAD index, and **(F)** leaf area. Data are means ± s.d.; n = 6 and 20 plants for each treatment for biometric and SPAD measurements, respectively. Asterisks denote significant differences (*, $P < 0.05$; ***, $P < 0.001$) between untreated controls and OP treated plants.

(50 μM OP) or an inhibitory effect (100 μM OP). OP had a small but significant effect on uptake at higher LATS associated concentrations (500 μM NO_3^-), but only at low OP dose (1 μM OP). At concentrations of 10, 50, and 100 μM OP, uptake of 500 μM NO_3^- decreased (**Supplementary Figure 1**).

ATPase Activity

The ability of OP to act as an inhibitor of membrane bound ATPases was evaluated using 3-week-old root and shoot tissues. The inhibitory effect of OP on ATPases in mammals is well characterized, but the inhibitory effect has not been tested in plants. To evaluate the potential ATPase inhibition in plants by OP, total protein and microsomal membranes were extracted. ATPase activity assays were performed using a dose curve of OP from 0.0001 to 1,000 μM. Sodium-orthovanadate is a strong inhibitor of P-Type ATPases in both plants and animals (Miao and Liu, 2012) and significantly inhibited ATPase activity in all experimental conditions. ATPase in the protein fraction was strongly inhibited by OP at very high concentrations (1 mM) and only in roots (**Supplementary Figure 2**).

Nitrate and Nitrogen Assimilation in Proteins and Amino Acids

Nitrogen assimilation was evaluated through the measurement of NO_3^-, total protein content and free amino acids content (**Figure 3**, **Supplemental Table 1**). Nitrate content in untreated high N and low N controls was not statistically significant different in shoots. However, the differences between the two treatments were highly significant in roots (−33%, $p < 0.001$). In plants treated with OP, NO_3^- content increased in leaves (12%) in high N conditions. Shoots of OP treated plants in low N conditions showed no change in NO_3^- while roots had a significantly lower NO_3^- content (−21%) (**Figure 3**). Protein content was not influenced by N limitation and OP treatment in leaves, while it increased significantly in roots. Low N decreased proteins in control and OP treated leaves of 29% and 44% compared to untreated high N control. On the contrary, low N determined a 1.5- and 3.9-fold increase of protein content in control and OP treated roots, respectively (**Figure 3** and **Table 1**).

The total amino acid concentration of shoots and roots of high N plants was differently affected by OP. While in leaves OP decreased significantly ($p < 0.05$) its content by 30% compared to the untreated shoots, in roots OP increased the amino acids content by 45% ($p < 0.05$). Total amino acids were strongly influenced by N limitation and OP treatment with significant low N × OP interaction. Low N decreased free amino acids of 61%, on average, in both organs ($p < 0.01$); low N + OP determined the opposite effect, with a significant increase ($p < 0.01$) of 176% and 78% in shoots and roots, respectively, compared to the low N without OP.

Asparagine was quantitatively the major amino acid representing about 71% and 55% of total free amino acids in shoots and roots of high N plants, respectively (**Table 1**). Low nitrate strongly affected this amino acid content, decreasing it to 13% and 7% of the high N values in shoots and roots, respectively. High N + OP had the same effects on shoots and roots total amino acids (probably because of the high asparagine contribution to the total amino acids content); while at low N, OP strongly increased asparagine content only in leaves (+2.8-fold, $p < 0.01$). This same effect induced by low N × OP treatment in shoots was also observed for aspartate (+ 90%), glutamine, glutamate (+ 110%), monoethanolamine (MEA) (+ 150%), glycine (+ 100%), serine (+ 270%), threonine (+ 860%), minor amino acids (+ 230%), and, specifically, branched chain amino acids (BCAAs) which belong to minor amino acids (+ 130%) (**Table 1**). Alanine and GABA underwent a very strong increase in their content under low N + OP, both in shoots and roots compared to low N controls (**Table 1**). Alanine increased by 3.6- and 4.3-fold in shoots and roots, respectively; while GABA showed a 4.3- and 5.2-fold increase in shoots and roots, respectively.

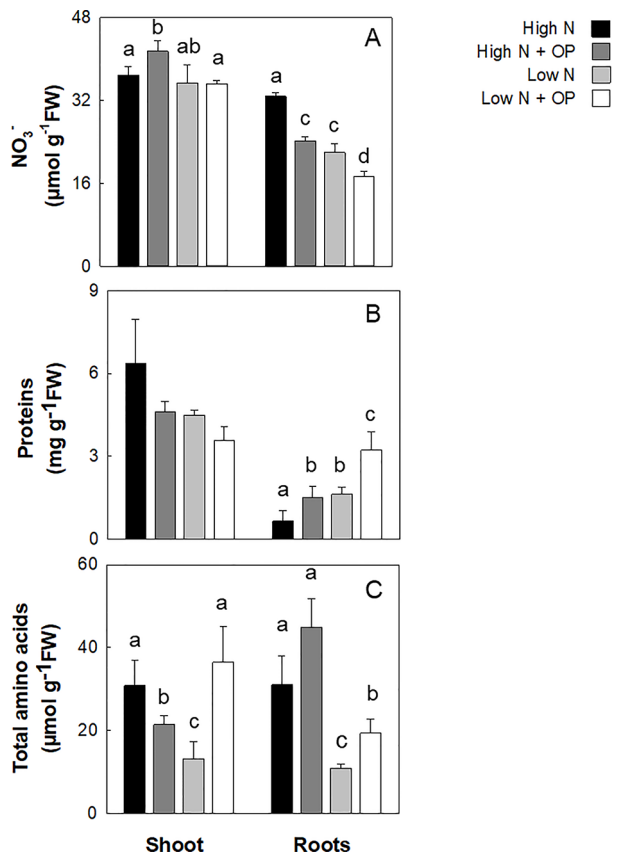


FIGURE 3 | Nitrate **(A)**, protein **(B)**, and total amino acid **(C)** content in maize plants hydroponically grown in control (10 mM NO3-, high N) and stress (1 mM NO3-, low N) nitrogen conditions with and without 1 µM omeprazole (OP). Data are means ± s.d.; n = 3 biological replicates for each treatment. Different letters are significantly different ($p < 0.05$, LSD-test).

OP Effects on NR Activity and Activation State

Total NR activity in maize shoots and roots of shock frozen and stored at −80°C plant material was 4.02 and 4.22 µmol NO_2^- h^{-1} g^{-1} FW, respectively (**Table 2**). The activation state strongly increased under OP treatment independently of N nutrition and organ, even if not significantly in shoots (**Table 2**). On the contrary, in fresh harvested plant shoots under high N the NR activity was on average 12.14 µmol NO_2^- h^{-1} g^{-1} FW, and it significantly increased ($p < 0.01$) of 18% and 48% under 1 and 10 µM OP, respectively (**Figure 4A**). The activation state of fresh harvested control shoots was 72.8%, and the OP treatment increased this value of 60% and 39% under 1 and 10 µM OP, respectively (**Figure 4B**).

The effect of OP treatment was tested on purified protein from Arabidopsis. OP 50 µM was able to increase the enzyme catalytic efficiency and the specificity for NO_3^- (as substrate) resulting in an increased V_{max} and decreased K_m (**Figure 5**). This suggests that OP helps in maintaining adequate affinity of enzyme toward its substrate as well as its catalytic rate though a possible physical interaction with NR.

Carbohydrates, Starch, and Citric Acid Cycle Content

Starch content was equivalent in shoots and roots under high N treatment (5.6 µmol G_{eq} g^{-1} FW on average) and low N (11.6 µmol G_{eq} g^{-1} FW on average). OP treatment resulted in an increase of 2.0- and 1.8-fold of starch content in high N shoots and roots,

TABLE 1 | Ions, hydrogen peroxide, malondialdehyde (MDA), carbohydrates, proteins, free amino acids, and polyphenols in shoots and roots of maize plants hydroponically grown in control (high N) and stress (low N) nitrogen conditions with and without 1μM omeprazole (OP). Values are mean ± s.d. (n = 3).

	Shoot								Root			
	High N	High N +OP	Low N	Low N +OP	High N	High N +OP	Low N	Low N +OP
Carbohydrates (μmol g^{-1} FW)								
Glucose	3.33 ± 1.10 ^{c}	4.22 ± 0.53 ^{c}	5.30 ± 0.23 ^{b}	6.71 ± 0.70 ^{a}	5.05 ± 0.37 ^{bc}	4.83 ± 0.22 ^{c}	6.40 ± 1.25 ^{b}	8.99 ± 0.91 ^{a}
Fructose	2.53 ± 0.19 ^{a}	1.76 ± 0.02 ^{b}	1.93 ± 0.63 ^{ab}	2.76 ± 0.94 ^{a}	2.35 ± 0.63 ^{a}	0.66 ± 0.48 ^{b}	2.20 ± 0.66 ^{ab}	1.35 ± 0.25 ^{b}
Starch	5.57 ± 1.52 ^{c}	11.03 ± 2.98 ^{b}	10.06 ± 3.52 ^{bc}	38.87 ± 8.05 ^{a}	5.67 ± 1.13 ^{c}	10.49 ± 3.52 ^{b}	13.17 ± 7.47 ^{bc}	43.90 ± 9.00 ^{a}
Sucrose	1.12 ± 0.10 ^{c}	0.82 ± 0.03 ^{d}	1.44 ± 0.14 ^{b}	2.29 ± 0.23 ^{a}	1.80 ± 0.48 ^{a}	0.84 ± 0.22 ^{b}	1.17 ± 0.13 ^{b}	2.30 ± 0.62 ^{a}
Soluble proteins (mg g^{-1} FW)								
Soluble proteins	6.35 ± 1.60 ^{a}	4.61 ± 0.37 ^{a}	4.49 ± 0.18 ^{a}	3.58 ± 0.49 ^{b}	0.65 ± 0.38 ^{c}	1.52 ± 0.41 ^{b}	1.63 ± 0.25 ^{b}	3.22 ± 0.67 ^{a}
Amino acids (μmol g^{-1} FW)								
Total amino acid	30.85 ± 5.99 ^{a}	21.45 ± 2.12 ^{b}	13.20 ± 4.10 ^{c}	36.44 ± 8.57 ^{a}	31.03 ± 6.96 ^{b}	44.97 ± 6.76 ^{a}	10.88 ± 0.93 ^{d}	19.33 ± 3.35 ^{c}
Alanine	1.31 ± 0.26 ^{b}	0.92 ± 0.08 ^{c}	2.07 ± 0.70 ^{b}	7.54 ± 1.84 ^{a}	0.92 ± 0.24 ^{d}	2.94 ± 0.42 ^{b}	1.76 ± 0.30 ^{c}	7.48 ± 1.61 ^{a}
Asparagine	21.88 ± 4.35 ^{a}	14.57 ± 1.58 ^{b}	2.84 ± 0.92 ^{d}	7.97 ± 1.77 ^{c}	17.19 ± 4.08 ^{a}	24.07 ± 3.52 ^{a}	1.29 ± 0.17 ^{b}	1.54 ± 0.32 ^{b}
Aspartate	0.58 ± 0.11 ^{b}	0.43 ± 0.03 ^{c}	0.57 ± 0.17 ^{bc}	1.08 ± 0.24 ^{a}	1.42 ± 0.36 ^{ab}	1.66 ± 0.38 ^{a}	1.00 ± 0.10 ^{b}	1.20 ± 0.15 ^{ab}
BCAAs	0.38 ± 0.14 ^{b}	0.29 ± 0.02 ^{b}	0.34 ± 0.05 ^{b}	0.79 ± 0.12 ^{a}	0.75 ± 0.13 ^{a}	0.87 ± 0.09 ^{a}	0.76 ± 0.09 ^{a}	0.79 ± 0.12 ^{a}
GABA	0.03 ± 0.01 ^{bc}	0.03 ± 0.00 ^{c}	0.04 ± 0.01 ^{b}	0.18 ± 0.05 ^{a}	0.08 ± 0.02 ^{b}	0.12 ± 0.03 ^{ab}	0.04 ± 0.01 ^{c}	0.18 ± 0.04 ^{a}
Glutamate	1.65 ± 0.32 ^{b}	1.12 ± 0.07 ^{c}	1.72 ± 0.57 ^{bc}	3.57 ± 0.75 ^{a}	2.65 ± 0.42 ^{ab}	2.88 ± 0.59 ^{ab}	2.10 ± 0.24 ^{b}	3.22 ± 0.41 ^{a}
Glutamine	0.96 ± 0.20 ^{b}	0.30 ± 0.02 ^{c}	0.69 ± 0.23 ^{b}	2.24 ± 0.73 ^{a}	3.92 ± 0.98 ^{b}	7.81 ± 1.08 ^{a}	1.24 ± 0.10 ^{c}	1.38 ± 0.24 ^{c}
Glycine	1.45 ± 0.23 ^{bc}	1.41 ± 0.09 ^{c}	2.15 ± 0.59 ^{b}	4.26 ± 1.03 ^{a}	0.82 ± 0.09 ^{a}	0.53 ± 0.06 ^{b}	0.23 ± 0.03 ^{c}	0.43 ± 0.06 ^{b}
Minor amino acids	0.80 ± 0.22 ^{b}	0.62 ± 0.05 ^{b}	0.72 ± 0.15 ^{b}	2.35 ± 0.41 ^{a}	1.56 ± 0.31 ^{ab}	2.05 ± 0.29 ^{a}	1.45 ± 0.16 ^{b}	1.48 ± 0.25 ^{b}
Monoethanolamine	0.27 ± 0.06 ^{b}	0.20 ± 0.02 ^{b}	0.24 ± 0.09 ^{b}	0.60 ± 0.15 ^{a}	0.46 ± 0.11 ^{a}	0.43 ± 0.10 ^{a}	0.30 ± 0.01 ^{b}	0.38 ± 0.04 ^{a}
Ornithine	0.24 ± 0.05 ^{a}	0.17 ± 0.08 ^{abc}	0.11 ± 0.01 ^{c}	0.15 ± 0.01 ^{b}	0.90 ± 0.15 ^{a}	0.90 ± 0.08 ^{a}	0.40 ± 0.07 ^{b}	0.32 ± 0.03 ^{b}
Proline	0.30 ± 0.06 ^{ab}	0.34 ± 0.03 ^{ab}	0.30 ± 0.01 ^{b}	0.38 ± 0.04 ^{a}	0.27 ± 0.01 ^{a}	0.33 ± 0.06 ^{a}	0.22 ± 0.01 ^{b}	0.35 ± 0.08 ^{a}
Serine	1.29 ± 0.25 ^{b}	1.27 ± 0.11 ^{b}	1.69 ± 0.63 ^{b}	6.21 ± 1.65 ^{a}	0.71 ± 0.17 ^{b}	1.11 ± 0.20 ^{a}	0.71 ± 0.06 ^{b}	1.19 ± 0.22 ^{a}
Threonine	0.09 ± 0.02 ^{b}	0.07 ± 0.02 ^{bc}	0.05 ± 0.02 ^{c}	0.49 ± 0.15 ^{a}	0.15 ± 0.04 ^{ab}	0.22 ± 0.04 ^{a}	0.16 ± 0.02 ^{b}	0.18 ± 0.04 ^{ab}
Ions and hydrogen peroxide (μmol g^{-1} FW)								
Acetate	3.48 ± 0.30 ^{a}	3.10 ± 0.37 ^{a}	3.27 ± 0.72 ^{a}	1.95 ± 0.18 ^{b}	6.05 ± 0.33 ^{a}	5.76 ± 0.68 ^{a}	4.41 ± 0.62 ^{b}	4.32 ± 0.20 ^{b}
Chloride	62.51 ± 2.95 ^{a}	52.53 ± 2.11 ^{b}	72.69 ± 16.06 ^{a}	48.63 ± 1.59 ^{c}	32.08 ± 3.54 ^{b}	29.27 ± 1.62 ^{b}	41.95 ± 4.41 ^{a}	42.83 ± 6.09 ^{a}
Malate	2.32 ± 0.12 ^{c}	1.64 ± 0.07 ^{d}	3.85 ± 0.93 ^{a}	2.73 ± 0.05 ^{b}	1.05 ± 0.10 ^{c}	0.64 ± 0.11 ^{d}	2.09 ± 0.25 ^{b}	2.74 ± 0.18 ^{a}
Nitrate	36.92 ± 1.56 ^{b}	41.51 ± 1.94 ^{a}	35.34 ± 3.48 ^{b}	35.19 ± 0.74 ^{b}	32.76 ± 0.72 ^{a}	24.18 ± 0.78 ^{b}	21.93 ± 1.70 ^{b}	17.38 ± 1.00 ^{c}
Oxalacetate	2.16 ± 0.10 ^{a}	1.97 ± 0.25 ^{a}	0.93 ± 0.14 ^{c}	1.21 ± 0.12 ^{b}	4.65 ± 0.42 ^{a}	3.78 ± 0.23 ^{b}	3.96 ± 0.64 ^{ab}	3.57 ± 0.13 ^{b}
Sulphate	1.82 ± 0.08 ^{b}	2.02 ± 0.03 ^{a}	2.07 ± 0.32 ^{ab}	1.28 ± 0.03 ^{c}	7.84 ± 0.60 ^{c}	7.08 ± 0.45 ^{c}	12.26 ± 1.07 ^{a}	10.57 ± 0.16 ^{b}
Hydrogen peroxide	3.10 ± 0.66 ^{c}	3.23 ± 0.31 ^{c}	7.38 ± 0.64 ^{a}	4.70 ± 0.34 ^{b}	2.74 ± 0.34 ^{a}	2.70 ± 0.57 ^{a}	2.63 ± 0.23 ^{a}	1.81 ± 0.23 ^{b}
Other compounds								
MDA (nmol g^{-1} FW)	28.70 ± 0.51 ^{b}	34.32 ± 4.93 ^{a}	24.12 ± 3.50 ^{c}	28.45 ± 0.87 ^{bc}	81.00 ± 10.67 ^{a}	83.91 ± 8.57 ^{a}	51.23 ± 5.75 ^{b}	48.78 ± 2.32 ^{b}
Polyphenols (mg$_{GAE}$ g^{-1} FW)	2.34 ± 0.19 ^{a}	1.99 ± 0.08 ^{b}	2.35 ± 0.23 ^{a}	1.74 ± 0.13 ^{c}	1.15 ± 0.13 ^{a}	1.35 ± 0.18 ^{a}	1.34 ± 0.21 ^{a}	1.32 ± 0.06 ^{a}

Means in the same rows with different letters are significantly different ($p < 0.05$, Student's t-test).

TABLE 2 | Carbon and nitrogen metabolism enzymes and antioxidant enzymes activities in shoots and roots of maize plants hydroponically grown in control (high N) and stress (low N) nitrogen conditions with and without 1 μM omeprazole (OP). Values are mean ± s.d. (n = 3).

	Shoot				Root			
	High N		High N +OP		High N		Low N +OP	
Carbon and nitrogen metabolism enzymes (μmol h^{-1} g^{-1} FW)								
AGPase	39.82 ± 6.49	a	40.17 ± 3.24	a	11.53 ± 3.09	c	26.33 ± 3.33	b
C-Citrate synthase	27.71 ± 5.97	a	26.01 ± 4.97	a	20.29 ± 5.81	ab	15.92 ± 4.80	b
M-Citrate synthase	19.01 ± 3.05	a	15.88 ± 4.74	ab	11.54 ± 1.76	b	14.12 ± 3.47	ab
G6P DH	15.76 ± 2.01	a	14.94 ± 4.85	ab	12.71 ± 3.91	ab	10.28 ± 3.18	b
Glucokinase	4.84 ± 1.26	a	3.79 ± 0.23	a	5.29 ± 1.28	a	4.95 ± 1.08	a
Glutamate DH (NAD)	11.44 ± 2.60	ab	11.02 ± 0.55	a	5.29 ± 1.55	b	7.07 ± 2.11	b
Isocitrate DH (NAD)	5.85 ± 0.96	a	4.64 ± 1.13	a	5.33 ± 1.17	a	6.27 ± 4.21	a
Isocitrate DH (NADP)	9.77 ± 1.43	a	9.17 ± 1.21	a	7.77 ± 2.16	a	7.57 ± 0.81	a
NR activity	4.37 ± 0.87	a	3.38 ± 0.48	a	4.61 ± 1.85	a	3.86 ± 0.92	a
NR Activation state (%)	65.1 ± 24.5	a	114.4 ± 34.9	a	68.6 ± 24.8	a	118.2 ± 44.4	a
PEP Carboxylase	145 ± 29	ab	187 ± 51	a	104 ± 32	ab	63.4 ± 16.7	b
Pyruvate kinase	171 ± 15	a	166 ± 5	a	118 ± 13	b	142 ± 29	ab
Antioxidant enzymes (mmol h^{-1} g^{-1} FW)								
APX	1.08 ± 0.19	b	1.67 ± 0.16	a	1.87 ± 0.26	a	1.57 ± 0.70	ab
CAT	1.59 ± 0.29	a	1.93 ± 0.12	a	1.89 ± 0.03	a	1.67 ± 0.23	a
GR	1.52 ± 0.18	ab	1.58 ± 0.03	a	1.37 ± 0.10	b	1.51 ± 0.08	ab
SOD	4.49 ± 1.65	a	4.01 ± 1.77	a	4.86 ± 0.52	a	2.99 ± 2.22	a

	Root							
	High N		High N +OP		Low N		Low N +OP	
Carbon and nitrogen metabolism enzymes (μmol h^{-1} g^{-1} FW)								
AGPase	45.20 ± 6.51	a	46.00 ± 5.04	a	51.09 ± 4.86	a	41.72 ± 10.70	a
C-Citrate synthase	33.00 ± 5.27	a	33.83 ± 3.37	a	35.43 ± 3.12	a	31.14 ± 3.99	a
M-Citrate synthase	24.33 ± 3.91	a	23.55 ± 2.76	a	24.96 ± 4.48	a	21.36 ± 2.06	a
G6P DH	8.47 ± 0.46	a	9.10 ± 0.42	a	8.21 ± 1.60	a	10.61 ± 1.95	a
Glucokinase	45.59 ± 7.31	a	29.69 ± 2.88	b	25.69 ± 7.90	b	17.78 ± 3.43	c
Glutamate DH (NAD)	14.54 ± 3.40	a	18.93 ± 3.19	a	24.36 ± 7.26	a	18.35 ± 4.37	a
Isocitrate DH (NAD)	67.00 ± 6.48	a	63.12 ± 5.41	a	58.00 ± 8.47	a	64.35 ± 13.35	a
Isocitrate DH (NADP)	3.87 ± 1.59	a	4.20 ± 1.42	a	4.65 ± 1.88	a	4.19 ± 0.64	a
NR activity	79.2 ± 22.8	ab	116 ± 22	a	77.1 ± 9.9	b	113 ± 27	ab
NR Activation state (%)	25.8 ± 4.3	a	29.5 ± 6.3	a	29.8 ± 7.1	a	32.8 ± 8.4	a
PEP Carboxylase	49.2 ± 9.8	a	53.1 ± 11.0	a	59.5 ± 14.8	a	50.8 ± 9.7	a
Pyruvate kinase								
Antioxidant enzymes (mmol h^{-1} g^{-1} FW)								
APX	1.12 ± 0.30	b	1.93 ± 0.34	a	1.22 ± 0.35	b	0.92 ± 0.03	b
CAT	1.49 ± 0.41	b	1.93 ± 0.40	ab	1.74 ± 0.19	b	2.03 ± 0.10	a
GR	1.61 ± 0.13	b	1.67 ± 0.19	ab	1.67 ± 0.21	ab	1.80 ± 0.07	a
SOD	4.21 ± 1.51	a	4.41 ± 0.65	a	3.84 ± 1.64	a	3.20 ± 0.64	a

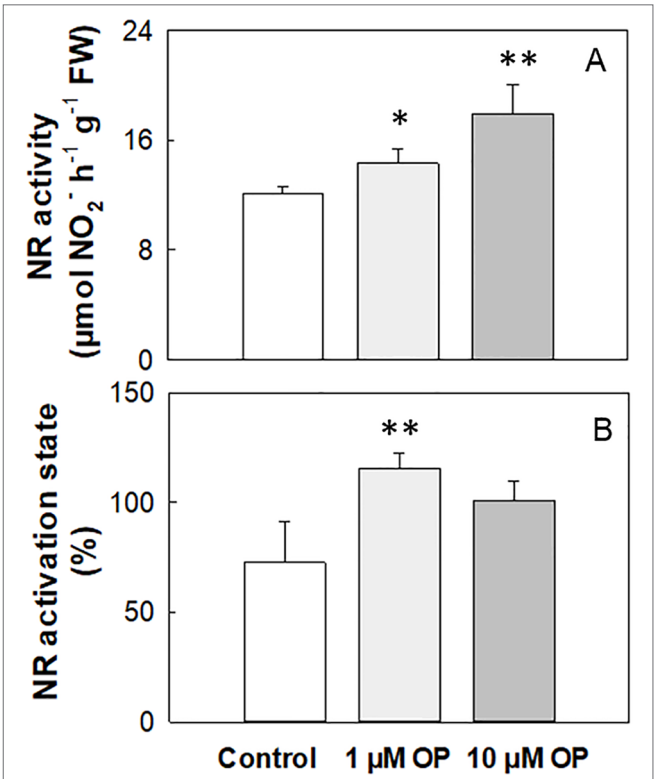


FIGURE 4 | Nitrate reductase activity **(A)** and activation state **(B)** of shoots from maize (*Zea mays* L.) plants hydroponically grown in control (10 mM NO_3^-, high N) and stress (1 mM NO_3^-, low N) nitrogen conditions with (black bars) and without 1 μM omeprazole (OP) (white bars). Data are means ± SD; n = 3 biological replicates for each treatment. Asterisks denote significant differences (*, P < 0.05; **, P < 0.01) between different OP treated plants.

respectively; while a much stronger effect was exerted at low N with 3.9- and 3.3-fold increases in the two organs, respectively (**Table 1**). Sucrose levels were decreased in OP treated high N shoots (−30%, $p < 0.01$) and roots (−50%, $p < 0.05$). A strong increase was observed for sucrose and hexoses under OP treated low N conditions ($p < 0.05$). OP treatment caused a shift in primary metabolism that shunted sugars into starch storage in both low and high N conditions. In shoots under low N and OP conditions, this was a consequence of a 2.8-fold increase of ADP-glucose pyrophosphorylase (AGPase) activity (**Table 2**).

OP treatment also perturbed the citric acid cycle. Levels of oxaloacetate and malate were evaluated (**Table 1**). Oxaloacetate was 2.1 and 4.2 μmol g^{-1} FW in shots and roots of high N plants, respectively, independently of OP. OP significantly decreased (−40%, $p < 0.01$) acetate content only in low N shoots. Malate was 2.3 and 3.9 μmol g^{-1} FW in shots of control high and low N plants, respectively; while it was 45% and 54% lower in control high and low N roots compared to relative shoots. OP reduced malate content in shoots both high N and low N (−29%); but it had an opposite effect on roots, decreasing its content at high N (−39%) while increasing it at low N (+31%) (**Table 1**). Acetate, even not belonging to the citric acid cycle, had a similar trend to that of oxaloacetate, with the difference that low N did affect control roots (−27%, $p < 0.05$) but not shoots (**Table 1**).

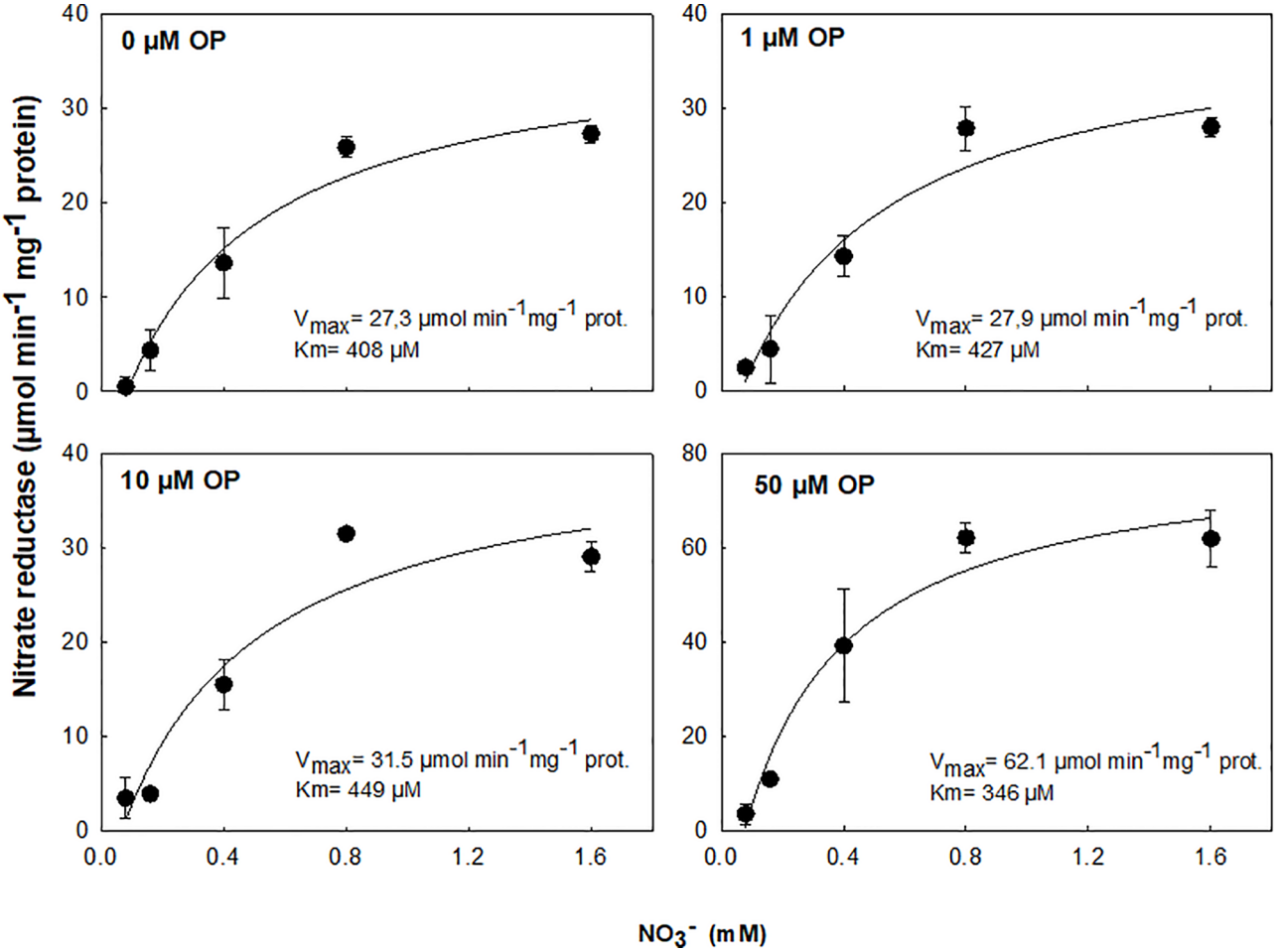


FIGURE 5 | *In vitro* activity assay of purified NADH-Nitrate Reductase (NR) from *Arabidopsis thaliana* in cell-free extracts with 0.08, 0.16, 0.4, 0.8, and 1.6 mM NO_3^- and 0, 1, 10, or 50 µM omeprazole (OP). Data are means ± s.d. (shown by vertical bars when larger than the symbol); n = 3 from independent experiments. Lines were fitted by linear regression.

OP Effects on Hydrogen Peroxide, MDA, Polyphenols, and Antioxidant Enzymes

Formation of hydrogen peroxide (H_2O_2) under nitrogen deficiency is indicative of reduced efficiency of electron transport systems and can act as a redox signal (Kandlbinder et al., 2004; Tewari et al., 2007). Nitrogen stress induced formation of H_2O_2. Its concentrations in low N controls were 7.38 µmol g^{-1} FW in shoots and 2.63 µmol g^{-1} FW in roots. The H_2O_2 content in low N shoots was 2.38-fold higher than the high N controls. OP reduced H_2O_2 content by 34% in roots and shoots (**Table 1**). Malondialdehyde (MDA) content was 69% higher in shoots and 185% higher in roots under low N conditions. However, OP treatment had no effect on the MDA concentration (**Table 1**).

Polyphenols play a key role in antioxidant activity and membrane protection in plants (Woodrow et al., 2017). Their content was, on average, 2.34 and 1.24 mg GAE g^{-1} FW in shoots ad roots, respectively. OP had no effect on roots, while it significantly decreased the content of these metabolites both in high N shoots (−15%, $p < 0.05$) and low N shoots (−26%, $p < 0.01$) (**Table 1**).

Catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) activities were fairly constant independently of N nutrition, OP treatment and organ, being, on average 1.8, 1.6, and 4.0 U g^{-1} FW, respectively (**Table 1**). On the contrary, ascorbate peroxidase (APX) varied in dependence on N nutrition and OP treatment. In high N control shots and roots, it was 1.1 U g^{-1} FW. Low N increased APX activity in shoots (+73%) but not in roots. OP significantly affected this enzyme activity only in plants grown under high N, increasing it of 54 ($p < 0.01$) and 72% ($p < 0.05$) in shoots and roots, respectively, compared to controls (**Table 1**).

Gene Expression

NRT2.2 and *NRT2.1* are the main genes controlling the high-affinity nitrate uptake in maize (Garnett et al., 2013). OP treatment increased the root expression of *ZmNRT2.1* of threefold in high N and almost fourfold in low N conditions. Shoot expression of *ZmNRT2.1* was not significantly influenced by OP in high N conditions while it was half of untreated controls in low N conditions (**Figure 6A**). The *ZmNAR2.1*

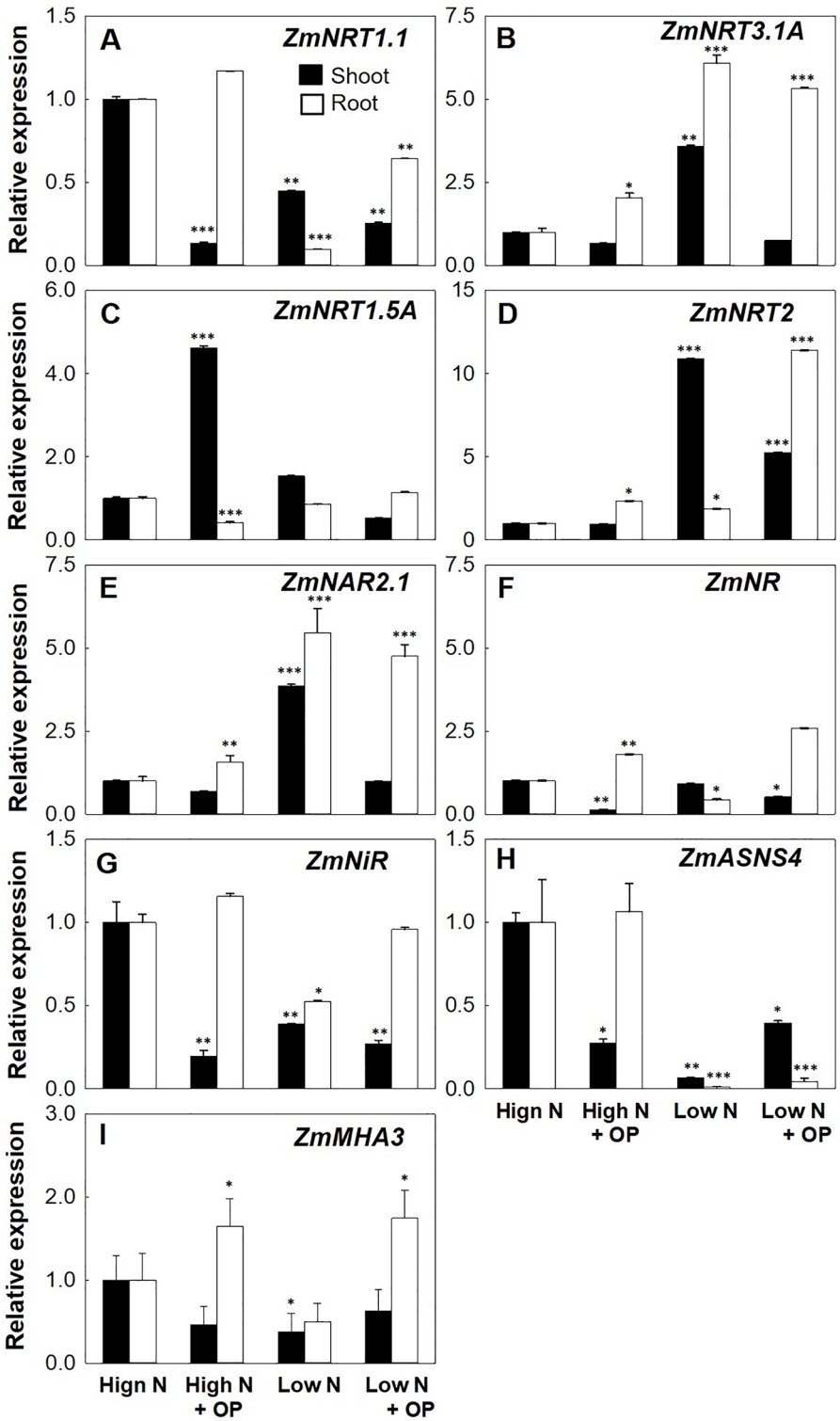


FIGURE 6 | Nitrogen uptake and assimilation genes expression of maize plants hydroponically grown in in control (10 mM NO_3^-, high N) and stress (1 mM NO_3^-, low N) nitrogen conditions with and without 1 μM omeprazole (OP) in shoot (black bars) and root (white bars). Relative gene expression (in terms of fold change) of **(A)** nitrate transporter 1.1 *ZmNRT1.1*, **(B)** nitrate transporter 1.3A *ZmNRT1.3A*, **(C)** nitrate transporter 1.5A *ZmNRT1.5A*, **(D)** nitrate transporters 2.1 and 2.2 *ZmNRT2*, **(E)** NAR2-like partner protein *ZmNAR2*.1, **(F)** nitrate reductase *ZmNR*, **(G)** nitrite reductase *ZmNiR*, **(H)** asparagine synthetase *ZmASN4*, and **(I)** the plasma membrane MHA3 ATPase *ZmMHA3*. Data are means of relative expression measurements based on the ΔΔCt Method ± s.d.; n = 3 biological replicates for each treatment. Asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between different each treatment and controls under high N according to Student's T-Test.

and *NAR2*-like gene *ZmNRT3.1A* coding for accessory protein complexes with NRT2.1, implicated in NRT2.1 localization or stabilization at the PM as well as involved in iHATS signaling (Okamoto et al., 2006; Yong et al., 2010; Chen et al., 2017), were highly induced by OP. Low N conditions induced the expression of these two genes in both roots and shoots, with OP inducing a twofold increase in high N conditions. Similar to *ZmNRT2.1* expression in low N with OP, *ZmNAR2.1* and *ZmNRT3.1A* were decreased in shoots under low N and OP. *ZmNPF6.3/NRT1.1,* which is expressed in root tissues as well as in young shoot tissues and is involved in nitrate uptake from soil and its translocation to shoot (Noguero and Lacombe, 2016) and that can functions also as nitrate sensor promoting and controlling root system architecture (Medici and Krouk, 2014), was highly increased by OP in particular in root both under high N and low N. Also *ZmNPF7.3/NRT1.5*, coding for a low-affinity bidirectional nitrate transporter involved in xylem loading and root-to-shoot translocation of nitrate (Zheng et al., 2016), was highly expressed in leaves of high N plants under OP treatment, too.

Cytosolic NRs reduce nitrate translocated from the roots into nitrite and are controlled transcriptionally and posttranslationally (Krapp, 2015). Low N conditions resulted in a down regulation of *ZmNR* in roots (**Figure 6H**). OP treatment reduced the expression of NR in shoots for both low and high N conditions. In roots, OP treatment significantly increased NR expression twofold in high N conditions and 2.5-fold in low N conditions. Nitrite reductase gene (*ZmNiR*) demonstrated a similar pattern of expression under OP treatment with reduced expression in shoots and increased expression in roots (**Figure 6I**). The asparagine synthetase ZmASN4 is one of four isoforms in maize responsible for N assimilation. Asparagine is the amide having the higher N/C ratio (2N:4C) and as the major form of transport for N in plants is involved in assimilation, distribution, and remobilization (Krapp, 2015). In low N condition *ZmASN4* was severely down regulated in roots and shoots. *ASNS4* is ubiquitously expressed in all tissues and is transcriptionally downregulated in maize under nitrogen starvation (Todd et al., 2008; Zanin et al., 2015). In low N plants treated with OP, shoot expression was higher. Interestingly, under high N and OP treatment, shoot expression of *ZmASN4* was significantly downregulated, likely indicating a negative feedback regulation.

PCA, Heat Map, and Correlation Analyses

To obtain an overview of the effects of OP × nitrogen nutrition on growth and biochemical parameters of maize seedlings, a PC analysis (PCA) was carried out. In shoots, the first two PCs were related with Eigen values > 1 and explained 84.6% of the total variance with PC1 and PC2 accounting for 51.5% and 33.2%, respectively (**Figure 7A**). PC1 was positively correlated to nitrate, sulphate, asparagine, oxaloacetate and acetate, proteins, fresh and dry weight, enzymatic activities, and polyphenols. PC1 was also negatively correlated to amino acids, in particular alanine, serine, minor amino acids, GABA, glutamate and glutamine, MEA, starch, and soluble sugars. Moreover, PC2 was positively correlated to hydrogen peroxide, malate, APX, catalase, chloride, and NR activity. Furthermore, the PCA scatter-plot split the samples into three main groups, with high N and high N + OP clustered together clustered in the fourth quadrant in the positive side of PC1, completely separated from the other two treatments. They showed the highest nitrate and chlorophyll content (SPAD), but also the highest MDA concentration and GR and other carbon enzymes activities. Low N was clustered in the second quadrant, in the negative side of PC2, showing the lowest fresh weight, dry weight, chlorophyll content but also the lowest MDA content (**Figure 7A**). Low N + OP cluster in the third quadrant, in the negative side of PC1, had the highest total and minor amino acid, included glutamate, glutamine, aspartate, GABA, and MEA, in addition to starch and soluble sugars content.

In roots the first two PCs were related with Eigen values > 1 and explained 80.3% of the total variance. Nitrogen nutrition contributed to the clear separation on PC1, which described 46.1% of the variability, while the treatment with OP contributed to separation on PC2, which described 34.2% of the variability (**Figure 7B**). PC1 was positively correlated to total amino acids, in particular amides and ornithine, but also MDA, SOD, and GDH activities; while it was negatively correlated to glucose, malate, soluble proteins, starch, sulphate and chloride, GR, and PEPC activities. Moreover, PC2 was positively correlated to fresh weight and dry weight, proline, glutamate, NR activation, GABA, BCAAs, serine, and threonine; while it was negatively correlated to fructose, mitochondrial and cytosolic citrate synthase activities, pyruvate kinase, and NR activities. The score plot of the PCA divided the four treatments in different quadrants with high N and high N + OP, in the fourth and first quadrant, respectively, but without a sharp division between the two treatments. High N showed the highest nitrate and OAA, but also the highest hydrogen peroxide and SOD activities; while high N + OP showed the major total amino acid content, in particular glutamine, aspartate, and minor amino acids. Low N + OP was clustered in the second quadrant, in the negative side of PC1, and showed the major carbohydrate and protein content, glutamate, proline and GABA content, and the highest PEPC, catalase, and GR activities. Low N was clustered in the third quadrant, in the negative side of PC2, and showed the highest sulphate content and NR activity, but also the lowest amino acid content, dry and fresh weight (**Figure 7B**).

A heat map providing the biochemical and physiological changes of maize plants in response to nitrogen × OP was displayed in **Figure 8**. The heat-map identified two main clusters in both shoot and root, with nitrogen being the main clustering factor in both shoots and roots, followed by OP. In particular, high N treatments clustered separated from the other two low N treatments in shoots because of their higher fresh and dry weight, SPAD index, asparagine, oxaloacetate, carbon enzymes activity and nitrate content (**Figure 8A**), and in roots because of their higher MEA, glycine, acetate, ornithine, MDA, total amino acid content, in particular asparagine and aspartate (**Figure 8B**). Indeed, two separated subclusters could be defined under both the first and the second clusters which

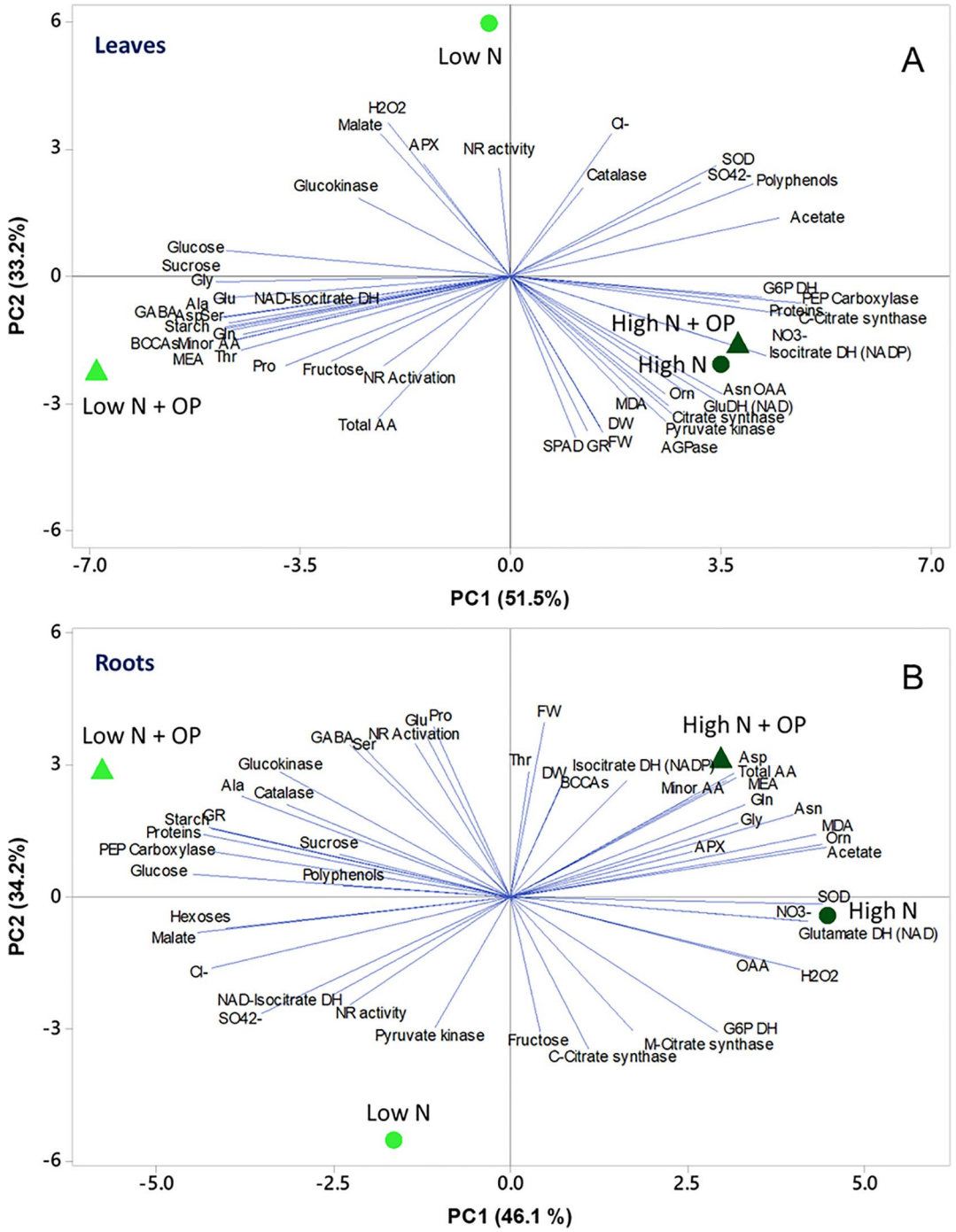


FIGURE 7 | Principal component analysis (PCA) scatter plots based on the first two principal components (PC1 and PC2) generated from the analyzed physiological parameters in maize plants for shoot **(A)** and root **(B)** under control (10 mM NO_3^-, high N) and stress (1 mM NO_3^-, low N) nitrogen conditions with and without 1 μM omeprazole (OP).

illustrated the nitrogen × OP interaction. In particular, the OP application at high N in the shoots separated from that with high N without OP because of the higher nitrate, SPAD index, PEPC, but also the highest MDA, catalase and GR activity, and lowest sugar content and NR activity (**Figure 8A**). While low N + OP subclustered separately from low N without OP in the shoots because of the highest total and minor amino acid content, with the exception of asparagine, highest carbohydrate content, and lowest protein and carbon metabolism enzymes activity (**Figure 8A**). In the roots, low N + OP clustered

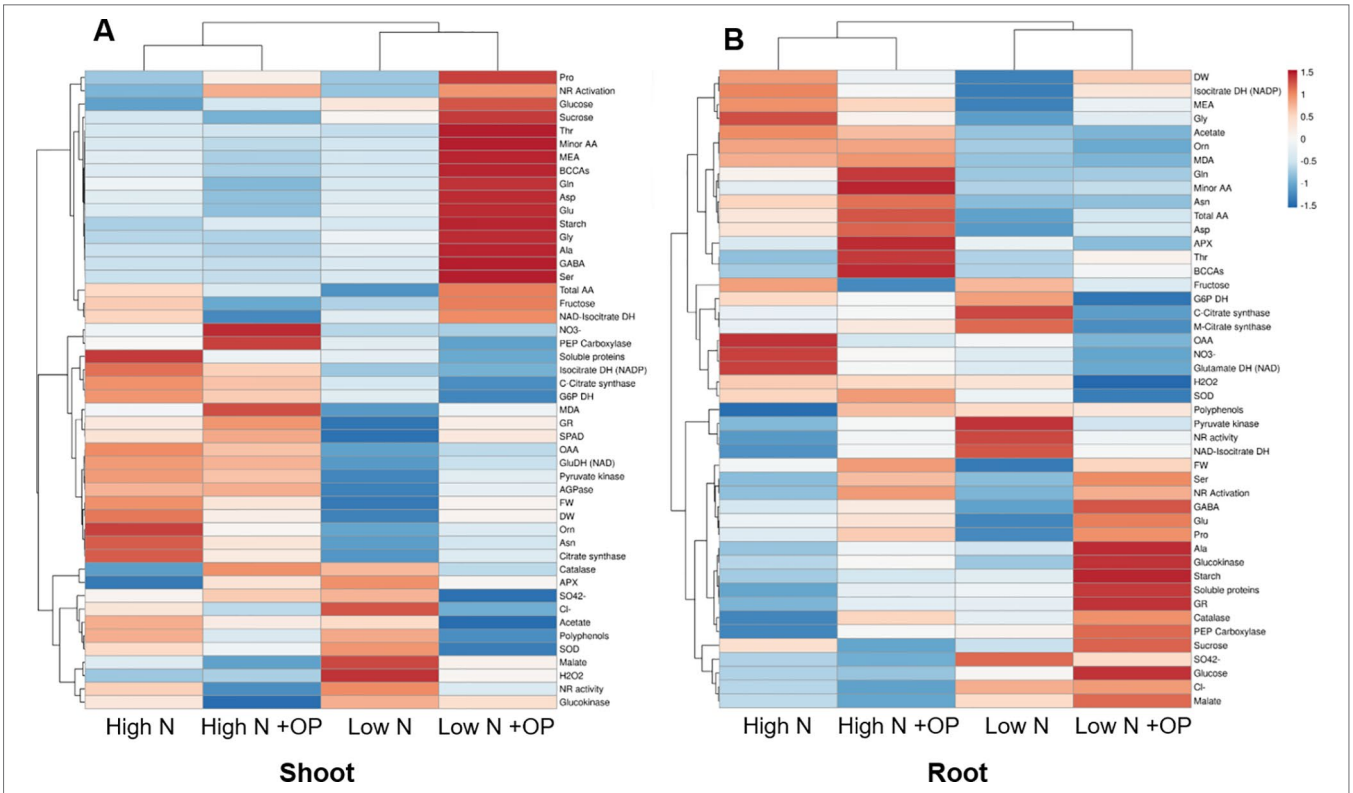


FIGURE 8 | Cluster heat map analysis summarizing the maize shoot **(A)** and root **(B)** responses to high and low nitrogen concentration in the nutrient solution and omeprazole (OP) treatment. Plants were hydroponically grown in control (10 mM NO_3^-, high N) and stress (1 mM NO_3^-, low N) nitrogen conditions with and without 1 μM OP. The heat map was generated using the https://biit.cs.ut.ee/clustvis/online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage. Data presented are means of biological replicates; biometric measurements (FW of shoots, DW of shoots, FW of roots, DW of roots, leaf area) (n = 6), SPAD index (n = 20), and other biochemical measurements (n = 3).

separately from low N without OP because of the highest carbohydrate and protein content, PEPC, glucokinase and GR activities, GABA, alanine, glutamate, and proline content (**Figure 8B**).

The correlation analysis showed in roots a negative correlation between the level of nitrate and soluble proteins ($r = 0.86$; $p < 0.001$), but a positive correlation between fresh weight and free amino acids ($r = 0.73$; $p < 0.001$). Particularly in roots under low N, there was a negative correlation between the level of asparagine and soluble proteins ($r = 0.75$; $p < 0.05$), but a positive one between malate and free amino acids ($r = 0.85$; $p < 0.05$) and glutamate and soluble proteins ($r = 0.91$; $p < 0.05$).

DISCUSSION

The targets and effects of OP in mammals have been well characterized for many decades (Clissold and Campoli-Richards, 1986; Massoomi et al., 1993). Recent reports have established that OP applications in plants elicit two major phenotypes: growth promotion and salt tolerance (Van Oosten et al., 2017a; Rouphael et al., 2018; Carillo et al., 2019d; Cirillo et al., 2019). Recently it has been proved that also OP can enhance mechanical stress tolerance (Okamoto et al., 2018). In this study, we further investigated if mechanisms directly involved with nitrate uptake and assimilation could be perturbed or activated by OP treatment contributing to the growth promotion phenotype. Previous experiments indicated that OP treatment increased nitrate content in tomato (Van Oosten et al., 2017a; Rouphael et al., 2018), basil (Carillo et al., 2019a; Carillo et al., 2019d), and in *Arabidopsis thaliana* (unpublished results). This led to the hypothesis that OP may affect the uptake and assimilation of nitrogen thus enhancing growth. Our results indicate that OP does indeed enhance NUE through nitrogen assimilation and this small molecule also perturbs several primary and secondary metabolic pathways. Here, we have characterized the primary phenotype under N stress with OP and many of the metabolites that are altered upon treatment. Furthermore, we have evidence that OP directly interacts with NR, enhancing assimilation through an increased affinity for the substrate and constitutive activation of the enzyme.

Nitrogen Stress and Omeprazole Treatment

While we did not observe a significant growth enhancement in maize by OP at 1 μM, we did observe that it alleviates much of the growth penalty imposed by severely limiting available nitrogen in

the nutrient solution and is reflected in the fresh and dry weights of maize leaves and roots (**Figure 1**). Generally, OP treatment almost completely restored growth in N-limiting conditions (**Figures 1A–D**).

In plants grown under low N, the free amino acids decreased both in shoots and roots, while proteins decreased in shoots and increased in roots compared to controls. OP appeared to further decrease the content of proteins in the leaves while stimulating the synthesis of free amino acids, in particular glutamine and asparagine and their transport to the roots. The two amides are used by the plant as a long-distance nitrogen transport molecule, given their high N/C ratio. When formed, they can be loaded into the phloem and sent to the roots to be converted to glutamate and then used as amine donor for the synthesis of all other protein amino acids.

In addition, both in shoots and roots alanine and GABA strongly increased under low N × OP treatment. This could be an important mechanism of biochemical pH-stat for the viability of maize plant under N limitation. In fact, the synthesis of alanine trough the decarboxylation of malate to pyruvate operated by malic enzyme and the decarboxylation of glutamate to GABA by mean of glutamate decarboxylase are proton-consuming reactions buffering cytosolic acidosis (Limami et al., 2008; Carillo, 2018). Moreover, alanine transaminated to pyruvate can be converted to acetyl-Coenzyme A in the mitochondrial matrix, while GABA shunt can provide NADH and/or succinate to tricarboxylic acid (TCA) cycle exerting an anaplerotic function (Bouché and Fromm, 2004) that sustains plants under N stress (Hare and Cress, 1997; Carillo, 2018). Therefore, OP induced GABA and alanine synthesis under low N could be crucial to ensure plant viability under this abiotic stress, supplying carbon skeletons and ATP for enhancing the synthesis of amino acids and proteins especially in the roots (Carillo et al., 2019b). This may increase root growth and improve the absorption and assimilation of nutrients from the soil. Accordingly, OP treatment significantly decreased nitrate content in the roots compared to the controls. This result is consistent with the reported role of GABA and alanine in stress mitigation against abiotic stresses (Michaeli and Fromm, 2015; Diab and Limami, 2016; Carillo, 2018). However, it is not excluded that OP influence on GABA and alanine concentrations may just be a downstream consequence of the increased N uptake or mobilization. In fact, as previously seen for amides, it seems that OP favors mechanisms of mobilization of the N resources already present in the shoot tissues to synthesize new amino acids to transport to root. This is probably obtained by diverting the available energy from protein or polyphenols synthesis to the synthesis of new amino acids in shoots. Moreover, it is likely that exists a trade-off between polyphenols and ROS, which represent energy, and carbon plus nitrogen metabolism as indicated by PCA. OP seems to favor carbon and nitrogen metabolism. This point deserves further investigation.

Omeprazole's Mechanism of Action in Nitrogen Use Efficiency

We evaluated the ability of OP to act as an ATPase inhibitor in maize. The inhibitory effect was only observed at concentrations 100- 1,000-fold higher than 1 μM dose that alleviated N starvation. Previous experiments in tomato have shown that concentrations in excess of 30 μM inhibit growth (Van Oosten et al., 2017a) and we have observed similar results in Arabidopsis (unpublished results). In animals, omeprazole and other benzimidazole PPIs interact with three cysteines in the beta loop of gastric Type IIC ATPases. Plants lack this class of ATPases (Axelsen and Palmgren, 1998; Hasegawa, 2013) and amino acid sequence analysis of mammalian Type IIC ATPases, in the region where OP binds, does not demonstrate any significant similarity with any regions in plant ATPases (unpublished results). While our results indicate that the concentration used to elicit enhanced salt tolerance (Van Oosten et al., 2017; Carillo et al., 2019d; Cirillo et al., 2019) and enhanced NUE does not affect total ATPase activity, it is clear that OP has an inhibitory effect at high concentrations. The phenotypes observed in this study and others at low OP concentrations are likely not due to OP having a direct effect on ATPase activity. The inhibitory effect of OP on ATPase activity in plants merits further study, since plants lack the Type IIC ATPases or similar proteins.

In maize, low doses appear to increase NUE. The enhanced ability to utilize limiting N conferred by OP treatment could be due to a combination of two major factors: uptake and reductive assimilation, in particular NO_3^- reduction. We assayed root uptake in young root segments (cut roots) and observed no increased uptake on OP treatment (**Supplementary Figure 1**), even if the expression of key genes involved in nitrate uptake and plasma membrane ATPase, responsible for generating the proton motive force necessary for nitrate uptake, were upregulated (**Figure 6**). However, while OP treatment did not increase the rate of uptake, it did affect key steps in nitrate assimilation. OP treatment did increase assimilation through the reduction of nitrate to nitrite through a direct effect on NR. NR enzyme from OP treated plants was found to be in the constitutively active state, regardless of N status in the environment (**Figure 4**, **Table 2**). When OP was used in an *in vitro* enzyme assay using purified NR from *Arabidopsis thaliana,* higher concentrations of OP were able to increase the catalytic efficiency and the specificity for the substrate nitrate of the enzyme resulting in an increased V_{max} and decreased K_m. This suggests that OP helps in maintaining adequate affinity of the enzyme toward its substrate as well as its catalytic rate (**Figure 5**). These two results indicate that OP likely directly and physically interacts with NR. This physical interaction that increases the affinity of the enzyme for its substrate may also interfere with inhibition of NR *in vivo*. NR undergoes a partial kinase-dependent inhibition, due to a phosphorylation, followed by an interaction with Mg ions and recruitment of 14:3:3 proteins, which decreases the enzyme activity and makes it more susceptible to proteolytic degradation (Kaiser et al., 1999). Nitrate in the cytosol is also able to protect the enzyme against proteolytic degradation (Campbell, 1999).

Moreover, even if OP did not directly increase nitrate uptake in young root segments (cut roots), it could be responsible for a direct stimulation of root biomass, that is root surface and length by OP-induced endogenous phytohormones like auxin as suggested by Rouphael et al. (2018) or by OP-induced GABA

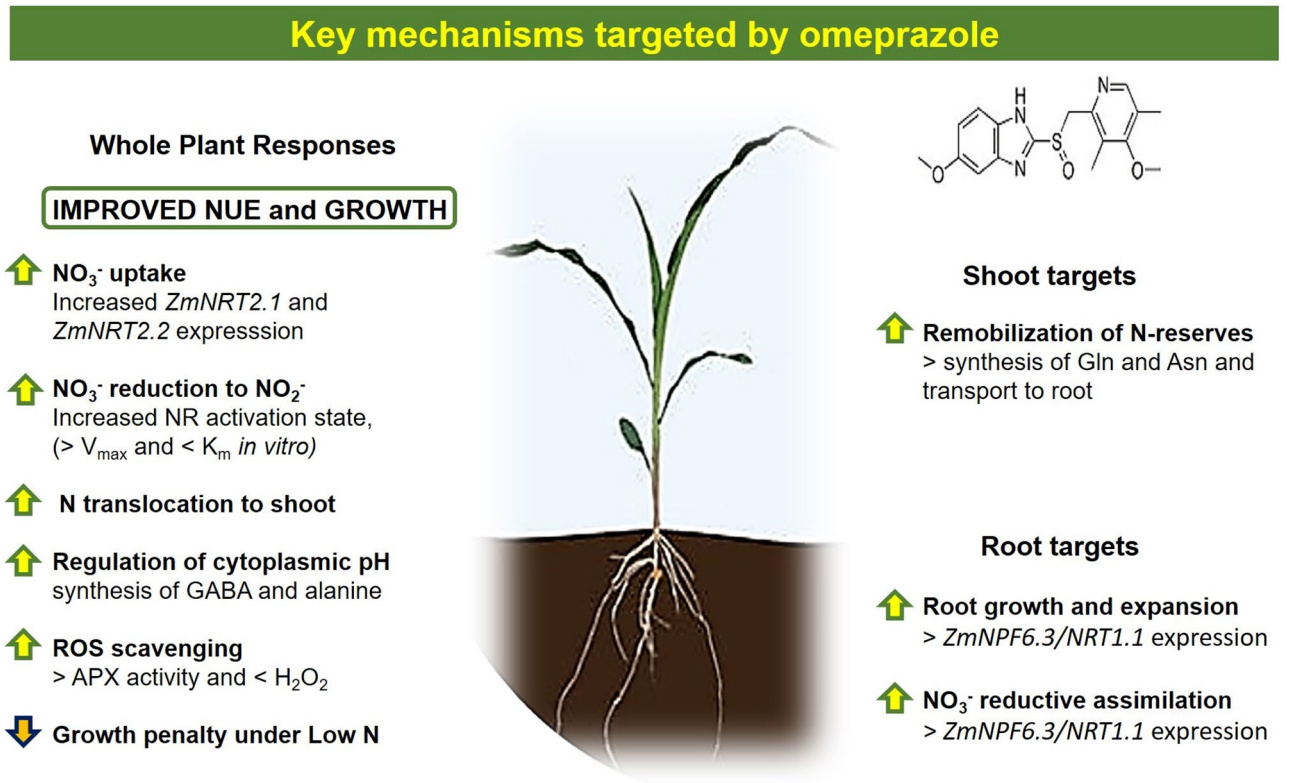


FIGURE 9 | Summary of main key mechanisms targeted by omeprazole in corn.

(Van Oosten et al., 2017a). In support of this hypothesis, a strong increase of expression of *ZmNPF6.3/NRT1.1* in roots of OP treated plants was found. The phosphorylated form of NPF6.3 can be responsible for NPF6.3 dependent regulation of both nitrate and auxin transport and therefore for lateral root growth and expansion (Bouguyon et al., 2015). The increase of root dimension and biomass and therefore of available root surface could improve/maximize the efficiency of root, in terms of soil exploration and nutrient mobilization and acquisition, avoiding the loss of nitrate, which is highly soluble in water and prone to leaching, from the root zone (**Figure 9**). Indeed, a measurement of the hormonal balance within the plant upon OP application would be of great interest and could help to better understand the results obtained. Preliminary evidence suggests that OP interacts with plant hormones involved in both growth and stress responses and therefore indirectly with NUE (unpublished results).

Implications of Omeprazole as a Regulator of NUE

Omeprazole treatment in maize plants alleviates the growth limitations imposed by low nitrogen in the environment (**Figures 1** and **2**). The changes in primary and secondary metabolism (**Figures 7** and **8**) are significant and the potential for enhancing NUE in field conditions needs to be determined. While benzimidazoles have been used in agriculture for decades as fungicides (Lucas et al., 2015), it is uncertain if public opinion and the current regulatory climate would accept a novel plant growth regulator like omeprazole and/or its derivatives. However, with the advances in gene editing, the targets of OP (**Figure 9**) make excellent candidates for gene editing with the aim of enhancing NUE in crop species. Our results show that it is possible to perturb the physiological process in the plant in such a way that uptake and assimilation can be enhanced through mechanisms present in the plant. Understanding how to regulate these processes is essential to enhancing NUE and subsequently developing sustainable crops with lower environmental impacts.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MO, ED'A, and PC contributed equally to this work. MO and PC contributed to the experimental design and wrote the first

draft of article. MO, ED'A, AR, VC, PW, and PC performed the experiments. MO and PC analyzed the data. MO, YG, AM, and PC supervised and completed the writing. All authors have read and approved the article.

FUNDING

Financial support by the Access to Research Infrastructures activity in the Horizon2020 Programme of the EU (EPPN2020 Grant Agreement 731013) is gratefully acknowledged. Authors also acknowledge the University of Campania "Luigi Vanvitelli" Programma VALERE: Vanvitelli per la Ricerca.

ACKNOWLEDGMENTS

The authors would like to thank Stefania Grillo and Giorgia Batelli of the National Research Council of Italy's Institute of Biosciences and Bioresources for the access to plant growth facilities and laboratories, and Cédric Cassan of the UMR1332 Biologie du Fruit et Pathologie INRA Bordeaux for his technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01507/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 February 2020
doi: 10.3389/fpls.2020.00072

Effects of Seed-Applied Biofertilizers on Rhizosphere Biodiversity and Growth of Common Wheat (*Triticum aestivum* L.) in the Field

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OPEN ACCESS

Edited by:
Carlos Alberto Silva, Universidade Federal de Lavras, Brazil

Reviewed by:
Orlando Carlos Huertas Tavares, Universidade Federal Rural do Rio de Janeiro, Brazil
Carlos Vergara, Universidade Federal Rural do Rio de Janeiro, Brazil

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *20 November 2019*
Accepted: *17 January 2020*
Published: *26 February 2020*

Citation:
Dal Cortivo C, Ferrari M, Visioli G, Lauro M, Fornasier F, Barion G, Panozzo A and Vamerali T (2020) Effects of Seed-Applied Biofertilizers on Rhizosphere Biodiversity and Growth of Common Wheat (Triticum aestivum L.) in the Field. Front. Plant Sci. 11:72. doi: 10.3389/fpls.2020.00072

In order to reduce chemical fertilization and improve the sustainability of common wheat (*Triticum aestivum* L.) cultivation, maintaining at the same time high production and quality standards, this study investigated the effects of three commercial biofertilizers on rhizosphere bacterial biomass, biodiversity and enzymatic activity, and on plant growth and grain yield in a field trial. The wheat seeds were inoculated with the following aiding microrganisms: (i) a bacterial consortium (*Azospirillum* spp. + *Azoarcus* spp. + *Azorhizobium* spp.); and two mycorrhizal fungal-bacterial consortia, *viz*. (ii) *Rhizophagus irregularis* + *Azotobacter vinelandii*, and (iii) *R. irregularis* + *Bacillus megaterium* + *Frateuria aurantia*, and comparisons were made with noninoculated controls. We demonstrate that all the biofertilizers significantly enhanced plant growth and nitrogen accumulation during stem elongation and heading, but this was translated into only small grain yield gains (+1%–4% vs controls). The total gluten content of the flour was not affected, but in general biofertilization significantly upregulated two high-quality protein subunits, i.e., the 81 kDa high-molecular-weight glutenin subunit and the 43.6 kDa low-molecular-weight glutenin subunit. These effects were associated with increases in the rhizosphere microbial biomass and the activity of enzymes such as β-glucosidase, α-mannosidase, β-mannosidase, and xylosidase, which are involved in organic matter decomposition, particularly when *Rhizophagus irregularis* was included as inoculant. No changes in microbial biodiversity were observed. Our results suggest that seed-applied biofertilizers may be effectively exploited in sustainable wheat cultivation without altering the biodiversity of the resident microbiome, but attention should be paid to the composition of the microbial consortia in order to maximize their benefits in crop cultivation.

Keywords: plant growth-promoting rhizobacteria, vesicular arbuscular mycorrhizae, soil bacterial biodiversity, 16S rDNA sequencing, gluten composition, sustainable agriculture

INTRODUCTION

The use of microbial inoculants is of strategic interest for their potential to replace chemical fertilizers and pesticides in agricultural systems, and improve environmental sustainability.

Plant-aiding microorganisms, often referred to as plant growth-promoting rhizobacteria (PGPR) (Gupta et al., 2015) and arbuscular mycorrhizal fungi (AMF) (Igiehon and Babalola, 2017), interact with plants roots (Hayat et al., 2010) by enhancing growth, mineral nutrition, drought tolerance, and disease resistance (Nadeem et al., 2013).

Bacteria can beneficially contribute to plant growth *via* N_2-fixation and solubilization of low mobile nutrients. Biological N_2-fixation is carried out by various symbiotic and nonsymbiotic bacteria (Shridhar, 2012).

Symbiotic PGPR fix atmospheric N_2 mainly within plant roots, with many genera involved, such as *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium*. The latter can enter plant roots intercellularly and colonize the xylem of wheat, rice, corn, and other nonlegume crops without forming real symbiotic structures (Cocking, 2003), although *Azorhizobium caulinodans* is known to form both stem and root nodules in *Sesbania rostrata* (Robertson et al., 1995).

Nonsymbiotic N_2 fixation is carried out by free-living diazotrophic bacteria, such as *Azospirillum*, *Azoarcus*, *Azotobacter, Burkholderia, Gluconacetobacter, Clostridium,* and *Pseudomonas* (Bhattacharyya and Jha, 2012; Singh, 2018). The absence of symbiosis with plants supports their common use in biofertilizers formulation. These bacteria can improve the uptake efficiency of nitrogen in many crops, thanks to the nitrogenase activity and soil N mineralization (Chauhan et al., 2015). In addition, *Azotobacter* and *Azospirillum* stimulate root hair formation, and lateral and adventitious root initiation through hormonal (auxins) exchange (Vejan et al., 2016; Zeffa et al., 2019).

Some PGPR are also known as phosphate- and potassium-solubilizing bacteria through rhizosphere acidification (Afzal and Bano, 2008; Meena et al., 2014). Among these, *Bacillus megaterium* and *Frateuria aurantia* were reported as efficient P- and K-mobilizing bacteria, respectively, thus being potentially exploitable in crop cultivation (Subhashini, 2014; Ghaffari et al., 2018).

AMF are nonpathogenic fungi living in symbioses with roots of a large number of spontaneous and cultivated plants, supplying them with mineral nutrients and water, particularly in natural environments (Solaiman and Mickan, 2014). Fostering AMF-plant symbiosis through inoculation, can significantly improve nutrients accumulation, the plant physiological processes and biomass accumulation (Mitra et al., 2019), besides root growth promotion and abiotic stresses mitigation (Begum et al., 2019).

However, many biotic and abiotic factors may affect the ability of plant-aiding microorganisms to successfully colonize the rhizosphere (Ahmad et al., 2011), and hence impact on these effects (Kloepper and Beauchamp, 1992; Castro-Sowinski et al., 2007).

In a context of environmental protection and increased demand for chemical-free food products, increasing numbers of commercial biofertilizers have come onto the market in recent years, containing either single or associated PGPR strains or mycorrhizal fungi (Owen et al., 2015).

There is considerable evidence for the positive effects of PGPR on plant growth under controlled conditions, and their often ineffectiveness in the open field (Dobbelaere et al., 2003; Dal Cortivo et al., 2017), possibly due to the use of strains unable to adequately colonize plant roots and/or to compete with the resident rhizobiome (Lugtenberg and Dekkers, 1999; Bloemberg and Lugtenberg, 2001; Kamilova et al., 2005). Aiding microorganisms need to be introduced into agroecosystems in sufficient quantities to efficiently colonize plant roots, as this step is crucial for their success (Rao, 1993; Bishnoi, 2015). Following biofertilization, it can be expected positive or negative competition with the indigenous bacteria population, or no interaction, depending on survival strategies (Schwieger and Tebbe, 2000; Bacilio-Jimenez et al., 2001; Brimecombe et al., 2007). In this regard, essential bacterial behaviors include tolerance to nutrient- or water-limited conditions, affinity for root exudates, and competition with the resident rhizobacteria through the secretion of antibiotics (Paul and Nair, 2008; Nadeem et al., 2013; Kaur et al., 2017). Little information is as yet available on the effects of PGPR-AMF consortia introduced into the resident bacterial community structure. Effective use of biofertilizers therefore rests on a better understanding of their effects on soil microbial communities, and hence of the role they play in soil biodiversity and plant health (Roesti et al., 2006; Hart et al., 2017).

Previous studies have shown the positive effects of PGPR on plant productivity, particularly under stress conditions, suggesting their potential role in a climate change scenario where extreme events, such as floods and droughts, occur with greater frequency in cultivated land (Egamberdieva and Adesemoye, 2016). However, little is so far known of their effects on the quality of cereal grains.

Our previous investigations in open fields documented the ability of a N-fixing bacteria consortium (i.e., *Azospirillum* + *Azoarcus* + *Azorhizobium*), when applied as foliar spraying inoculum during tillering, to improve root growth and N accumulation in common wheat (Dal Cortivo et al., 2017). Similarly, a AMF-bacteria consortium of *Rhizophagus irregularis* + *Azotobacter vinelandii* was found to enhance root growth and mineral uptake in this crop (Dal Cortivo et al., 2018).

In this framework, the current multidisciplinary study investigated a spectrum of effects of different commercial biofertilizers (consisting of PGPRs alone or in association with AMFs) on rhizosphere enzymatic processes, microbial biomass, and biodiversity (using high-throughput next-generation sequencing—NGS), and on growth, grain yield and quality (gluten content and composition, not previously investigated) in common wheat. The aim was to gather information on the potential advantages of their use as seed inoculums instead of postemergence foliar spraying on a widely-cultivated crop and on the mechanisms involved, and to assess safety issues with respect to their interaction with the resident rhizobiome.

MATERIALS AND METHODS

Biofertilizers

The following three biofertilizers were applied to fungicide-free seeds of common wheat (*Triticum aestivum* L.) immediately before sowing: (i) TN: TripleN® (Mapleton Agri Biotec, Mapleton, Australia), at 0.02 g kg^{-1} of seeds, containing three PGPRs *Azorhizobium* spp., *Azoarcus* spp., and *Azospirillum* spp. (1×10^{10} CFU g^{-1}); (ii) R-N: Rhizosum N® (Biosum Technology, Madrid, Spain), at 0.25 g kg^{-1} of seeds, containing the AMF *Rhizophagus irregularis* (previously known as *Glomus intraradices*) (2% w/w) and *Azotobacter vinelandii* (1×10^{10} CFU g^{-1}); and (iii) R-PK: Rhizosum PK® (Biosum Technology, Madrid, Spain), at 0.375 g kg^{-1} of seeds, containing the AMF *Rhizophagus irregularis* (2% w/w) together with *Bacillus megaterium* (0.66×10^{10} CFU g^{-1}) and *Frateuria aurantia* (0.33×10^{10} CFU g^{-1}). The inoculum doses followed the manufacturers' recommendations. The freeze-dried inoculum was mixed with ultrapure water (10 ml kg^{-1} of seeds) in order to enhance its adherence to the seed surface, and 5 ml kg^{-1} of seeds of Delfan Plus (Tradecorp, Madrid, Spain), which contains amino acids for early bacterial activation.

Experimental Design

The experiment was carried out in open field at the experimental farm of the University of Padua (Legnaro, Padua, NE Italy). The site has a deep, silty-loam soil (fulvi-calcaric-cambisol; USDA classification), pH 8.0, 1.7% organic matter, a CEC of 11.4 cmol (+) kg^{-1}, and a total N content of 1.1 g kg^{-1} (arable layer, beginning of the experiment). As regards other soil nutrients, available P and K were moderately high: 15.48 and 97 mg kg^{-1}, respectively; Mg was 247 mg kg^{-1} and Ca 2,619 mg kg^{-1}.

The three biofertilizers were compared with untreated controls (CO) in a completely randomized block experimental design (n = 3). Each plot/replicate measured 30 m^2 (10 × 3 m), and contained 24 rows of plants 12 cm apart. The previous crop was sugar beet. Soil tillage included 30-cm deep plowing followed by two harrowings. Presowing fertilization consisted in incorporating 32 kg ha^{-1} of N, 96 of P_2O_5 and 96 of K_2O into the soil through harrowing. The total amount of N, supplied throughout the crop cycle was 160 kg ha^{-1} as ammonium nitrate. The wheat var. Bologna (SIS, Bologna, Italy) was sown on November 3, 2016, and harvested on June 22, 2017. The crop was protected against weeds, insects and fungal pathogens by specific treatments, following local recommendations. To preserve mycorrhizal fungi's survival, plants were protected from fungal pathogens at the heading stage using active ingredients (i.e., Cyproconazole, Azoxystrobin and Prochloraz) recognized as nonharmful to AMF (Plant Health Care Incorporation, 2009).

Plant Analysis

Leaf chlorophyll content was monitored twice during the growing cycle, at stem elongation (GS35) (Zadoks et al., 1974) and complete ear emergence (GS59), with a SPAD 502 chlorophyll meter (Konica-Minolta, Hong Kong) on the last fully developed leaf of the main culm (10 plants per plot/replicate). The monitored plants were then harvested to determine fresh and dry shoot biomass (after oven-drying for 36 h at 105°C). Shoot N content was also determined according to the Kjeldahl method, and Ca, K, P, Fe, Mg, and Zn concentrations by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH, Kleve, Germany) on 0.4-g microwave acid-digested (7 ml HNO3 65% v/v and 1 ml H_2O_2 30% v/v) samples (Mileston ETHOS 900, Bergamo, Italy) according to the EPA method 3052 (USEPA, 1995). Measurement accuracy was ensured with certified reference materials (ERM-CD281 and BRC-402; JRC-IRMM, Geel, Belgium).

Root colonization across a 1-m-deep soil profile was assessed at full flowering (GS65) on May 3, 2017 using the coring method (n = 3; one core per plot). Soil cores 70 mm in diameter were collected from a central row of the plot at least 1 m from the border. Each core was split into 0.1 m subsamples, which were frozen at −18°C until washing. After separation by a hydraulic sieving-centrifugation device on a 500-µm mesh sieve, the roots were stored in a 15% v/v ethanol solution at 4°C until digitalization. Root images were processed with the KS 300 Rel. 3.0 software (Karl Zeiss, Munich, Germany), using a minimum area of 40 pixels as the threshold for background noise. Root length was determined by the FbL (FiberLength) algorithm, and the mean root diameter as the area-to-length ratio of root objects in each sample (Vamerali et al., 2003). Root length density (RLD) was expressed as cm of root per cm^3 of soil, and root surface density (RSD) as cm^2 of root per cm^3 of soil.

Grain Yield and Gluten Quality

Wheat grain yield was measured at maturity in the central area of each plot (n = 3) by collecting the grains with a plot combine harvester. The harvest index (grain-to-total shoot weight ratio) was determined in a checking area of 1 m^2 in each plot, together with the 1,000-kernel weight.

Gluten proteins were analyzed in 30-g seed samples (n = 3) gently milled by six pulses of 10 s each with a Knifetec 1095 (Foss, Hillerod, Denmark). Gliadins, high-molecular-weight glutenins (HMW-GS), and low-molecular-weight glutenin subunits (LWM-GS) were sequentially extracted from 30-mg subsamples, according to Visioli et al. (2018a) protocol. Relative quantification of HMW-GS, LMW-GS, and gliadins was performed spectrophotometrically by colorimetric Bradford assay (Bio-Rad, Hercules, CA, USA) at the 595 nm wavelength, with three technical replicates for each sample. Linear regression between absorbance and protein concentration was obtained through calibration with BSA (Bovine Serum Albumin) standards, and the results expressed as mg g^{-1} of wheat flour. Proteins belonging to the three gluten fractions were also separated by SDS-PAGE and subjected to densitometric analyses to evaluate possible variations in the amounts of single protein subunits, and the results expressed as mg/g^{-1} of flour (Visioli et al., 2018a).

Rhizosphere Microbial Biomass and Enzymatic Activity

Microbial biomass, enzymatic activity, and bacterial biodiversity were evaluated in the wheat rhizosphere (Rh) and in bulk soil

(BS) collected on December 16, 2016. Soil monoliths 0.2 m deep containing the root system of ∼30 plants for each biological replicate were randomly taken from the central area of each plot. Plants were gently extracted from the ground, and after removing most of the soil by shaking, the remaining rhizosphere soil adhering to the roots was carefully collected with a small sterile brush. The rhizosphere soil samples from the 30 plants of each replicate were pooled to obtain one sample of >2 g, which was placed in a sterile Falcon tube and immediately stored at −80°C until analysis. The reference bulk soil sample, without wheat roots, was collected from a 0.2-m-deep profile in uncultivated zones between the plots.

Soil microbial biomass was determined as double-strand DNA (dsDNA) content (Bragato et al., 2016) in 300-mg DW soil samples through DNA extraction with a 0.12 M, pH 8 Na_2HPO_4 buffer using bead beating and quantification with PicoGreen reagent, as described by Fornasier et al. (2014). Biomass was expressed as μg dsDNA g^{-1} soil D. W.

Nineteen types of enzymatic activity representing the various nutrient cycles were then determined in treated wheat and compared with untreated controls and bulk soil (n = 3). The enzymes examined were: arylsulfatase (aryS), alpha-glucosidase (alfaG), beta-glucosidase (betaG), alpha-galactosidase (alfa GAL), beta-galactosidase (beta GAL), alpha-mannosidase (alfa_MAN), beta-mannosidase (beta_MAN), glucuronidase (uroni), cellobiohydrolase (cell), xilosidase (xilo), chitinase (chit), leucine-aminopeptidase (leu), tripsin- and papain-like protease (trip), acid phosphomonoesterase (acP), phosphodiesterase (bisP), pirophosphate-phosphodiesterase (piroP), alkaline phosphomonoesterase (alkP), inositol-phosphatase (phytase) (inosit), and nonanoate-esterase (nona). Their activity was measured by a heteromolecular exchange procedure (Cowie et al., 2013) using a solution of lysozyme (3%) and bead beating, as described in Bardelli et al. (2017). All activities were determined in 300-mg DW soil samples and expressed as nmol of MUF (4-methyl-umbelliferyl) min^{-1} g^{-1} soil DW.

Rhizosphere Microbial Biodiversity Analysis by 16S rRNA Gene Sequencing

DNA was extracted from 0.5-g samples of Rh and BS with the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocols, visualized by electrophoresis on 0.8% (w/v) agarose gels to test for DNA integrity, and quantified by Nanodrop ND1000. 16S rDNA amplification, sequencing and data analysis were performed at GenProbio srl's DNA sequencing facility (www.genprobio.com), according to the protocol described by Visioli et al. (2018b).

Differences between samples in the relative abundances of the taxonomic units were ascertained by a one-way Analysis of Variance (ANOVA). Bacterial taxa with *P*-values <0.05 were selected and identified as the phylotypes or bacterial families that were significantly influenced by the biofertilizers being tested. Pyrosequencing reads were sent to GenBank to obtain their under accession numbers. They are available as bioproject PRJNA388660.

Statistical Analysis

An ANOVA was carried out on the dataset for all the parameters examined using the Statgraphics Centurion XI software (Adalta, Arezzo, Italy). Separation of means was set at $P \leq 0.05$ with the Newman-Keuls test.

To facilitate interpretation of the whole dataset, a factorial discriminant analysis (MDA, Multigroup Discriminant Analysis with Wilks' lambda and Pillai's trace tests) and a principal component analysis (PCA) were carried out to describe the plant- and microbiologically-related variables. Multivariate data normality was first verified by the Shapiro test. Before analysis, the data were standardized by subtracting the mean and dividing by the standard deviation within each variable. All analyses were performed with MS Excel XLSTAT (Addinsoft, Paris, France).

RESULTS

Climatic Conditions During the Trial

The climatic parameters recorded by the local meteorological station (ARPAV, Teolo, Italy) during the field trial showed that the average monthly temperature was quite similar to the 10-year mean (2007–2017), but large differences were found for precipitation. Compared with the historical mean, temperatures were lower in December and January, while rainfall was higher in November but markedly lower for the rest of the cycle, particularly during the winter (**Figure SI 1**). From December to June, overall precipitation was only 170 mm.

Rhizosphere Soil Microbial Biomass and Biodiversity, and Enzymatic Activity

As expected, the soil microbial biomass in cultivated soil was greater than in bulk soil. There was also greater microbial biomass in the rhizosphere of the inoculated plants than in that of the noninoculated controls, particularly with R-PK, although the difference was not statistically significant (**Table 1**).

The levels of activity of many of the rhizosphere enzymes investigated here were higher in inoculated plants than in bulk soil, but they were also seldom higher than in the noninoculated controls. The soil microbial enzymatic activity response allowed us to clearly separate treatments by principal coordinate analysis (PCoA) (**Figure 1**), which showed that the R-N and R-PK treatments were more distant from the untreated controls (CO). The two dimensions/variables of db-RDA (distance-based redundancy analysis) explained an overall variability of 81%, mostly attributed to the first (db-RDA1 = 59%, $P = 0.004$) than to the second variable (db-RDA2 = 21%, $P = 0.292$).

Comparison among the rhizospheres of the controls and treated plants showed that the enzymatic activity of beta-glucosidase, α-mannosidase, β;-mannosidase, and xylosidase was significantly higher with R-N and R-PK, i.e., the treatments including plant-aiding fungi, than in the biofertilizer containing only PGP rhizobacteria. The activity of the alkaline phosphomonoesterase was also significantly higher

TABLE 1 | Total microbial biomass (ug dsDNA g^{-1} dry soil; n = 3 ± S.E.) and enzymatic activity (nmol of MUF min^{-1} g^{-1} dry soil; n = 3 ± S.E.) in bulk soil (BS), and in the rhizosphere of *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs untreated controls (CO).

Enzymatic activity	Treatment									
	BS		CO		TN		R-N		R-PK	
AryS	10.3 ± 2.5	a	12.1 ± 2.2	a	15.2 ± 1.6	a	12.9 ± 0.9	a	12.2 ± 1.2	a
alphaG	1.11 ± 0.2	b	2.11 ± 0.5	ab	3.54 ± 0.5	ab	2.64 ± 1.24	ab	7.35 ± 2.90	a
betaG	4.38 ± 0.8	c	9.11 ± 0.4	b	10.03 ± 0.5	b	11.42 ± 0.6	ab	13.91 ± 1.1	a
alpha GAL	0.46 ± 0.1	b	1.42 ± 0.4	a	1.81 ± 0.2	a	1.77 ± 0.1	a	2.25 ± 0.01	a
beta GAL	1.3 ± 0.3	b	2.7 ± 0.4	a	3.0 ± 0.1	a	2.8 ± 0.1	a	2.8 ± 0.1	a
alpha MAN	0.09 ± 0.02	c	0.26 ± 002	b	0.34 ± 0.03	ab	0.39 ± 0.04	a	0.39 ± 0.03	a
beta MAN	0.13 ± 0.02	b	0.22 ± 0.01	b	0.34 ± 0.01	b	1.19 ± 0.02	a	0.95 ± 0.02	a
Uroni	1.6 ± 0.4	b	3.9 ± 0.2	a	3.8 ± 0.3	a	4.0 ± 0.4	a	4.2 ± 0.4	a
Cell	0.15 ± 0.04	b	0.52 ± 0.1	ab	0.47 ± 0.03	ab	0.77 ± 0.2	a	0.85 ± 0.04	a
Xilo	0.84 ± 0.2	d	1.46 ± 0.1	c	1.76 ± 0.1	bc	2.08 ± 0.2	ab	2.57 ± 0.05	a
Chit	2.3 ± 0.04	b	5.8 ± 0.1	a	5.2 ± 0.8	a	5.2 ± 1.3	a	3.6 ± 0.2	ab
Leu	35.2 ± 6.1	b	64.9 ± 5.9	a	70.1 ± 3.1	a	64.6 ± 3.9	a	70.9 ± 8.3	a
Trip	2.7 ± 0.4	a	4.5 ± 0.5	a	4.8 ± 0.4	a	3.8 ± 0.2	a	4.5 ± 0.7	a
acP	38.4 ± 8.1	a	48.8 ± 4.1	a	54.9 ± 1.5	a	53.1 ± 2.3	a	55.7 ± 2.9	a
bisP	39.8 ± 8.8	a	48.7 ± 7.7	a	54.8 ± 2.8	a	54.4 ± 4.6	a	52.6 ± 4.5	a
piroP	9.4 ± 2.1	a	11.9 ± 2.2	a	13.0 ± 0.9	a	14.9 ± 0.6	a	16.1 ± 2.1	a
alkP	264.4 ± 58	b	357.6 ± 46.5	b	438.5 ± 15.7	a	401.9 ± 27.8	a	375.5 ± 40.8	a
Inosit	0.68 ± 0.2	b	0.93 ± 0.1	ab	1.40 ± 0.1	a	1.15 ± 0.2	ab	1.40 ± 0.1	ab
Nona	68.5 ± 15.2	b	109.6 ± 14.7	ab	101.0 ± 11.4	ab	79.4 ± 13.5	b	131.3 ± 16.8	a
Microbial biomass	16.4 ± 2.2	b	20.5 ± 3.0	ab	21.6 ± 1.2	ab	22.9 ± 1.9	ab	27.4 ± 2.6	a

aryS, arylsulfatase; alfaG, alpha-glucosidase; betaG, beta-glucosidase; alfa GaL, alpha-galactosidase; beta GAL, beta-galactosidase; alpha MAN, alpha-mannosidase; beta MAN, beta-mannosidase; uroni, glucuronidase; cell, cellobiohydrolase; xilom, xylosisase; chit, chitinase; leu, leucine-aminopeptidase; trip, tripsin- and papain-like protease; acP, phosphomonoesterase; bisP, phosphodiesterase; piroP, pirophosphate phosphodiesterase; alkP, alkaline phosphomonoesterase; inosit, inositol phosphatase (Phytase); nona, nonanoate-esterase. Letters indicate statistically significant differences among treatments (Newman-Keuls test, P ≤ 0.05).

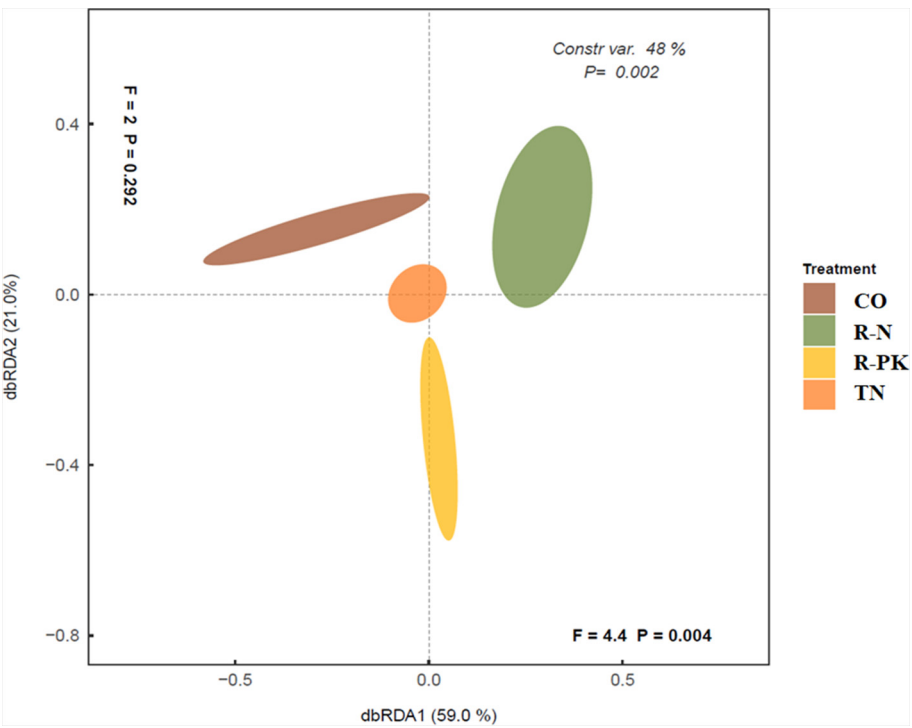


FIGURE 1 | Overall differences among rhizosphere hydrolytic enzyme activities determined by distance-based redundancy analysis (dbRDA) ordination among treatments (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) and noninoculated controls (CO). Percentages along the axes show the proportions of dissimilarity captured.

in treated plants, particularly with TN and R-N, and nonanoate-esterase activity was considerably enhanced with R-PK (**Table 1**).

Regarding bacterial biodiversity in the rhizosphere and the bulk soil, the total number of gene sequences (average of three replicates) ranged from 54,929 to 59,843. Bacterial diversity, measured as OTUs (Operational Taxonomic Units), and the calculated bacterial diversity indices, i.e., the Shannon diversity index and the Chao1 estimator of richness, revealed no significant differences among treatments. The rarefaction curves using the Chao and Shannon indices approached the plateau, indicating that further sequencing would not have resulted in additional OTUs (**Figure SI 2**). Differences in the microbial rhizosphere compositions were detected at both the phylum and family levels (**Figure 2**, **Table SI 1**). The major differences were between bulk soil and the rhizosphere, confirming the significant impact of soil root colonization on microbiota development. In particular, the *Bacteroidetes*, *Cyanobacteria*, *Proteobacteria,* and *Fibrobacteres* phyla were significantly more abundant in the rhizosphere, and *Actinobacteria*, *Acidobacteria*, *Gemmatimonadetes,* and *Chloroflexi* in the bulk soil (**Figure 3**). Some differences were also found between the rhizospheres of the controls and the inoculated plants: seed treatment with R-PK greatly stimulated the abundance of *Cyanobacteria* (+ 24% vs controls), TN the bacteria of the *Flavobacteriaceae* family, which reached 3.34% of the total community vs 1.99% of controls, and R-N the *Planctomycetaceae* family (**Table SI 1**). Compared with controls, R-PK reduced the abundance of the *Verrucomicrobiaceae* family, and R-N the *Gaiellaceae*, although these were present at <1%.

Wheat Growth and Grain Yield

Wheat plant growth was appreciably enhanced following seed inoculation with biofertilizers, as evidenced by the greater shoot biomass at both the stem elongation (GS35, fifth node detectable) and complete ear emergence (GS59) stages, although on the second observation the R-PK treatment was the only significant ($P \leq 0.05$) (**Figures 3A, B**). Leaf chlorophyll content of the youngest developed leaf, which decreased with plant aging from

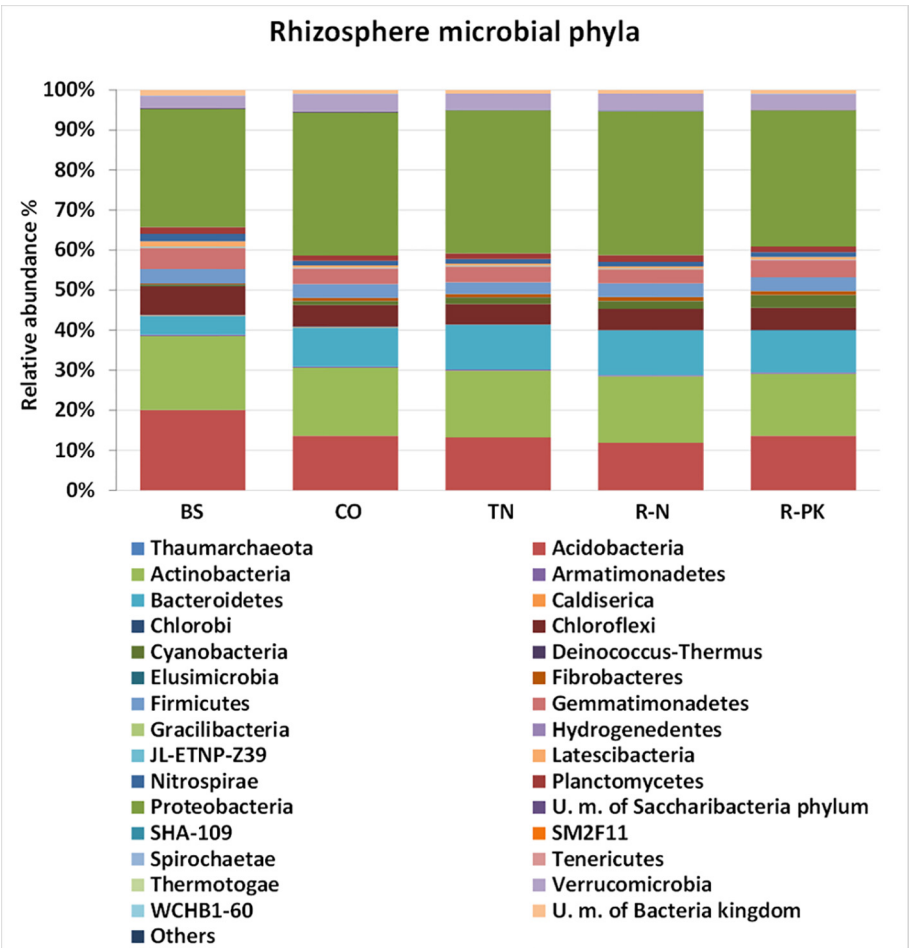


FIGURE 2 | Microbial community composition (%; n = 3 ± s.e.) at the phylum level based on 16S rDNA reads in bulk soil (BS) and the rhizosphere of *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) and noninoculated controls (CO) (Newman-Keuls test, $P \leq 0.05$). Only taxa >0.6% are shown.

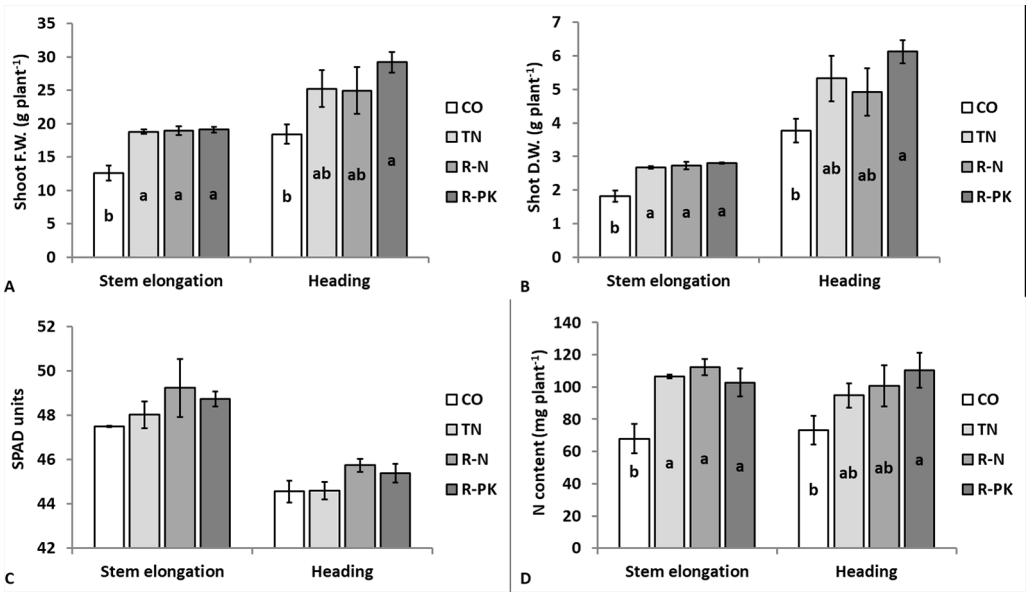


FIGURE 3 | Shoot fresh **(A)** and dry **(B)** weight, leaf chlorophyll content **(C)**, and shoot nitrogen content **(D)** (n = 3 ± s.e.) in *Triticum aestivum* plants inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs untreated controls (CO). Letters indicate statistically significant differences among treatments (Newman-Keuls test, $P \leq 0.05$).

stem elongation to heading, was not affected by seed inoculation, although it was slightly improved by R-N and R-PK (**Figure 3C**). However, inoculated plants accumulated a greater amount of nitrogen above ground as a result of both greater biomass and nitrogen concentration, with significant improvements for all treatments at stem elongation, and for R-PK at the heading stage (**Figure 3D**). At this stage, the shoot concentrations of other nutrients (i.e., K, P, Ca, Mg, Fe, and Zn) were generally lower with plant inoculation, but the overall content (uptake) of Ca, K and Zn improved significantly, particularly with R-PK, due to better plant growth (**Table 2**).

Wheat yield and its components, i.e., the harvest index (HI), and thousand kernel weight (TKW), were very stable across treatments, with small nonsignificant improvements in the inoculated wheat ($P > 0.05$), mainly with R-PK. The average values were: HI = 43%, TKW = 31 g, and yield = 756 g m^{-2}.

Destructive root investigations at complete flowering revealed similar root patterns among the inoculated plants and controls in terms of the average whole root profile. However, significant benefits in root length density (RLD) and root area density (RAD) were found in the arable layer with the TN treatment ($P > 0.05$). On the other hand, the two biofertilizers containing AMF (i.e., R-N and R-PK) led to slight decreases in root length, area and diameter (**Table 3**).

Effects of Biofertilizers on Gluten Content and Composition

Biofertilizers had no significant effects on flour gluten content, which was similar with noninoculated controls, although there was a general slight increase in gliadins and a reduction in glutenins, particularly the high-molecular-weight glutenin subunits (HMW-GS) (**Table 4**). Maximum variations were found with the R-PK treatment, which resulted in a 7% increase in gliadins, and an 8% reduction in glutenins. SDS-PAGE revealed that all the inocula led to a significant increase in the 81 kDa HMW-GS and the 43,6 kDa LMW-GS (**Figures 4A, B**), both playing a key role in technological quality. No significant changes in the composition of the gliadin subunits were observed (**Figure 4C**).

Principal Component Analysis

The PCA identified two synthetic components which explained an overall variability of 99.85%, mostly attributed to the first one (F1 = 95.16%; F2 = 4.69%) (**Figure 5**). Most of the relevant variables (loadings > |0.4|) i.e., gluten subunits, rhizosphere bacterial composition and shoot growth were assigned to F2, while gliadins and root parameters (both length and surface area density) were the most representative ones in F1.

The direction of the vector of each variable indicated generally good correlations among the variables plotted very closely together, i.e., SPAD, shoot biomass, and shoot nitrogen uptake, which were negatively correlated with the abundances of the bacterial phyla *Acidobacteria*, *Proteobacteria,* and *Actinobacteria*. The abundance of *Cyanobacteria* was correlated with LMW-GS and gliadin contents, while HMW-GS content was correlated with root growth and the abundances of *Proteobacteria* and *Actinobacteria*.

The centroid position and cluster separation in the discriminant analysis (**Figure 5**) summarize wheat response to the three biofertilizers, and show that TN and R-N treatments promoted root growth, while R-PK mainly affected gluten composition. In this way, the PCA highlighted the effects on root parameters as the most relevant impact of bacterial inoculation.

TABLE 2 | Shoot element (Ca, Calcium; Fe, Iron; K, Potassium; Mg, Magnesium; P, Phosphorus; Zn, Zinc) concentrations and contents (mean ± S.E.; n = 3) at the heading stage of *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs untreated controls (CO).

Treatment	Concentration (mg kg^{-1})						Content (mg plant^{-1})					
	Ca		Fe		K		Ca		Fe		K	
CO	3200 ± 313	a	262 ± 80.5	a	27768 ± 1395	a	12.1 ± 1.91	b	0.98 ± 0.25	a	105 ± 6.54	b
TN	2476 ± 37	b (-23)	197 ± 24.3	a (-25)	21083 ± 1151	b (-24)	13.2 ± 2.71	ab (+9)	1.04 ± 0.23	a (+6)	112 ± 27.84	ab (+7)
R-N	3004 ± 140	ab (-6)	189 ± 20.7	a (-28)	22211 ± 632	b (-20)	14.8 ± 1.62	ab (+22)	0.93 ± 0.24	a (-6)	109 ± 14.46	ab (+4)
R-PK	2684 ± 100	ab (-16)	210 ± 14.1	a (-20)	19410 ± 578	b (-30)	16.4 ± 0.32	a (+36)	1.28 ± 0.06	a (+30)	119 ± 3.25	a (+14)
	Mg		P		Zn		Mg		P		Zn	
CO	1894 ± 139	a	2764 ± 291	a	29.7 ± 2.04	a	7.14 ± 1.22	a	10.4 ± 0.88	a	0.11 ± 0.01	b
TN	1185 ± 19	b (-37)	2064 ± 122	b (-25)	20.7 ± 0.72	b (-30)	6.31 ± 1.29	a (-12)	11.0 ± 2.76	a (+5)	0.11 ± 0.03	ab (-1)
R-N	1414 ± 65	b (-25)	2171 ± 100	b (-21)	29.5 ± 4.63	ab (-1)	6.95 ± 0.76	a (-3)	10.7 ± 2.06	a (+2)	0.14 ± 0.03	ab (+29)
R-PK	1242 ± 25	b (-34)	1850 ± 170	b (-33)	22.9 ± 2.03	b (-23)	7.60 ± 0.32	a (+7)	11.3 ± 0.51	a (+9)	0.14 ± 0.01	a (+25)

Letters indicate statistically significant differences among treatments within same element (Newman-Keuls test, P ≤ 0.05). In brackets: % variation for each treatment compared with controls.

TABLE 3 | Root length density (RLD), root surface density (RSD), and diameter (D) (mean ± S.E.; n = 3) at the flowering stage as averages of different soil layers in *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs noninoculated controls (CO).

Root parameter	Treatment	Soil depth (m)								
		0-1			0-0.5			0.5-1		
RLD (cm cm^{-3})	CO	3.02	a		4.14	b		1.89	a	
	TN	3.16	a	(+5)	4.68	a	(+13)	1.63	a	(-14)
	R-N	2.72	a	(-10)	3.93	b	(-5)	1.50	a	(-21)
	R-PK	2.73	a	(-9)	3.71	b	(-10)	1.76	a	(-7)
RSD (cm^2 cm^{-3})	CO	42.0	a		54.9	ab		29.0	a	
	TN	46.2	a	(+10)	66.7	a	(+21)	25.7	a	(-12)
	R-N	37.3	a	(-11)	51.5	b	(-6)	23.1	a	(-21)
	R-PK	37.3	a	(-11)	48.3	b	(-12)	26.4	a	(-9)
D (μm)	CO	293	a		268	a		317	a	
	TN	303	a	(+4)	284	a	(+6)	322	a	(+1)
	R-N	287	a	(-2)	263	a	(-2)	311	a	(-2)
	R-PK	282	a	(-4)	261	a	(-2)	303	a	(-4)

Letters indicate significant differences among treatments within the same parameter and soil depth (Newman-Keuls test, P ≤ 0.05). In brackets: % variation in inoculated plants vs noninoculated controls.

TABLE 4 | Total gliadins, low-molecular-weight (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS), total glutenins (mg g^{-1}; n = 3; ± S.E.) and glutenin/gliadin and HMW/LMW ratios in grains of *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs noninoculated controls (CO).

Treatment	Total gliadins (mg g^{-1})			LMW-glutenins (mg g^{-1})			HMW-glutenins (mg g^{-1})			Total glutenins (mg g^{-1})			Glutenins/Gliadins			HMW/LMW		
CO	24.0 ± 0.4	a		4.1 ± 0.2	a		3.1 ± 0.4	a		7.2 ± 0.4	a		0.30 ± 0.02	a		0.75 ± 0.11	a	
TN	24.3 ± 1.3	a	(+1)	4.4 ± 0.1	a	(+7)	2.7 ± 0.2	a	(-13)	7.1 ± 0.2	a	(-3)	0.30 ± 0.02	a		0.62 ± 0.05	a	(-25)
R-N	23.1 ± 0.7	a	(-4)	4.2 ± 0.1	a	(+2)	2.6 ± 0.1	a	(-16)	6.8 ± 0.2	a	(-4)	0.29 ± 0.02	a	(-3)	0.62 ± 0.04	a	(-25)
R-PK	25.7 ± 0.8	a	(+7)	4.4 ± 0.2	a	(+7)	2.2 ± 0.2	a	(-29)	6.6 ± 0.3	a	(-8)	0.26 ± 0.02	a	(-13)	0.50 ± 0.11	a	(-38)

Letters indicate significant differences among treatments within the same parameter (Newman-Keuls test, P ≤ 0.05). In brackets: % variation in inoculated plants vs noninoculated controls.

DISCUSSION

Biofertilizers represent a sustainable tool for improving crop yield, as beneficial bacteria and fungi can exert several positive effects on plant nutrition and growth in many crops, including wheat (Basu et al., 2017; Igiehon and Babalola, 2017). Although this is generally clear under laboratory and controlled conditions, the application of plant-aiding microorganisms in open field may be constrained by poor agronomic response. Two of the biofertilizers studied here, i.e., TN composed of a bacteria consortium (*Azospirillum* spp. + *Azoarcus* spp. + *Azorhizobium* spp.), and R-N, a fungal-bacterial consortium (*Rhizophagus irregularis* + *Azotobacter vinelandii*), have already been successfully applied in open field by foliar spraying before stem elongation (Dal Cortivo et al., 2017; Dal Cortivo et al., 2018). In the present study, the biofertilizers were applied as seed inoculants, which is less costly than canopy spraying, with the aim to investigate their potential agronomic effects together with the environmental/ecological impact, which

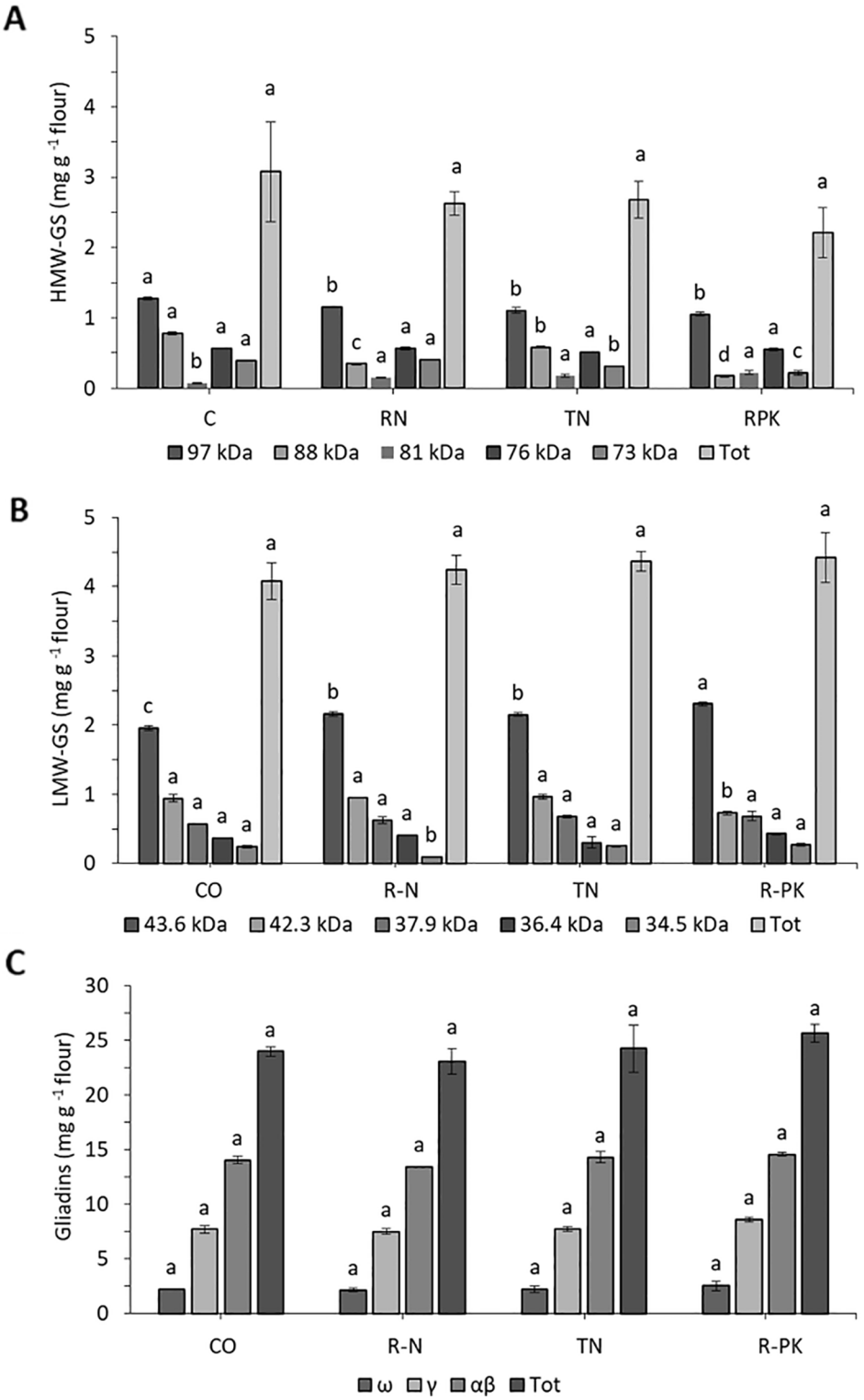
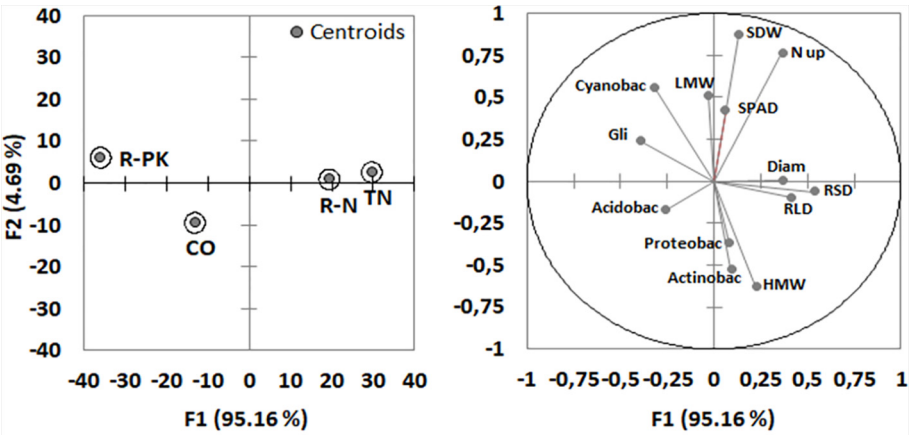


FIGURE 4 | High-molecular-weight glutenin (HMW-GS, **A**), low-molecular-weight glutenin subunits (LMW-GS, **B**), and gliadins **(C)** (mg g^{-1} D. W. flour; n = 3; mean ± s.e.) as represented by different kDa bands revealed with SDS-PAGE in *Triticum aestivum* plants inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs noninoculated controls (CO). Letters indicate significant differences among treatments within the same band (Newman-Keuls test, $P \leq 0.05$).



Abbreviation	Variable	F1	F2
HMW	HMW-Glutenins	0.225	**-0.626**
LMW	LMW-Glutenins	-0.031	**0.513**
Gli	Gliadins	**-0.399**	0.245
Acidobac	Acidobacteria	-0.255	-0.168
Actinobac	Actinobaceria	0.090	**-0.520**
Cyanobac	Cyanobacteria	-0.320	**0.560**
Proteobac	Proteobacteria	0.079	-0.365
SPAD	SPAD	0.063	**0.430**
SDW	Shoot dry weight	0.131	**0.876**
N up	Nitrogen uptake	0.362	**0.767**
RLD	Root length density	**0.417**	-0.096
Diam	Average root diameter	0.362	0.008
RAD	Root surface area density	**0.537**	-0.062

FIGURE 5 | Principal component analysis (PCA; top right) with variable loadings (values > |0.4| in bold; bottom) and discriminant analysis (DA; top left) for *Triticum aestivum* inoculated with biofertilizers (TN, TripleN; R-N, Rhizosum N; R-PK, Rhizosum PK) vs noninoculated controls (CO). Circles in the PCA include 75% of cases.

is related to the interaction with the resident microbial community, and the possible mechanisms of interaction with the wheat plants. Using this application method, we expect endophytic PGPR to colonize the root surface and intercellularly colonize the internal plant tissues of different plant organs, and AMF to colonize the plant roots starting with the first rootlet of the germinating seeds, thereby contributing to plant nutrition and growth (Lodewyckx et al., 2002; Smith and Read, 2008; Pagnani et al., 2020). Indeed, efficient colonization of root tissues by PGPR bacteria included in TN, as well as by *Rhizophagus irregularis* contained in R-N and R-PK, has been already documented by electron microscopy (ESEM) in our previous studies (Dal Cortivo et al., 2017; Dal Cortivo et al., 2018). This fits with the appreciable increases in shoot growth and accumulation of minerals, particularly nitrogen, detected across the growing season, although these did not translate into significant increases in wheat yield in our field experiment. The different plant responses may be attributed to the microbial composition of the biofertilizers, as TN mainly stimulated root growth, while R-N and R-PK enhanced the uptake of low-mobile nutrients, such as Ca, K, and Zn, mainly through improved plant growth.

The spectrum of effects of seed inoculation on wheat reported here are similar to previous literature dealing with various growing conditions (Turan et al., 2012; Piccinin et al., 2013; Nadeem et al., 2014). Growth enhancements may be attributed to the N-fixing and nutrient-solubilizing abilities of the applied microorganisms, and to the production of growth-promoting substances, such as IAA (indole-3-acetic acid). PGPR can also modify the level of phytohormones involved in plant senescence through the production of the enzyme ACC-deaminase, and toxins, like rhizobitoxine, which limits ethylene synthesis (Stamenković et al., 2018).

As a result of the associations among a mycorrhizal fungus and P- and K-solubilizing bacteria, such as *Bacillus megaterium* and *Frateuria aurantia* (Elkoca et al., 2010; Velázquez et al.,

2016; Ezawa and Saito, 2018), the R-PK treatment was found to engender the best plant growth and nutrient uptake responses, but seldom together with a reduction in nutrient concentrations in the plant tissues, although this is expected where soil fertility is high (Richardson et al., 2009). Mycorrhizal fungi are known to form a highly-developed hyphal network that absorbs nutrients, particularly phosphates, up to several centimeters from the root adsorption zone, often leading to a decrease in fine root length (Liu, 2009; Ezawa and Saito, 2018), as in this trial.

We found a clear positive effect on root growth of inoculation with TN, resulting in greater volumetric root length density in the arable layer. In fact, *Azospirillum* bacteria, included in the formulation of TN, are recognized as stimulating root length and area through the release of auxins, thereby increasing nitrogen and low-mobile nutrients, and water uptake (Bhattacharyya and Jha, 2012). Several studies have reported root growth enhancements, particularly at the early plant stages, but this may be not a stable response in mature plants and across years, as the PGPR-plant association in open field is strongly affected by adverse environmental conditions (e.g., excessive precipitation) after soil/plant inoculation (Basaglia et al., 2003; Dal Cortivo et al., 2017). However, this experiment confirms that soil conditions and fertility were probably favorable to the onset of the plant-PGPR signals that precede colonization in competition with the resident microbiome (Videira e Castro et al., 2016), allowing wheat to benefit in terms of root growth.

Wheat seed inoculation also had a clear positive impact on the rhizosphere microbiome, at least relatively soon after sowing (about 6 weeks). There was a general increase in total microbial biomass and some soil enzymatic activities, demonstrating enhanced microbial metabolism, mainly when the inoculum contained both plant-aiding bacteria and AMF. The increased enzymatic activities included beta-glucosidase, which hydrolyzes cellobiose to free glucose (Zang et al., 2018), alfa-mannosidase, beta-mannosidase, and xylosidase, which is produced by both endophytic bacteria and fungi, and which hydrolyzes mannans and xylans as the main components of lignified organic materials together with cellulose and lignin (Nankai et al., 2002; Robl et al., 2013). Alkaline nonophosphoesterase, a key enzyme for organic P degradation into inorganic phosphate available for plant uptake, particularly under P-limiting conditions (Acuña et al., 2016), was also generally upregulated by inoculation. Nonanoate-esterase activity was increased significantly by the R-PK treatment, although other contributing enzymes act on ester bonds, including esterases and proteases.

An important issue to be considered in plant/soil inoculation is the possible impact on the resident microbiome. In our study, there was a considerable higher bacterial biodiversity in the rhizosphere compared with bulk soil, confirming the essential role played by root presence in the soil microbiome. On the other hand, we found small differences between the rhizospheres of the treated plants vs the noninoculated controls in terms of bacterial biodiversity, suggesting that all the inocula applications studied here are safe. Nevertheless, inoculation modified the abundances of specific rhizosphere bacteria phyla. Interestingly, the R-PK inoculum highly stimulated the *Cyanobacteria*, which are known to form associations with wheat roots to alleviate nitrogen deficiency and enhance the rhizosphere microbial biomass (Karthikeyan et al., 2007). As *Cyanobacteria* are also able to produce plant growth-promoting substances, it has been suggested they be included as inoculants for rice and maize (Prasanna et al., 2016). Bacteria belonging to the *Flavobacteriaceae* family, which were stimulated by the TN treatment, may also be beneficial for wheat in this experiment, as they proved to have important ecological functions contributing to organic matter turnover and pesticide decomposition (Wolińska et al., 2017), which in part helps explain the increase in some soil enzymatic activities (**Table 1**).

The main agronomic result obtained in this study was the greater nitrogen uptake in inoculated plants, although this did not translate into significant gains in grain yield, and no effects on grain quality in terms of gluten content were observed, in contrast with the findings of some authors which tested the effects of PGPR inoculation in ancient *Triticum* varieties (Pagnani et al., 2020). Accumulation of gluten proteins is a complex process involving spatial and temporal regulation. Environmental conditions, such as heat and drought stress, as well as the dose and application timing of nitrogen fertilizers can affect significant changes in gluten composition (Flagella et al., 2010; Visioli et al., 2018a). However, very little information is available on possible changes in gluten protein composition in response to biofertilizer application. Stępień and Wojtkowiak (2013) showed that gluten content, particularly of HMW-GS and LMW-GS, increased in spring and winter wheat cultivars with organic fertilizers, regardless of the addition of effective microorganisms. A novel finding in this study is the significant upregulation of certain LMW-GS and HMW-GS, the latter being polymeric proteins involved in dough strength and elasticity (Sissons, 2008). In particular, the 81 kDa HMW-GS is the Bx subunit codified by the locus *Glu-B1* closely correlated with the technological quality of flour (Sissons, 2008), and the LMW-2 asset, in which the LMW-GS with the highest molecular weight (~44 kDa) is the most abundant subunit of this class (D'Ovidio and Masci, 2004) and is generally upregulated by N supply (Visioli et al., 2018a). Hence, the upregulation of both 81 kDa HMW-GS and 43.6 kDa LMW-GS with all the biofertilizers tested, particularly R-PK, may be attributed to the N-fixing and nutrient-solubilizing contribution of the applied microorganisms or the changes induced in the bacteria groups during grain filling.

CONCLUSION

This study has shown that bacteria or bacteria-AMF consortia tested can be safely applied as seed inocula, as they did not alter the bacterial taxa associated with wheat roots allowing the resident microbial biodiversity to be preserved. The benefits of seed inoculation included enhancement of the rhizosphere microbial biomass and of the activity of enzymes involved in organic matter decomposition and nutrient release, especially when the biofertilizer contains the AMF *Rhizophagus irregularis*. We confirmed the importance of diazotrophic bacteria (i.e., *Azoarcus*, *Azospirillum*,

Azorhizobium) in enhancing plant nitrogen nutrition and root growth. Despite with moderate effects, the biofertilizers tested here are also expected to alleviate nutrient deficiency, particularly P and Fe, which is of great importance in the worldwide spread alkaline soils, like in our site. Seed inoculation is therefore suitable for aiding the fertilization of wheat, and possibly of other cereals in organic agriculture, and may also provide beneficial environmental effects in reducing N losses.

We did not find any improvements in grain yield, although significant agronomic benefits in poor soil, low-input agriculture or under abiotic stress conditions cannot be excluded. A new finding was the increase in gluten quality due to upregulation of specific gluten protein subunits, which can be exploited in the wheat food chain.

Currently, the most suitable option seems to be the association between bacteria and AMF, but future research is required to gain further specific insights into biofertilizer composition in order to exploit the synergistic action of various bacteria and mycorrhizal fungi, and understand their behavior under reduced soil resource availability.

DATA AVAILABILITY STATEMENT

The sequencing data generated by this study can be found in the NCBI under accession number bioproject PRJNA388660.

AUTHOR CONTRIBUTIONS

TV conceived and designed the experiment, and revised the MS. CC and MF carried out the experiment and analyzed the data. GV and ML carried out gluten protein analyses and metagenomics analyses, and FF performed soil enzymatic activities analysis. GB performed the statistical analysis of data. CC wrote the first draft of the paper. AP contributed to improve the *Introduction* and the *Discussion* sections. All authors contributed to interpreting and discussing the results, and read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors wish to thank Adriano Massignan, Francesco Munari, and Simone Cassanego for helping with data collection, and Tessa Say for revising the English text. East Balt Italia Bakeries is gratefully acknowledged for the financial support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00072/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 April 2020
doi: 10.3389/fpls.2020.00396

Synergisms of Microbial Consortia, N Forms, and Micronutrients Alleviate Oxidative Damage and Stimulate Hormonal Cold Stress Adaptations in Maize

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 16 September 2019
Accepted: 19 March 2020
Published: 24 April 2020

Citation:
Moradtalab N, Ahmed A, Geistlinger J, Walker F, Höglinger B, Ludewig U and Neumann G (2020) Synergisms of Microbial Consortia, N Forms, and Micronutrients Alleviate Oxidative Damage and Stimulate Hormonal Cold Stress Adaptations in Maize. Front. Plant Sci. 11:396. doi: 10.3389/fpls.2020.00396

Aims: Low soil temperature in spring is a major constraint for the cultivation of tropical crops in temperate climates. This study aims at the exploitation of synergistic interactions of micronutrients, consortia of plant growth-promoting microorganisms and N forms as cold-stress protectants.

Methods: Maize seedlings were exposed for two weeks to low root zone temperatures at 8–14°C under controlled conditions on a silty clay-loam soil (pH 6.9) collected from a maize field cultivation site. A pre-selection trial with fungal and bacterial PGPM strains revealed superior cold-protective performance for a microbial consortium of *Trichoderma harzianum* OMG16 and *Bacillus spp.* with Zn/Mn supplementation (CombiA^{+}), particularly in combination with N-ammonium as a starting point for the characterization of the underlying physiological and molecular mechanisms.

Results: In nitrate-treated plants, the cold stress treatment increased oxidative leaf damage by 133% and reduced the shoot biomass by 25%, related with reduced acquisition of phosphate (P), zinc (Zn) and manganese (Mn). The supplying of N as ammonium improved the Zn and Mn nutritional status and increased the ABA shoot concentration by 33%, as well as moderately increased detoxification of reactive oxygen species (ROS). Moreover, use of N as ammonium also increased the root auxin (IAA) concentration (+76%), with increased expression of auxin-responsive genes, involved in IAA synthesis (*ZmTSA*), transport (*ZmPIN1a*), and perception (*ZmARF12*). Additional inoculation with the microbial consortium promoted root colonization with the inoculant strain *T. harzianum* OMG16 in combination with ammonium fertilization (+140%). An increased ABA/cytokinin ratio and increased concentrations of jasmonic (JA) and salicylic acids (SA) were related to a further increase in enzymatic and non-enzymatic ROS detoxification. Additional supplementation with Zn and Mn further increased shoot

IAA, root length and total antioxidants, resulting in the highest shoot biomass production and the lowest leaf damage by oxidative chemical species.

Conclusion: Our results suggest the mitigation of cold stress and reduction of stress priming effects on maize plants due to improved ROS detoxification and induction of hormonal stress adaptations relying on the strategic combination of stress-protective nutrients with selected microbial inoculants.

Keywords: synergisms, plant growth-promoting microorganisms, ammonium, colding, phytohormones, ROS detoxification

INTRODUCTION

The cultivation of tropical and subtropical crops in agricultural production systems under temperate climates continuously increases and is further promoted by global warming. Under these conditions, short vegetation periods due to low temperatures in early spring remain a major challenge for crops, such as maize, tolerating soil temperatures not much lower than 15°C for normal germination and early growth (Cutforth et al., 1986; Kaspar and Bland, 1992). This is further complicated by the more widespread adoption of no-tillage or conservation tillage, leading to a slower seedbed warming in spring (Hayhoe et al., 1996).

As a consequence of cold stress (5−15°C), poor field establishment due to inhibition of root development, impaired uptake and translocation of water and nutrients can translate into poor vegetative growth, low-stress resistance and finally reduction of yield (Duncan and Hesketh, 1968; Muldoon et al., 1984; Imran et al., 2013). Besides, the maize shoot meristem is directly affected since it remains belowground even until the V6 stage (Stone et al., 1999). Impairment of root growth particularly limits the acquisition of phosphate (P) and micronutrients, such as zinc (Zn), manganese (Mn) and iron (Fe) due to low soil mobility, leading to induced nutrient deficiencies (Engels and Marschner, 1996; Bradacova et al., 2016; Moradtalab et al., 2018). Due to the importance of micronutrients as co-factors for enzymatic and non-enzymatic detoxification of reactive oxygen species (ROS), oxidative stress appears in consequence of ROS overproduction, which causes a severe damage to membranes, organelles, and cell functions (Cakmak, 2000; Gong et al., 2005). Impairment of photosynthesis due to oxidative leaf damage and impaired auxin production related to zinc limitation are factors further contributing to inhibition of root growth, impaired nutrient acquisition and limited plant regrowth (Moradtalab et al., 2018).

Natural cold stress adaptations are weakly expressed in tropical and subtropical-originated plant species. Most breeding programs toward improved cold tolerance use Flint maize inbred lines as a source of adaptation, originally based on the Northern Flint race adapted to cold temperate regions of Northeastern America (Riva-Roveda et al., 2016). Temporary growth and rapid recovery from limitations due to low photosynthesis rates have been described as major adaptation traits of these genotypes (Riva-Roveda et al., 2016). As a complementary approach, adapted fertilization for supplementation of critical nutrients, such as phosphate (P) and micronutrients or application of stress-protective biostimulants are discussed as mitigation strategies to cope with cold-stress (Bradacova et al., 2016; Gómez-Muñoz et al., 2018; Moradtalab et al., 2018):

(i) Placement of P starter fertilizers as ammonium phosphates close to the seed is meanwhile regarded as a standard measure for maize cultivation in temperate climates (Nkebiwe et al., 2016). Both, P and ammonium N are applied close to the seedling root, where rhizosphere acidification, induced by preferential ammonium uptake (Marschner and Römheld, 1983) can increase the solubility of critical nutrients, such as P, Zn Mn, Fe, and Cu, with particular importance on soils with neutral to alkaline pH (Neumann and Römheld, 2002; Jing et al., 2010).

(ii) The application of stress-protective nutrients, such as Zn, Mn, Fe, B, Cu, or Si by seed treatments or starter fertigation to promote oxidative stress defense, is another strategy with proven beneficial effects on cold tolerance in maize (Imran et al., 2013; Bradacova et al., 2016; Moradtalab et al., 2018).

(iii) Also, inoculation with plant growth-promoting microorganisms (PGPMs) is discussed as a potential measure to promote early growth and field establishment of sensitive crops under challenging environmental conditions (Kumar and Verma, 2018). In the context of cold tolerance, the selection of so-called psychrotolerant PGPM strains with the ability to propagate also at soil temperatures below 15°C and sometimes even close to the freezing point, may provide a significant advantage (Selvakumar et al., 2008a,b; Subramanian, 2011). The same holds true for the use of microbial consortia as plant inoculants, combining different PGPM strains with complementary properties and differing stress tolerance (Nuti and Giovannetti, 2015; Woo and Pepe, 2018). A common feature of the described mitigation strategies is the mode of application of the aforementioned agricultural inputs. This may offer opportunities for the development of multifunctional products, combining beneficial properties and exploiting potential synergisms.

Based on this hypothesis, the aim of this study was to explore the synergistic interactions and the combined application of PGPMs, micronutrients (Zn and Mn), and the use of N as

ammonium and nitrate on the recovery and early growth of maize after two-weeks exposure to low root zone temperatures at 8–14°C on a silty clay-loam field soil, collected from a maize cultivation site.

A pre-selection trial was conducted with a range fungal and bacterial PGPM strains based on *Penicillium sp.* with cold-protective properties (Gómez-Muñoz et al., 2018), a cold-tolerant strain of *Bacillus atrophaeus* (ABI02) and a microbial consortium product (MCP), based on a combined formulation of *Trichoderma harzianum* OMG16 and *Bacillus spp.* with Zn/Mn supplementation (CombiA). The CombiA consortium was selected according to the MCP concept, by combining different as microbial strains with complementary properties as discussed an approach to increase the efficiency and the flexibility of PGPM-based production strategies under variable environmental conditions (Woo and Pepe, 2018). *Trichoderma-Bacillus* combinations are among the well-documented examples in this context. Although co-cultivation of *Trichoderna* and *Bacillus* strains on artificial growth media was frequently characterized by antagonisms (Gyu Kim et al., 2008), in many plant species, including *Oryza sativa* (Ali and Nadarajah, 2014), *Triticum aestivum* (Karuppiah et al., 2019), *Cicer arietinum* (Zaim et al., 2018), *Solanum melongena* and *Capsicum annuum* (Abeysinghe, 2009), synergistic beneficial effects were reported after co-inoculation. This included stimulation of germination and growth promotion, as well as biocontrol effects against fungal pathogens, such as *Rhizoctonia, Fusarium* and *Pythium*, known as important damping-off diseases in cold and wet soils with potential relevance also for the present study. Testing seven different fungal and bacterial inoculants, Mpanga et al. (2019b) found superior root growth-promoting properties and improved nutrient acquisition after CombiA inoculation in maize under P limitation. Similarly, superior cold-protective performance in terms of biomass production and reduction of oxidative leaf damage was reported for the CombiA consortium in the previous selection trial conducted in this study (**Supplementary Table S1**).

To evaluate the mode of action of the selected cold stress mitigation strategies, we hypothesized that (i) ammonium-dominated N supplying will increase the availability of critical nutrients, such as P, Zn, Mn, Fe via rhizosphere acidification on the investigated soil with neutral pH and additionally stimulate rhizosphere interactions with the selected inoculants as previously reported by Mpanga et al. (2019a), (ii) The starter application of micronutrients will additionally provide co-factors (Zn, Mn) for the systems of ROS detoxification already before the onset of the cold stress treatments. The improved micronutrient status will support the expression of adaptive responses to cold stress by mitigation of oxidative damage, as similarly reported by Bradacova et al. (2016) and Moradtalab et al. (2018), (iii) The selected microbial inoculants will interact with plant hormonal homeostasis, supporting nutrient acquisition by stimulation of root growth, induction of stress priming effects and provide protection against pathogen attack.

To dissect the investigated mitigation strategies to low temperatures, the monitoring of physiological stress indicators, hormonal homeostasis and expression of related genes was conducted for the single and combined application of the selected cold-stress protectants.

MATERIALS AND METHODS

Plant Cultivation

For all experiments, maize (*Zea mays* L. cv. Rolandinio) plants were grown for six weeks under greenhouse conditions. For the preparation of the growth substrate, a silty clay loam field soil pH 6.9 (see **Table 1** for soil properties) was freshly collected from the Ap horizon of a maize cultivation site at the Hohenheim University experimental station Ihinger Hof (48°45' N, 8°56' E, Renningen, Germany), air-dried and sieved using 2 mm mesh size. The soil substrate was mixed with quartz sand (30% w/w) to improve the soil structure. Fertilization was performed with

TABLE 1 | Chemical and physicochemical properties of the soil sampled at the Hohenheim University experimental station Ihinger Hof Renningen, Germany), 2016.

Determination	Unit	Value
pH-Value ($CaCl_2$)		6.9
Available P CAL-Extract VDLUFA)	mg/kg	82.9
Availabke K CAL-Extract VDLUFA)	mg/kg	141.1
Mg ($CaCl_2$)	mg/kg	190
Humus	mg/kg	22000
Carbon $_{total}$	mg/kg	12800
N	%	0.177
S	%	0.054
Sand (63–2000 μm)	%	2.9
Silt (2–63 μm)	%	66.8
Clay (<2μm)	%	30.3
Carbonate (Scheibler)	mg/kg	1.1
Fe (CAT-Extract)	mg/kg	126
Cu (CAT-Extract)	mg/kg	4.22
Mg (CAT-Extract)	mg/kg	215
Mn (CAT-Extract)	mg/kg	404
Zn (CAT-Extract)	mg/kg	2.92
K (CAT-Extract)	mg/kg	97.2
P (Olsen)	mg/kg	49.2
B (ICP-OES KW)	mg/kg	17.8
Ca (ICP-OES KW)	mg/kg	4.600
Fe (ICP-OES KW)	mg/kg	26.912
K (ICP-OES KW)	mg/kg	4.407
Cu (ICP-OES KW)	mg/kg	23.1
Mg (ICP-OES KW)	mg/kg	5.630
Mn (ICP-OES KW)	mg/kg	991
P (ICP-OES KW)	mg/kg	990
Zn (ICP-OES KW)	mg/kg	62.6
Base saturation (Co-hexamine VDLUFA)	%	80
Calcium (Ca) exchangeable (CoHexamin)	cmol(c)/kg	13.7
KAK pot (co-hexamine VDLUFA)	cmol(c)/kg	20.6
Potassium (K) exchangeable (CoHexamin)	cmol(c)/kg	0.42
Magnesium (Mg) exchangeable (CoHexamin)	cmol(c)/kg	2.37
Sodium (Na) exchangeable (CoHexamin)	cmol(c)/kg	0

$Ca(H_2PO_4)_2$: 80 mg kg^{-1} dry matter (DM) P; K_2SO_4: 150 mg kg^{-1} DM K and $MgSO_4$: 50 mg kg^{-1} DM Mg. Nitrogen was applied at a rate of 100 mg kg^{-1} DM N, either as a commercial Ca-nitrate formulation, (YaraLiva® CALCINIT®, YARA GmbH & Co., KG, Germany) or as stabilized ammonium sulfate (NovaTec® Solub 21, COMPO EXPERT GmbH, Germany).

Thereafter, 2 L plastic pots were filled with 1.8 kg of the substrate prior to sowing, which was conducted with five seeds per pot and reduction to one seedling after germination. During the first two weeks, plants were grown under ambient greenhouse temperature conditions (ranging from 18−25°C) and as soon as the plants were established, they were exposed to low root zone temperature (RZT in a thermostatic root-cooling device, described by Bradacova et al. (2016). The root-cooling system is based on an immersion water bath circulator (Thermomix 1480, Frigomix 1497, Braun, Melsungen, Germany) connected to a pipe system, installed in moist peat culture substrate to circulate the cooling fluid through the moist peat layer. For adjustment of the reduced RZT regime (two weeks-cold stress at 8−14°C), the culture vessels were immersed into the cooled peat bed followed by 2-weeks recovery at ambient greenhouse temperature. Soil and air temperatures were constantly recorded by a LogTag device (**Supplementary Figure S1**). Every second day, soil moisture was adjusted gravimetrically to 70% of the substrate water-holding capacity (18.3% w/w).

Microbial Inoculants

To select the most effective cold stress-protectants, five different treatments were tested in a pre-screening experiment: (i) Seed dressing with a commercial Zn/Mn formulation (0.2 ml Lebosol® Mn500 SC and 0.1 ml Lebosol® Zn700 SC 100 g^{-1} seeds, Lebosol® Dünger GmbH, Ermstein, Germany); (ii) fertigation with 10^9 spores Kg^{-1} DM ABI02, a cold-tolerant *Bacillus atrophaeus* strain (ABITEP, Berlin, Germany) combined with Zn/Mn seed dressing, (iii) fertigation with 10^8 spores Kg^{-1} DM Biological fertilizer OD (BFOD), a *Penicillium* sp. PK112 formulation (Bayer Crop Science Biologicals GmbH, Malchow, Germany) with documented cold−stress protecting effects combined with Zn/Mn seed dressing, (iv) Fertigation with 2.5×10^7 cfu Kg^{-1} DM Combi A^+, a microbial consortium formulation with Zn (13% w/w) + Mn (9% w/w) + *Trichoderma harzianum* OMG16 (9×10^9 spores g^{-1}) + Vitabac (1×10^{11} cfu g^{-1}, as a mixture of *Bacillus licheniformis, B. megaterium, B. polymyxa, B. pumilis,* and *B. subtilis,* Bactvita GmbH, Straelen, Germany). Untreated variants exposed to cold stress and ambient greenhouse temperature were included as controls. In the follow-up experiments, only CombiA with ($CombiA^+$) and without Zn/Mn supplementation ($CombiA^-$) were used as inoculants.

The first inoculation was performed one week after sowing (WAS), followed by a 2nd application one day prior to exposure to low RZT 13 days after sowing (DAS) and a final application at the start of recovery phase (29 DAS). All microbial inoculants were applied through soil drenching with a dispenser pipette into the top-soil close to the seedlings roots.

Assessment of Leaf Damage, Biomass, Root Length, and Rhizosphere pH

At the final harvest (6 WAS), cold stress-induced oxidative leaf damage (chlorosis/necrosis, anthocyanin formation) was quantified by counting the number of damaged leaves $plant^{-1}$. Root systems were excavated, and rhizosphere soil was collected by shaking-off root adhering soil and mixed in a plastic bag. Thereafter, pH was determined according to VDLUFA (1991). Fresh weight (FW) of the shoot and root tissue was measured and finally dry weight (DW) was recorded after oven-drying at 65°C. For root length determination, washed roots previously stored in 30% ethanol, were separated, submerged in a water film on transparent Perspex trays, and subsequently digitalized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). The root length of the digitalized samples was measured by the use of the WinRHIZO root analysis system (Reagent Instruments, Quebec, QC, Canada).

Analysis of Shoot Nutrient Contents

To assess the plant mineral nutrient status, dried shoot material was homogenized using a grinding mill (Labor Scheibenschwingmühle TS-100A, Sieb Technik GmbH, Mühlheim-Ruhr, Germany). After grinding, 250 mg of plant material was ashed in a muffle furnace for five hours at 500°C. After cooling to room temperature, the samples were extracted as described by Moradtalab et al. (2018). Spectrophotometrical determination (Hitachi U-3300, Hitachi Ltd., Corporation Japan) of orthophosphate was conducted by molybdo-vandate method of Gericke and Kurmis (1952). Potassium and Ca were determined by flame emission photometry (ELEX 6361, Eppendorf, Hamburg, Germany). Magnesium, Fe, Mn, Zn, and Cu concentrations were measured by atomic absorption spectrometry (ATI Unicam Solaar 939, Thermo Electron, Waltham, United States).

Superoxide Dismutase and Peroxidase Activity

Extraction and determination of superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC1.11.1.7) activities were optimized for root and shoot tissues of maize according to the method described (Moradtalab et al., 2018). Spectrophotometrical determination (Spectrophotometer U-3300, Hitachi, Tokyo, Japan) of SOD activity was performed based on the nitro-blue tetrazolium (NBT) method at a wavelength of 650 nm (Beauchamp and Fridovich, 1971). The activity of POD was determined at 470 nm using the tetra-guaiacol assay described by Hajiboland and Hasani (2007).

Analysis of Metabolites and Antioxidants

Details for metabolite determinations have been described previously by Moradtalab et al. (2018). For the determination of soluble sugars, leaf and root samples were homogenized in 100 mM phosphate buffer (pH 7.5) at 4°C. After centrifugation at $12000 \times g$ for 15 min, the supernatant was used for the determination of total soluble sugars by the anthrone-sulfuric acid method of Yemm and Willis (1954). Total phenolics

concentration was determined spectrophotometrically at 750 nm, using the Folin method (Hajiboland et al., 2017). For proline analysis, samples were homogenized with 3% (v/v) sulfosalicylic acid and the homogenate was centrifuged at 3,000 × *g* for 20 min. Proline was determined spectrophotometrically at 520 nm after acetic acid and acid ninhydrin derivatization (Bates et al., 1973). The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method was used to evaluate the free radical scavenging activity of antioxidants in the plant tissue (Panico et al., 2009).

UHPLC-MS Analysis of Phytohormones

Frozen maize tissue samples (shoot, roots) of 1 g were ground to a fine powder with liquid nitrogen and extracted twice with 2.5 ml of 80% methanol in falcon tubes. Thereafter, the samples were further homogenized by ultrasonication (Micra D-9 homogenizer, Art, Müllheim Germany) for 1 min and 15 s at 10,000 rpm. Two milliliters of the methanol extracts were transferred to microtubes and centrifuged at 5,645 × *g* for 5 min. Thereafter, 350 µl of the supernatant was mixed with 700 µl ultra-pure water and centrifuged at 5,645 × *g* for 5 min. The supernatant was cleaned through a filtration membrane (Chromafil R O-20/15MS) and transferred to HPLC vials. UHPLC-MS analysis was carried out on a Velos LTQSystem (Thermo Fisher Scientific, Waltham, MA, United States) fitted with a Synergi Polar column, 4 µ, 150 × 3.0 mm, (Phenomenex, Torrance, CA, United States). The injection volume was 3 µL and the flow rate was adjusted to 0.5 ml min^{-1} for gradient elution with mobile phase (A): water and 5% acetonitrile; mobile phase (B): acetonitrile and a gradient profile of 0–1 min, 95% A, 5% B, 11–13 min, 10% A, 90% B, 13.1 min, 95% A, 5% B, 16 min 95% A, 5% B). All standards were purchased from Sigma Aldrich, (Sigma Aldrich, St. Louis, MO, United States) including (±)-jasmonic acid; 3-indoleacetic-acid, gibberellic acid, (±) abscisic acid; *trans*-zeatin; salicylic acid (Moradtalab et al., 2018).

Expression of Hormonal Target Genes

The expression of selected target genes was analyzed to evaluate potential interactions of applied cold stress protectants with hormonal signaling. Relative expression of the IAA efflux transporter *ZmPIN1a (PINFORMED)*, the auxin response factor 12 (*ZmAFR12*), tryptophan synthase (*ZmTSA*), the auxin early response gene *ZmAuxIAA*, the abscisic acid responsive element-binding factor 2 (*ZmABF2*) and the isopentenyl transferases (*ZmIPT4* and *ZmIPT5*) were determined. Elongation factor-1alpha (*EF1α*) and *tubulin* were selected as reference genes. The results are expressed according to *EF1α* with particularly stable expression under cold stress (Tang et al., 2017). Isolation of mRNA and RT-qPCR quantification of relative transcript abundances was performed from frozen root material by GenXPro GmbH, Frankfurt am Main, Germany.

Rhizosphere Tracing of *Trichoderma harzianum* OMG16

For the quantification of the *T. harzianum* strain OMG16 (DSMZ accession no.: 32722) in the maize root endosphere, roots were thoroughly cleaned with a soft brush and water to remove residual soil particles, shortly dried between paper towels and cut into small pieces. Approximately 80 mg fine roots were placed in 2 mL tubes containing 1.0 mm silica spheres including one single 0.25-inch ceramic bead (MP Biomedicals, France) and 400 µL peqGOLD lysis buffer (VWR Peqlab, Germany). Root tissue was homogenized for 3 × 30 s at a speed of 6 m/s in a FastPrep 24 bead-beating system (MP Biomedicals). After each cycle samples were cooled on ice for 1 min. DNA was subsequently extracted utilizing the peqGOLD Fungal DNA Kit (VWR Peqlab), following the manufacturer's instructions. DNA was eluted in a TE buffer (pH 8.0) and checked on 0.8% TAE agarose gels. DNA concentrations were determined using a Qubit® 3.0 Fluorometer and the Qubit dsDNA HS Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Germany). A *T. harzianum* OMG16-specific primer pair, designed from OMG16 genomic DNA sequences were used for qPCR quantification of *T. harzianum* OMG16 DNA in the DNA samples according to the method described by Mpanga et al. (2019a).

Statistical Analysis

The study was carried out in a completely randomized design. Data are presented as means ± SD. For statistical analysis of significant differences between treatment groups, a one-way ANOVA followed by a Tukey-test ($p < 0.05$) was performed using the SAS software 9.4 (SAS Institute Inc., Cary, NC, United States). Pairwise comparisons (*t*-Test, $p < 0.05$) were conducted with the Sigma Plot 13 software package (SYSTAT Software Inc., Erkrath, Germany).

RESULTS

Cold-Protective Effects of the CombiA + Consortium as Related to N Forms

The first experiment addressed the impact of ammonium fertilization versus nitrate supply on the cold-protective performance of the CombiA^{+} consortium. The form of N had no significant effect on the shoot biomass of non-stressed control plants (**Table 2**). No macro- or micronutrient deficiencies were recorded, irrespective of the N fertilization regime (**Supplementary Table S2**), while shoot P and Zn accumulation were significantly increased in the ammonium variant (**Supplementary Table S2**), associated with a decline in rhizosphere pH by 0.6 units as compared with nitrate fertilization (**Table 2**). The two weeks cold-stress period decreased shoot biomass production of the plants with nitrate supply by 25% (**Table 2**), associated with a 133% increase in oxidative leaf damage, indicated by chlorosis, necrosis, and stress-induced anthocyanin formation (**Figures 1**, **2**).

Compared with nitrate fertilization, oxidative leaf damage was significantly lower in the ammonium variant and there was no significant decline in shoot biomass (**Table 3**). The Zn and Mn-nutritional status in cold-stressed plants with nitrate supply dropped close to the deficiency threshold

TABLE 2 | Shoot dry weight (DW), oxidative leaf damage (number of damaged leaves plant^{-1}), Zn and Mn shoot concentrations and rhizosphere pH of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9.

N-Form	Stress factor	Treatment	Shoot DW[g]	Oxidative leaf damage [Leaf number]	Zn [μg g^{-1}DW]	Mn [μg g^{-1}DW]	pH Mean value ($CaCl_2$)	pH Δ (Rhizo-Bulk soil)
Nitrate	No-Cold	Ctrl	6.0 ± 0.6 a	2.4 ± 0.54 c	46.0 ± 0.8 b	50.5 ± 1.9 b	7.3 ± 0.02 a	+0.4
	8–14°C	Ctrl	4.5 ± 0.5 b	5.6 ± 0.89 a	24.4 ± 3.9 c	34.8 ± 5.9 c	7.2 ± 0.03 b	+0.3
		Combi A$^+$	5.8 ± 0.7 a	3.4 ± 0.554 b	47.8 ± 5.6 b	48.0 ± 1.3 b	7.0 ± 0.05 c	+0.1
Ammonium	No-Cold	Ctrl	6.6 ± 0.2 a	1.6 ± 0.53 c	59.0 ± 2.0 a	51.0 ± 2.6 b	6.7 ± 0.02 d	−0.2
	8–14°C	Ctrl	6.2 ± 0.4 a	3.6 ± 0.52 c	59.4 ± 3.8 a	53.7 ± 1.3 ab	6.7 ± 0.02 d	−0.2
		Combi A$^+$	6.9 ± 1.1 a	2.4 ± 0.53 bc	57.6 ± 5.9 a	52.9 ± 3.4 ab	6.6 ± 0.03 e	−0.3

Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. Data represent the means and SD of five replicates. In each row, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

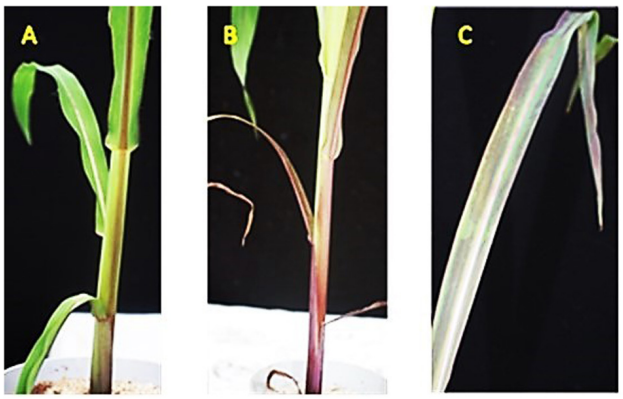


FIGURE 1 | A Undamaged leaves of non-stressed maize plants grown for four weeks under ambient greenhouse temperature (18–25°C), **(B,C)** Oxidative leaf damage and symptoms of P limitation (chlorosis, necrosis, stress-anthocyanins) of cold stressed- plants exposed to two weeks of reduced root zone temperature (8–14°C).

but remained in the sufficiency range in combination with ammonium fertilization (**Table 2** and **Supplementary Table S2**), associated with a lower rhizosphere pH. Nevertheless, ammonium-induced rhizosphere acidification had no effect on the P-nutritional status, which declined below the deficiency threshold in the cold-stressed plants (**Supplementary Table S2**).

The application of the microbial consortium product CombiA$^+$ significantly increased shoot biomass production in the cold-stressed nitrate variant with the same trend in combination with ammonium supply (**Table 2**), which additionally increased total root length (**Figure 2**). Under both N form regimes, CombiA$^+$ application significantly reduced cold stress-induced oxidative leaf damage but only in the ammonium variant, the level of leaf damage was not significantly different from the non-stressed control (**Table 2**) and the P status reached the sufficiency range (**Supplementary Table S2**). No significant differences were recorded for root biomass and the root/shoot biomass ratio (**Figure 2**).

Synergistic Effects of N-Form Supply, Micronutrient Supplementation and PGPM Inoculants Adaptive Cold-Stress Responses in Maize

A second experiment was conducted to dissect the individual contributions of ammonium fertilization, Zn/Mn supplementation and CombiA application to the cold-protective effect at the physiological and molecular level. Micronutrient effects were identified by comparison of MCP inoculant formulations with (CombiA$^+$) and without additions of Zn/Mn (CombiA$^-$).

Plant Growth and MCP Root Colonization

Superior cold-protective performance by combined application of stabilized ammonium, Zn/Mn supply, and the PGPM consortium as compared with nitrate fertilization was confirmed also in the second experiment. This was reflected in the highest shoot biomass production (+48%), increased root length (+161%) and the lowest level of cold-stress induced oxidative leaf damage (-42%). Ammonium fertilization and particularly CombiA$^+$ application reverted cold-stress induced Zn limitation of the host plants, while root biomass remained unaffected (**Figure 3**).

A strain-specific primer was available for *Trichoderma harzianum* OMG16 in the CombiA formulation. This enabled rhizosphere tracing to evaluate the root colonization efficiency of the inoculant. Traces of *T. harzianum* OMG16 DNA were detectable also in the root samples of non-inoculated controls. A significant increase in OMG16 root colonization was recorded exclusively in CombiA-inoculated roots of maize plants with ammonium fertilization and was not affected by additional Zn/Mn supplementation (**Figure 4**).

Accumulation of Antioxidants and Cryoprotective Solutes

Cold stress increased the shoot concentration of proline with cryo-protective an anti-oxidative functions (Szabados and Savouré, 2010) by 67% under nitrate supply and by 200% in the ammonium variant. A significant increase in soluble sugar concentrations was recorded only in combination with nitrate fertilization. Finally, the highest shoot concentrations of

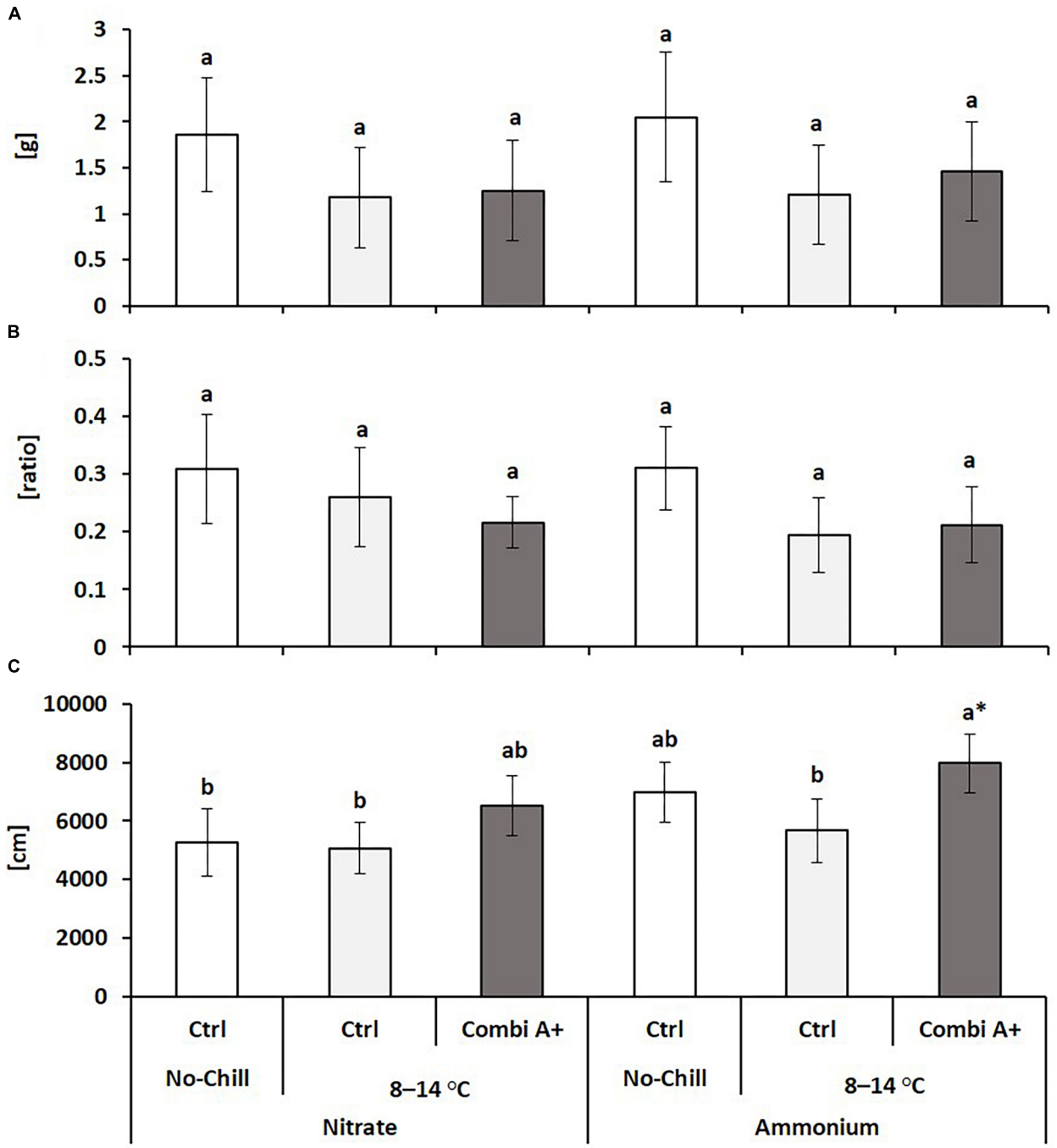


FIGURE 2 | Root dry weight **(A)**, root/shoot biomass ratio **(B)**, and total root length **(C)** of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14°C) on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. Data represent the means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$).

proline and soluble sugars accumulated in cold-stressed maize plants with CombiA inoculation and ammonium supply. This could be attributed to the presence of the MCP inoculant since additional Zn/Mn supplementation had no additional effects (**Table 3**).

Total phenolics and antioxidants increased in the shoot tissue of cold stressed maize plants particularly in combination with ammonium fertilization, while antioxidants in roots rather declined. Again, CombiA inoculation combined with ammonium supply resulted in the highest accumulation of phenolics and total

TABLE 3 | Tissue concentrations of sugars, proline, total phenolics and total antioxidants in maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9.

			Shoot				Root
N-Form	**Stress factor**	**Treatment**	**Sugar [mg g^{-1} FW]**	**Proline [mg g^{-1} FW]**	**Phenolics [mg g^{-1} FW]**	**Total antioxidants [%]**	**Total antioxidants [%]**
Nitrate	No-Cold	Ctrl	1.7 ± 0.2 d*	0.3 ± 0.04 d*	3.1 ± 0.1 d*	52.5 ± 0.94 d*	39.0 ± 2.5 c*
	8–14°C	Ctrl	2.3 ± 0.2 c	0.5 ± 0.05 c	3.9 ± 0.1 c	67.1 ± 1.89 c	25.7 ± 3.6 d
		Combi A^-	3.2 ± 0.4 b*	0.7 ± 0.04 b*	4.2 ± 0.4 c	91.4 ± 1.40 b*	62.3 ± 4.7 b*
		Combi A^+	2.9 ± 0.6 c	0.7 ± 0.03 b*	4.6 ± 0.2 bc*	85.7 ± 6.93 b*	69.6 ± 5.4 ab*
Ammonium	No-Cold	Ctrl	2.3 ± 0.2 c	0.2 ± 0.03 d*	3.9 ± 0.1 c*	58.0 ± 3.80 d*	39.4 ± 2.7 c
	8–14°C	Ctrl	2.5 ± 0.2 c	0.6 ± 0.03 c	4.9 ± 0.1 b	77.1 ± 3.63 bc	29.0 ± 4.0 cd
		Combi A^-	3.8 ± 0.1 a*	1.0 ± 0.04 a*	5.5 ± 0.5 ab	97.1 ± 1.97 a*	71.5 ± 5.3 b*
		Combi A^+	3.6 ± 0.2 a*	0.9 ± 0.04 a*	5.6 ± 0.4 a*	98.9 ± 5.92 ab*	87.1 ± 5.2 a*

*Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA$^-$; CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA$^-$ formulation without Zn/Mn; CombiA$^+$ formulation with Zn/Mn. Data represent the means and SD of five replicates. In each row, different letters indicate significant differences (Tukey-Test, $p < 0.05$). *significant in pairwise comparisons with RTZ untreated Ctrl (t-Test, $p < 0.05$).*

antioxidants, both, in shoot and root tissues. Additional Zn/Mn supplementation further increased the root concentrations of antioxidants (**Table 3**).

Enzymatic ROS Detoxification

Activities of superoxide dismutase and peroxidase were determined as key enzymes involved in the detoxification of cold-stress-induced production of reactive oxygen species. Accordingly, the lowest SOD and POD activities were recorded in the non-stressed controls but with higher values in the ammonium variants. Cold stress further increased SOD and POD in root and shoot tissues with higher levels in plants with ammonium fertilization as compared with nitrate supply. After all, the highest activities were found after CombiA inoculation in combination with ammonium supply. This effect could be mainly attributed to the presence of the MCP inoculant with a small additional impact of Zn/Mn supplementation (**Figure 5**).

Interactions With Hormonal Homeostasis

The form of N supply had no effects on the shoot concentrations of the growth hormones indole acetic acid (IAA), gibberellic acid (GA), and zeatin (CK) in non-stressed controlled plants, while the concentrations declined in the cold stress variants without significant differences between plants with nitrate or ammonium supply. The negative cold stress effect on shoot concentrations of IAA, GA, and CK was reverted by CombiA inoculation and more strongly expressed for the GA concentrations in plants with ammonium fertilization as compared with nitrate supply. Additional Zn/Mn supplementation further increased the IAA concentrations in the ammonium variants (**Figure 6**).

Ammonium fertilization increased the concentrations of the stress hormones abscisic acid (ABA) and jasmonic acid (JA) even in the shoot tissue of non-stressed control plants. Cold stress further increased the ABA levels particularly in the ammonium variant, while the concentrations of JA and salicylic acid (SA) declined. By contrast, JA and SA concentrations increased after CombiA inoculation while ABA increased only in the nitrate variant but not with ammonium supply. Additional effects of Zn/Mn supplementation in the CombiA$^+$ variants were not detectable (**Figure 6**).

In the roots of cold stressed plants, ammonium supply and particularly the combination with CombiA inoculation increased the IAA tissue concentrations by 75% and 131%, respectively (**Figure 7**), as similarly recorded also for the shoot tissue (**Figure 6**). Ammonium fertilization had no effect on the level of root cytokinins (zeatin) but root CK concentrations declined in response to CombiA application, contrary to the opposite effect, recorded in the shoot tissue (**Figure 6**). The decline in CK concentrations was particularly expressed in the variants with ammonium supply (-50%). CombiA also increased SA concentrations in the root tissue without additional effects by Zn/Mn supplementation (**Figure 7**). No treatment differences were detectable for root ABA concentrations. Jasmonic acid (JA) ranged below the detection limit.

In accordance with the increased IAA concentrations in the root tissue (**Figure 7**), expression of the tryptophan synthase gene (*ZmTSA*), involved in the biosynthesis of IAA and other indole compounds (Mano and Nemoto, 2012), was enhanced in response to ammonium fertilization and further increased in combination with CombiA inoculation. This was associated with a correspondingly increased expression of genes encoding for the auxin transporter *ZmPIN1* (Li et al., 2018) and the auxin response factor 12 (*ZmAFR12*) involved in IAA perception (**Figure 8A**). The *ZmAuxIAA5* gene was selected as a well-studied member of the auxin early response genes, found to be rapidly up-regulated by external auxin supply (Park and Hasenstein, 2016), to test responses to potential IAA production of the inoculants but in this case, no significant treatment differences were detectable (**Figure 8A**). Declining cytokinin concentrations recorded in the root tissue of CombiA-inoculated plants (**Figure 7**) were reflected in decreased expression of the genes encoding the isopentenyl transferases 4,5 (*ZmIPT4,5*) involved in cytokinin biosynthesis (**Figure 8B**). Ammonium

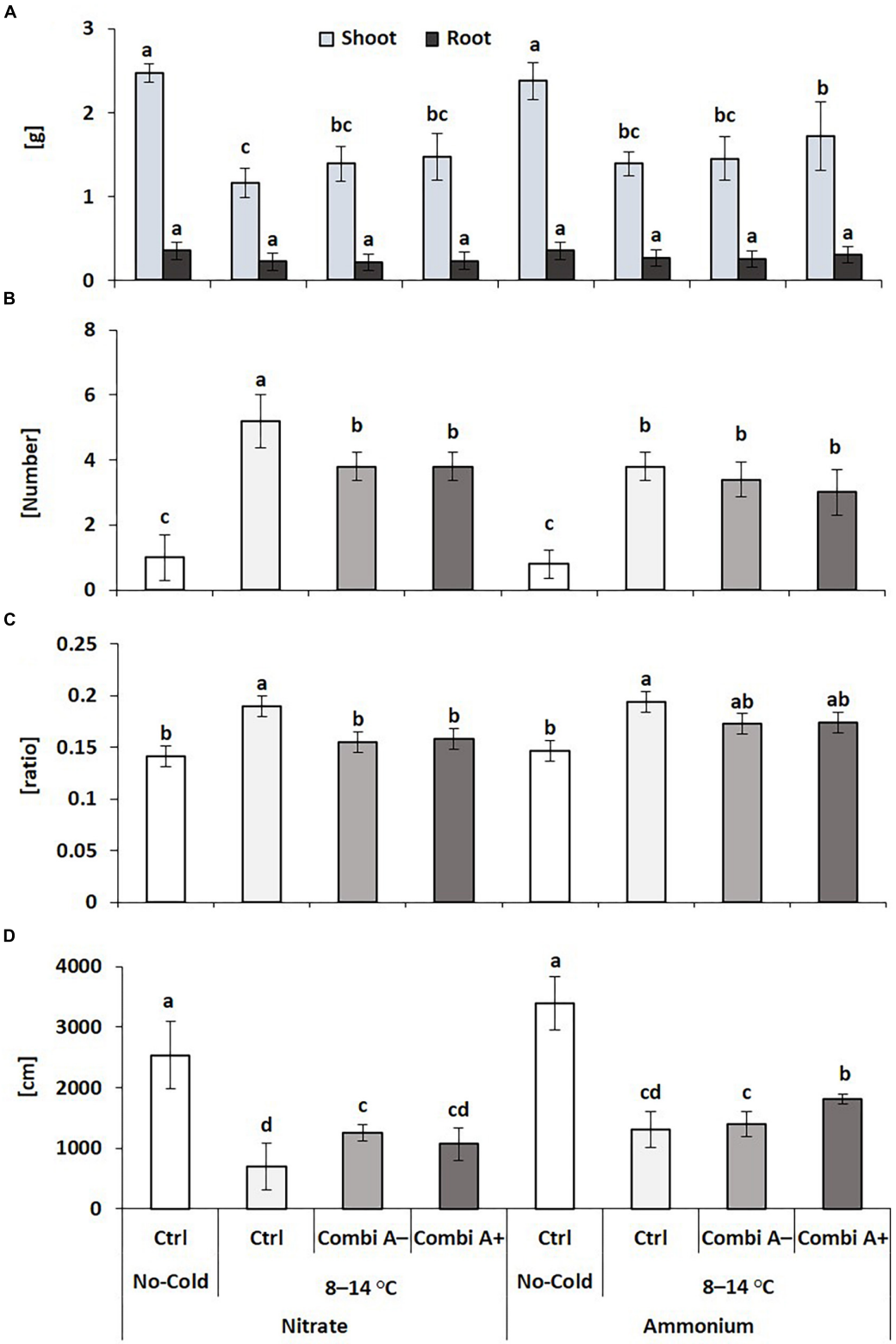


FIGURE 3 | Shoot and root dry weight **(A)**, oxidative leaf damage **(B)** root/shoot biomass ratio **(C)**, and root length **(D)** of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14°C) on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants including untreated control (Ctrl), Combi A^- (without Zn/Mn) and Combi A^+ (containing Zn/Mn) under nitrate or ammonium fertilization. Means and SD of five replicates. Different letters: significant differences (Tukey-Test, $p < 0.05$).

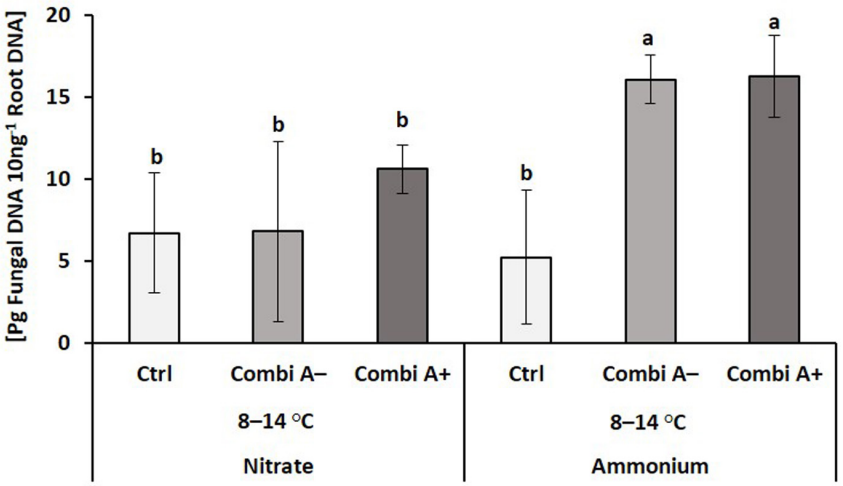


FIGURE 4 | Root colonization with *Trichoderma harzianum* OMG16 of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA$^-$; CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA$^-$ formulation without Zn/Mn; CombiA$^+$ formulation with Zn/Mn. Data represent the means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$).

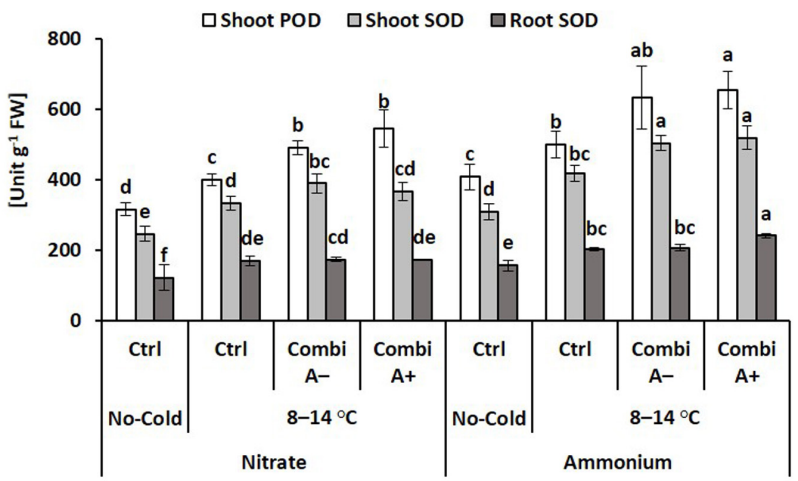


FIGURE 5 | Activities of peroxidase (POD) and superoxide dismutase (SOD) in maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA$^-$; CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA$^-$ formulation without Zn/Mn; CombiA$^+$ formulation with Zn/Mn. Bars represent the means and SD of five replicates. For each enzyme, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

fertilization and particularly the combination with CombiA$^+$ inoculation increased gene expression of the ABA-responsive ABA-binding factor2; ABF2 (**Figure 8B**).

DISCUSSION

Cold Protective Effects Induced by the Form of N Supply

In both experiments, a certain cold protective effect of stabilized ammonium supply compared with nitrate fertilization was indicated by a 27–36% decline of oxidative leaf damage (chlorosis, necrosis; **Figure 1**), detectable at the end of the 2-weeks cold stress period (**Table 2** and **Figure 3**). Ammonium fertilization counteracted cold-stress induced zinc and manganese deficiencies, which dropped to critical levels (Campbell and Plank, 2013) in the cold-stressed plants with nitrate supply (**Table 2** and **Supplementary Table S2**). Hence, micronutrient deficiencies (Zn, Mn, Fe) have been characterized as growth-limiting factors for cold-stressed maize plants also in previous studies, reverted by micronutrient supplementation via seed priming (Imran et al., 2013), seed dressing or fertigation (Bradacova et al., 2016; Moradtalab et al., 2018) prior to the onset of the stress period. Under ammonium fertilization,

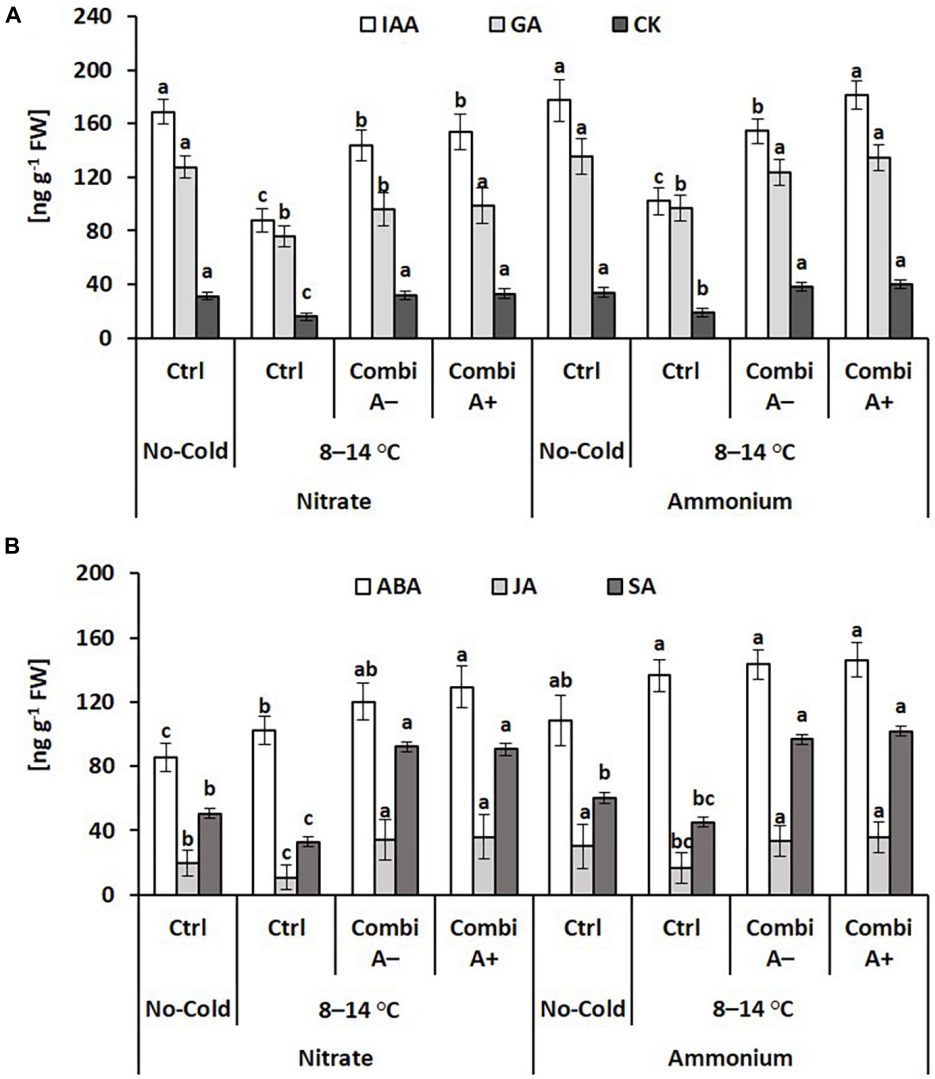


FIGURE 6 | Endogenous concentrations of growth **(A)**, and stress-related **(B)** phytohormones in the shoot tissue of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14°C) with (CombiA$^-$; CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA$^-$ formulation without Zn/Mn; CombiA$^+$ formulation with Zn/Mn. IAA = indole acetic acid: GA = gibberellic acid; CK = cytokinins (zeatin); ABA = abscisic acid; JA = jasmonic acid; SA = salicylic acid. Bars represent the means and SD of five replicates. For each hormone, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

increased shoot concentrations of Zn and Mn were related to the well-documented ammonium-induced rhizosphere acidification (Marschner and Römheld, 1983; Neumann and Römheld, 2002) by 0.6 pH units compared with nitrate supply (**Table 2**), which apparently increased Zn and Mn solubility in the rhizosphere. However, the rhizosphere acidification effect was obviously not sufficient to mobilize significant amounts of P, since the P status remained in the deficiency range (**Supplementary Table S2**).

Components of both, enzymatic and non-enzymatic ROS detoxification are particularly dependent on sufficient micronutrient supplying (Cakmak, 2000; Datnoff et al., 2007), providing enzymatic co-factors for superoxide dismutases (Zn, Mn, Fe, Cu), peroxidases (Fe) and enzymes involved in biosynthesis of phenolics with antioxidative potential (Mn, Cu). Consequently, Zn/Mn supplementation by seed dressing increased superoxide dismutase (SOD) activity, accumulation of phenolics and antioxidants associated with a decline in ROS accumulation and reduced oxidative leaf damage in cold-stressed maize plants (Bradacova et al., 2016; Moradtalab et al., 2018). Similarly, in this study, an improved Zn/Mn status in response to ammonium-induced rhizosphere acidification (**Table 2**) may be related with increased activities of superoxide dismutases (+ 25%) and peroxidases (+ 25%; **Figure 5**) and increased Mn-dependent shoot accumulation of phenolics (+ 26%; **Table 3**), while oxidative leaf damage declined by 27% (**Figure 3**). This was associated with increased shoot

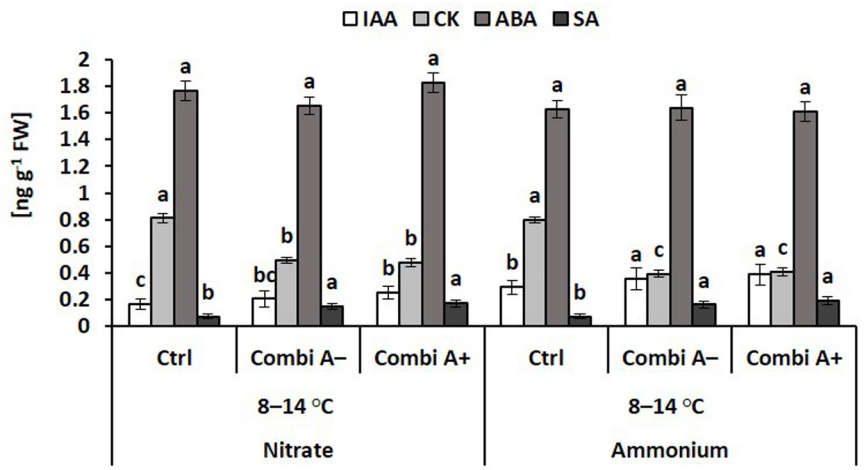


FIGURE 7 | Endogenous concentrations of phytohormones in the root tissue of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Low RZT variants (8–14°C) with (CombiA$^-$; CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA$^-$ formulation without Zn/Mn; CombiA$^+$ formulation with Zn/Mn. IAA = indole acetic acid; CK = cytokinins (zeatin); ABA = abscisic acid; SA = salicylic acid. Data represent the means and SD of five replicates. For each hormone, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

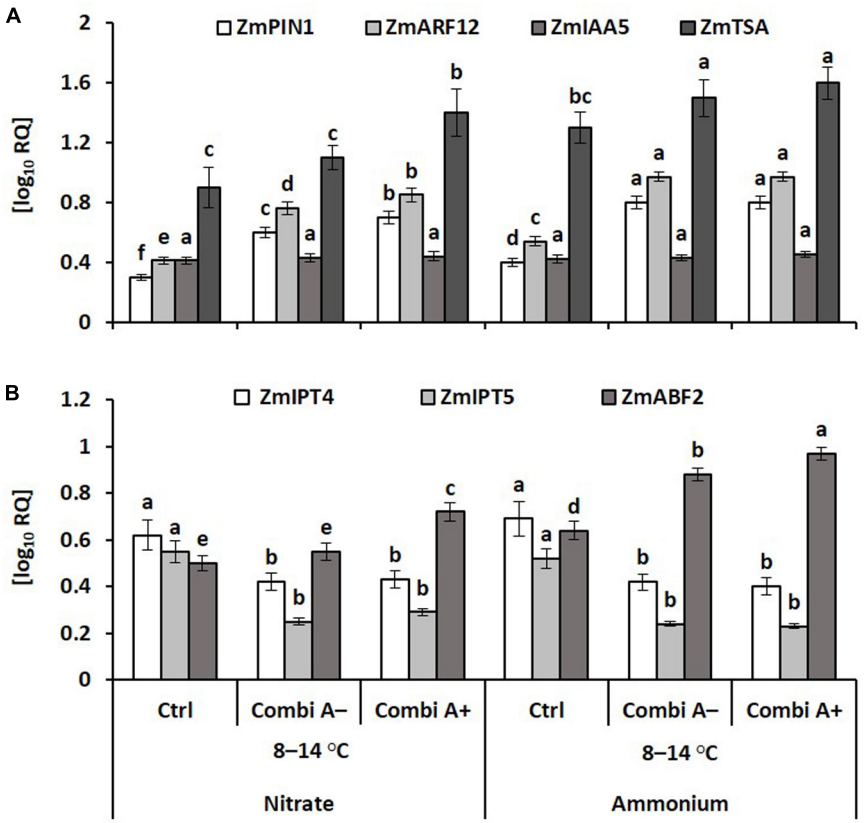


FIGURE 8 | Hormone-related transcript abundances of root tissue in maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14°C) on silty loam soil, pH 6.9. Low RZT variants including untreated control (Ctrl), Combi A$^-$ (formulation without Zn/Mn) and Combi A$^+$ (formulation with Zn/Mn) under nitrate or ammonium fertilization. Means and SD of five replicates. For each gene, different letters: significant differences (Tukey-Test, $p < 0.05$). **(A)** PIN1: PINFORMED1, ARF12: Auxin response factor12, IAA5: Aux/IAA-transcription factor5, TSA: tryptophan synthase, **(B)** IPT4,5: Isopentenyl transferases 4 and 5, ABF2: Abscisic acid-binding factor2.

concentrations of abscisic acid (ABA, **Figure 6**) as a central regulator of cold stress adaptations in plants (Szalai et al., 2011; Eremina et al., 2016). Direct links between ABA and enzymatic ROS detoxification in cold-stressed plants have been reported by Kumar et al. (2008), Szalai et al. (2011), Li and Zhang (2012), and Moradtalab et al. (2018), while Peuke et al. (1994) reported ammonium-induced stimulation in root to shoot translocation of ABA in *Ricinus* seedlings Accordingly, the cold-protective effect of ammonium fertilization observed in this study may be related with a stimulatory effect on ABA accumulation in the shoot tissue, which promoted the expression of enzymatic (SOD, POD, **Figure 6**) and non-enzymatic (phenolics, **Table 3**) ROS detoxification. Similarly, ammonium-induced induction of ABA accumulation and a relationship with improved oxidative stress defense was reported also in response to other abiotic stress factors such as drought and salinity (Hessini et al., 2013; Ding et al., 2016).

In the root tissue of cold-stressed plants, ammonium fertilization significantly increased the IAA concentration by 41% as compared with nitrate supply (**Figure 7**), with a similar trend for shoot IAA, which was not detectable in the absence of cold stress. In our study, this was related to a significantly increased expression of the *ZmPIN1a* gene (**Figure 6A**), encoding an auxin efflux carrier with functions in the lateral root formation in maize (Li et al., 2018). Increased gene expression was recorded also for the auxin response factor 12 (*ZmAFR12*) involved in IAA perception (**Figure 6A**) and upregulated in cold-stressed maize plants (Sobkowiak et al., 2014). Thus, a trend for increased root length development in the ammonium-treated plants was recorded in both experiments, although this difference was not statistically significant (**Figure 3**). Excessive production of ROS can promote the oxidative degradation of IAA. and resulted in a 50% reduction of IAA contents in Zn-deficient *Phaseolus vulgaris*, which was reverted by Zn fertilization (Cakmak et al., 1989), promoting enzymatic ROS detoxification (Cakmak, 2000). Similarly, a ROS-protective effect of higher SOD activities recorded in the root tissue of ammonium-treated plants (**Figure 5**) may counteract oxidative IAA degradation and provide an explanation for greater root IAA levels in cold-affected maize over nitrate-treated plants (**Figure 7**).

As an additional beneficial effect of ammonium fertilization, root colonization by the CombiA-PGPM strain *Trichoderma harzianum* OMG16 was increased in comparison with plants supplied with nitrate fertilizer (**Figure 4**). The reasons for this preference are not entirely clear but recently Mpanga et al. (2019a) found ammonium-induced promotion of root hair development in P-deficient maize plants, also identified as limiting nutrients in this study (**Supplementary Table S2**). This may provide additional infection sites, since preferential colonization of root hairs has been reported for various strains of *Trichoderma harzianum*, including T22 and OMG16 (Harman, 2000; Mpanga et al., 2019a). Additionally, the various cold stress-protective effects, induced by ammonium fertilization as described above, may improve the rhizosphere establishment of the inoculants by strengthening the host plant. Similarly, improved performance of a wide range of bacterial and fungal PGPMs, including single strain inoculants and microbial consortia in combination with stabilized ammonium fertilization, has been documented in various pot and field experiments under conditions of P limitation (Bradáčová et al., 2019a,b; Mpanga et al., 2019a,b).

Cold-Protective Effects of the CombiA Inoculation as Related to Zn and Mn Supplementation

For both forms of nitrogen fertilization, the inoculation with CombiA induced cold-protective effects in maize plants, which were still detectable after a two-weeks recovery period at soil temperatures ≥20°C. This may indicate not only direct stress mitigation, as indicated e.g., by reduced oxidative leaf damage recorded at the end of the 2-weeks cold stress period (**Table 2** and **Figure 3**) but also the induction of longer-lasting stress priming effects.

The most intense expression of cold protection in terms of increased shoot biomass production, reduced oxidative leaf damage, and stimulation of root growth, was recorded for the ammonium-CombiA^{+} combination (**Table 2** and **Figures 2**, **3**). The effects were detectable in both experiments conducted under controlled root zone temperatures (RZT), although shoot biomass production was different, probably due to differences in ambient air temperature at the greenhouse (**Supplementary Figure S1**).

The two-weeks cold stress treatments with 8–14°C RZT reduced the shoot dry matter production by 25–52% when nitrate was the N source (**Table 2** and **Figure 3**). This was associated with a significant decline in the shoot concentrations of the growth hormones IAA, GA, and CK (Zeatin) by 48, 41 and 49%, respectively (**Figure 6**), as previously reported also by Moradtalab et al. (2018). Reduction of shoot growth is regarded as a component of cold stress adaptations, which is actively regulated by a reduction of bioactive growth-promoting gibberellic acid (GA) levels, leading to an increased abundance of nuclear DELLA-protein growth repressors via a signaling pathway involving CBF/DREB1 transcription factors (Miura and Furumoto, 2013; Eremina et al., 2016). However, the decline of GA and IAA in cold stressed plants have been also related to cold-induced Zn-limitation (Moradtalab et al., 2018) since reduced GA and IAA levels are characteristic for Zn-deficient plants (Suge et al., 1986; Sekimoto et al., 1997; Cakmak et al., 1989). More recently, it was shown that various steps of GA biosynthesis depend on the presence of IAA (Ross et al., 2000) and Zn limitation promotes oxidative IAA degradation and impairs auxin transport (Cakmak et al., 1989; Shibasaki et al., 2009).

Interestingly, two weeks after recovery from the cold stress treatment, CombiA inoculation particularly in combination with ammonium fertilization, restored the concentrations of IAA, GA, and CK to the levels characteristic for non-stressed plants (**Table 4**). This was associated with the lowest level of oxidative leaf damage, increased shoot biomass production (**Table 2**, **Figures 2**, **3**), increased enzymatic (POD, SOD) and non-enzymatic (total antioxidants, phenolics, proline) ROS defense and accumulation of cryoprotectants (**Table 4** and

TABLE 4 | The ratio of abscisic acid/cytokinin (ABA/CK) and auxin/cytokinin (IAA/CK) concentrations in the root of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14°C) on silty clay loam soil, pH 6.9.

	8–14°C					
	Nitrate			Ammonium		
Ratio	Ctrl	Combi A$^-$	Combi A$^+$	Ctrl	Combi A$^-$	Combi A$^+$
ABA/CK	2.18 c	3.33 b	3.81 b	2.04 c	4.14 a	3.96 a
IAA/CK	0.21 c	0.42 b	0.53 b	0.37 c	0.91 a	0.96 a

Low RZT variants including untreated control (Ctrl), Combi A$^-$ (without Zn/Mn) and Combi A$^+$ (containing Zn/Mn) under nitrate or ammonium fertilization. Means of five replicates. In each row different letters: significant differences (Tukey-Test, $p < 0.05$).

TABLE 5 | Relative changes (%) of phenotypic and physiological responses in maize plants induced by stabilized ammonium fertilization (Ctrl ammonium), ammonium fertilization + PGPM inoculation (Ammonium + CombiA$^-$) and ammonium fertilization + PGPM inoculation + Zn/Mn supplementation (Ammonium + CombiA$^+$) after recovery (14 days) from two weeks exposure to low root zone temperatures (8–14°C) over plants supplied with N in the nitrate form.

Tissue	Factor	Ctrl (Ammonium)	Ammonium + Combi A$^-$	Ammonium + Combi A$^+$
Root	Length	n.s	+101	+161
	PGPM Colonization	n.s	+140	+143
	ROS Defense			
	Antioxidants	n.s	+178	+239
	SOD	+20	+22	+42
	Hormonal Effects			
	IAA	+75	+112	+131
	CK	n.s	−51	−50
	ABA	n.s	n.s	n.s
	SA	n.s	+123	+162
	ZmPIN1	+33	+167	+167
	ZmARF12	+32	+137	+137
	ZmIAA5	n.s	n.s	n.s
	ZmTSA	n.s	+67	+78
	ZmIPT4	n.s	−32	−35
	ZmIPT5	n.s	−56	−58
	ZmABF2	+28	+76	+94
Shoot	Biomass	n.s	n.s	+48
	Oxidative leaf damage	−27	−35	−42
	ROS Defense			
	SOD	+25	+52	+56
	POD	+25	+59	+64
	Phenolics	+26	+41	+44
	Antioxidants	n.s	+45	+47
	Cryoprotectants			
	Proline	n.s	+100	+80
	Sugar	n.s	+65	+57
	Hormonal Effects			
	IAA	n.s	+76	+106
	GA	n.s	+63	+78
	CK	n.s	+141	+153
	ABA	+33	+40	+43
	JA	+55	+208	+231
	SA	+38	+195	+211

SOD: Superoxide dismutase, POD: Peroxidase, IAA: Auxin, CK: Cytokinin, GA: Gibberellic acid, ABA: Abscisic acid, JA: Jasmonic acid, SA: Salicylic acid, PIN1: PINFORMED1, ARF12: Auxin response factor12, IAA5: Aux/IAA-transcription factor5, TSA: tryptophane synthase, IPT4,5: Isopentenyl transferases4,5, ABF2: Abscisic acid-binding factor2, n.s: not significant.

Figure 5), indicating an improved recovery from the cold stress treatment. Strengthening of ROS detoxification in the shoot tissue was not related to Zn and Mn supplementation by the CombiA$^+$ treatment since the same effect was observed also for CombiA$^-$ inoculation without additional micronutrient supply (**Tables 4**, **5**). In this case, the improved micronutrient supply, induced by ammonium fertilization (**Table 2**), was already sufficient to cover the requirements of the systems for ROS detoxification.

Also increased ABA production with the potential to trigger ROS defense was not detectable in CombiA treatments but was characteristic for sole ammonium supply (**Figure 6**). By contrast, CombiA inoculation was related to increased shoot accumulation of JA and SA (**Figure 6**). This points to induction of induced systemic resistance (ISR) via JA and SA signaling pathways, which is well documented for various *Trichoderma* and *Bacillus* strains, with stimulatory effects e.g., on the accumulation of phenolics and POD activity (García-Gutiérrez et al., 2013; Martínez-Medina et al., 2013, 2014, Shahzad et al., 2017). Unfortunately with the currently available data set, it is not possible to unfold the individual contributions of the selected *Trichoderma* and *Bacillus* inoculants to the ISR effect. This would require additional experiments with single-strain inoculations. Although abscisic acid is considered as a central regulator of cold stress responses in plants, it seems to regulate the adaptive expression of cold- related genes in cross talks involving also SA and JA (Szalai et al., 2011; Eremina et al., 2016). This may also explain the improved cold acclimation by CombiA inoculation via ISR-induced production of JA and SA (**Table 5**).

The only superior cold-protective feature related to the increased Zn/Mn supply provided by CombiA$^+$ in combination with ammonium fertilization recorded in this study was the increased accumulation of antioxidants in the root tissue (**Table 3**), which promoted root elongation (**Figures 2**, **3**). Since at the same time, root biomass production was not significantly affected, obviously fine root production was stimulated, characterized by a higher root length with the same root biomass after CombiA$^+$ application as compared to the CombiA$^-$ variant (**Figures 2**, **3**). This may indicate a protective effect against oxidative auxin degradation leading to root growth inhibition (Cakmak et al., 1989, Moradtalab et al., 2018). Generally, CombiA inoculation increased IAA concentrations not only in the shoot but also in the root tissue, associated with a decline in root CK (**Figure 5**). This was related to increased expression of auxin-responsive genes involved in IAA biosynthesis (*ZmTSA*), transport (*ZmPIN1A*) and IAA signal perception (*ZmARF12*), while the expression of genes involved in CK biosynthesis (*ZmIPT4* and *5*) declined (**Figure 6A**). By contrast, the expression of the *AuxIAA5* gene (*ZmIAA5*), reported to be rapidly activated by exogenous IAA

supply (Park and Hasenstein, 2016), was not changed by CombiA inoculation. This finding suggests that CombiA rather acted via signals interacting with internal IAA homeostasis of the host plant and not via microbial IAA production. Accordingly, Garnica-Vergara et al. (2015) found an auxin-independent activation of *PIN* genes (*PIN1, PIN2, PIN3, PIN7*), associated with increased lateral root formation in *Arabidopsis thaliana* by 6-pentyl-2H-pyran-2-one (6-PP), a major bioactive volatile organic compound (VOC) with potential cross-kingdom signaling functions, emitted by *Trichoderma* spp. Interestingly, increased levels of IAA and declining CK concentrations in the root tissue in response to *Trichoderma* inoculation detected in our study, have been observed also in earlier reports with melon seedlings and cherry rootstocks (Sofo et al., 2011; Martínez-Medina et al., 2014). This strongly suggests that the corresponding hormonal changes induced by CombiA inoculation (**Figures 5**, **6**) are likely caused by the *T. harzianum* OMG16 strain in the inoculum, associated with preferential root colonization in combination with ammonium fertilization (**Figure 4**). Similar effects on root growth and plant IAA homeostasis have been reported also for certain N-acyl homoserine lactones secreted by various rhizosphere bacteria for intercellular communication (quorum sensing; Hartmann et al., 2014).

Auxin production is considered as a central feature of *Bacillus* strains leading to root growth promotion via external supplementation of IAA by the inoculant (Borriss, 2015). Hence, IAA production has been reported for many *Bacillus* species, such as *B. subtilis* (Hashem et al., 2019), *B. megaterium* (Marulanda et al., 2009), *B. licheniformis* (Singh and Jha, 2016) and *B. velezensis* (Mpanga et al., 2019a), where IAA production was even stimulated in the presence of ammonium fertilizers. On the other hand, inactivation of genes responsible for bacterial tryptophan synthesis inhibited IAA formation and plant growth promotion (Idris et al., 2007). However, in our study, no upregulation of the *ZmIAA5* gene, activated by external IAA supplying was detectable (**Figure 8A**), suggesting that external IAA supplementation by the bacterial inoculants was not involved in the observed root growth increase, at least under the investigated cold stress conditions.

Declining CK concentrations induced by CombiA inoculation also had important consequences for the hormonal balances in the root tissue, known to be even more important for the hormonal regulation of physiological processes than the absolute concentrations of individual phytohormones (Nordström et al., 2004; Mueller and Leyser, 2011). The decline in root CK increased the IAA/CK ratio by factor 3 and doubled the ABA/CK ratio in plants with CombiA inoculation and supplying of N as ammonium (**Table 4**).

Since CKs are acting as potent hormonal antagonists of IAA, e.g., by inhibiting polar IAA transport mediated by PIN transporters (Fukaki and Tasaka, 2009), the increased IAA/CK ratio in CombiA treated plants (**Table 4**), may result in an improved shoot to root allocation of IAA, increased IAA activity with subsequent stimulation of root growth (**Figures 2**, **3**). Similarly, antagonistic effects of CK on ABA-mediated responses have been reported (Wilkinson et al., 2012; Pavlů et al., 2018). Therefore, the increased ABA/CK ratio may promote ABA-induced induction of cold-adaptations in the root tissue, although endogenous ABA concentrations were not changed (**Figure 8**), as similarly reported also for cold acclimation in durum wheat (Veselova et al., 2005). This is in line with increased gene expression of the ABA response factor *ZmABF2* particularly in the root tissue of CombiA^{+}-inoculated plants with ammonium supply (**Figure 6B**), known to be upregulated in cold-stressed maize plants (Sobkowiak et al., 2014).

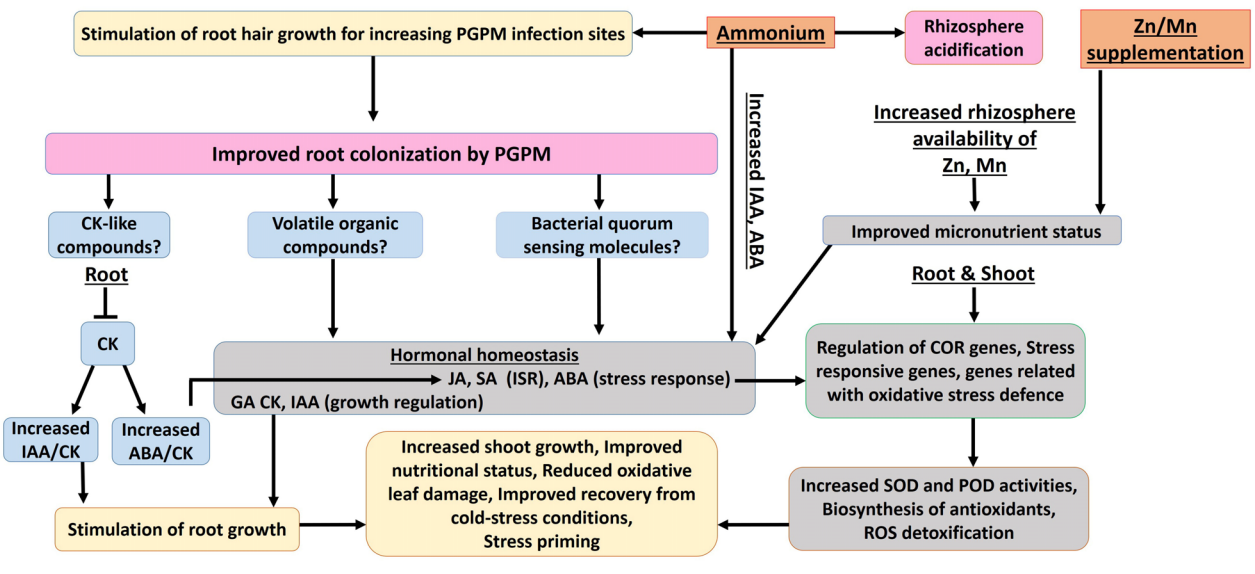


FIGURE 9 | Proposed interactions of stabilized ammonium fertilization, PGPM (CombiA) inoculation and Zn/Mn supplementation contributing to increased cold tolerance during the early growth of maize (for description of details see section 6.2 and 6.3).

Contribution of N Forms, Zn, Mn, and Microbial Inoculants to the Cold-Protective Maize Response

In summary, the results of the present study indicate a differential activation and stimulation of adaptive cold-stress responses, induced by the selected fertilization strategies. The various complementary and synergistic effects of ammonium fertilization, CombiA inoculation and ZnMn supplementation on cold stress adaptations in maize are schematically summarized in **Figure 9**, while **Table 4** provides an overview of the relative importance of the selected mitigation strategies (ammonium fertilization, PGPM inoculation, Zn/Mn supplementation) for the expression of cold-protective effects.

Effects of Ammonium Fertilization

In accordance with the hypothesis (i), ammonium-dominated fertilization stimulated rhizosphere acidification, which improved the availability and the nutritional status of critical nutrients such as Zn and Mn on the investigated soil with neutral pH (**Figure 9**), although the effect on P availability was marginal. The improved Zn and Mn-nutritional status, with important functions in oxidative stress defense, moderately increased the enzymatic and non-enzymatic ROS detoxification, counteracted oxidative IAA degradation and oxidative leaf damage (**Table 5**). Ammonium fertilization was also the major factor contributing to increased ABA concentrations in the shoot tissue (**Table 5**), as a central regulator of adaptive cold stress responses and stimulated root colonization with the PGPM inoculant CombiA (**Figure 9**).

Effects of PGPM (CombiA) Inoculation

Root growth promotion by stimulation of IAA biosynthesis and reduction of antagonistic cytokinins in the root tissue of the host plant, was a major feature provided by PGPM inoculation with CombiA. Additionally, PGPM inoculation was associated with typical responses of ISR signaling via induction of jasmonic and salicylic acid accumulation even in the shoot tissue and an increase in the ABA/cytokinin ratio in roots. This was related with a further increase in enzymatic (SOD, POD) and non-enzymatic (antioxidants, phenolics, proline) ROS detoxification expressed mainly in the shoot tissue, and consequently a further decline of oxidative leaf damage. The observed effects are in line with the assumptions of the initial hypothesis (iii).

Effects of Zn/Mn Supplementation

Partially in line with the hypothesis (ii), the additional supplementation with Zn and Mn mainly contributed to an additional increase of antioxidants and SOD activity in the root tissue. This was associated with increased IAA accumulation, reflecting a reduction of oxidative IAA degradation, which is typically induced under Zn deficiency related with high soil pH and impairment of root activity under cold stress. In consequence, further stimulation of root growth contributed to improved nutrient (P) acquisition, a generally improved plant nutritional status, improved plant performance and induced longer-lasting stress priming effects, still detectable two weeks after recovery from the cold stress treatments.

CONCLUSION

The combined use of N as ammonium, Mn, Zn and the Trichoderma/Bacillus inoculant is a suitable strategy to improve the tolerance of maize plants in the early growth stage to cold-stress conditions. This approach could be easily integrated into existing strategies for starter fertilization of maize production systems, such as seedbed fertilization with stabilized ammonium phosphates and micronutrient supplementation in combination with granulated spore formulations of the Trichoderma/Bacillus inoculant. Field performance of the agronomic practice proposed needs further evaluation in field trials, mirroring the already demonstrated effectiveness of single applications of micronutrients and silicon to improve the growth of maize plants (Imran et al., 2013; Moradtalab et al., 2018). Due to overlapping, adaptive plant responses to several abiotic stress factors, and additional biocontrol properties of the inoculants, even a wider spectrum of stress-protective effects might be expected.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NM and GN conceived and designed the experiments. NM and AA conducted the experiments, performed the analyses, and collected the data. GN, UL, FW, BH, and JG provided the facilities for analyses. GN, UL, JG, and FW read and edited the manuscript. All authors approved the final manuscript. NM and AA equally contributed to manuscript writing.

FUNDING

This work was supported by the European's Seventh Framework Programme (FP/2007–2013) under Grant Agreement no. 312117 and Di-Control project, no. 031B0514A under BonaRes Centre. Funded by the Federal Ministry of Education and Research (BMBF).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00396/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

MINI REVIEW
published: 12 May 2020
doi: 10.3389/fpls.2020.00426

From Lab to Field: Role of Humic Substances Under Open-Field and Greenhouse Conditions as Biostimulant and Biocontrol Agent

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The demand for biostimulants has been growing at an annual rate of 10 and 12.4% in Europe and Northern America, respectively. The beneficial effects of humic substances (HS) as biostimulants of plant growth have been well-known since the 1980s, and they can be supportive to a circular economy if they are extracted from different renewable resources of organic matter including harvest residues, wastewater, sewage sludge, and manure. This paper presents an overview of the scientific outputs on application methods of HS in different conditions. Firstly, the functionality of HS in the primary and secondary metabolism under stressed and non-stressed cropping conditions is discussed along with crop protection against pathogens. Secondly, the advantages and limitations of five different types of HS application under open-fields and greenhouse conditions are described. Key factors, such as the chemical structure of HS, application method, optimal rate, and field circumstances, play a crucial role in enhancing plant growth by HS treatment as a biostimulant. If we can get a better grip on these factors, HS has the potential to become a part of circular agriculture.

Keywords: humic acid, fulvic acid, foliar application, fertigation, circular economy, sustainable agriculture

OPEN ACCESS

Edited by:
Andrew Merchant, University of Sydney, Australia

Reviewed by:
Jose M. Garcia-Mina, University of Navarra, Spain
Barbara De Lucia, University of Bari Aldo Moro, Italy

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *28 January 2020*
Accepted: *24 March 2020*
Published: *12 May 2020*

Citation:
Jindo K, Olivares FL, Malcher DJP, Sánchez-Monedero MA, Kempenaar C and Canellas LP (2020) From Lab to Field: Role of Humic Substances Under Open-Field and Greenhouse Conditions as Biostimulant and Biocontrol Agent. Front. Plant Sci. 11:426. doi: 10.3389/fpls.2020.00426

INTRODUCTION

The function and application of biostimulants and biopesticides have garnered considerable interest due to their potential as environmentally sustainable resources for agricultural production. A number of national and international projects on biostimulant material have been launched in the framework of the circular economy by extracting the beneficial material from waste materials across different sectors of agriculture, livestock, water infrastructure, mining, and energy (Xu and Geelen, 2018). Notably, the projects BIO-FERTIL (Poland), BIOFECTOR (Germany), and HUMIC-XL (Netherlands) have highlighted the potential use of humic substances (HS) from waste material for plant growth, which can be a component of a local circular economy. To provide scientific evidences of the potential use of biostimulants, several reviews have been published recently

(Calvo et al., 2014; du Jardin, 2015; Van Oosten et al., 2017; Abbott et al., 2018; Bulgari et al., 2019; Juárez-Maldonado et al., 2019; Pylak et al., 2019). In general, HS, seaweed extracts, beneficial microorganisms, and chitosan and protein hydrolases are listed in the mentioned review papers. While the chitosan and protein hydrolases are becoming popular as a biostimulant in the last decade (Drobek et al., 2019), utilization of HS, composed of humic (HA) and fulvic acid (FA), has been recognized as a long-run product since the 1980s (Calvo et al., 2014). The underlying function of HS as biological activation for plant growth has been strongly related to the chemical composition (e.g., functional groups), hydrophobicity, and flexible conformational structure of HS (Muscolo et al., 2013; Canellas and Olivares, 2017). Whereas a large number of scientific publications are related to the impact of HS in hydroponic assays and under growth chamber conditions (Nardi et al., 2000, 2018; Russell et al., 2006), reports on its potentiality in the field and under greenhouse conditions are less explored, mainly due to the variety of underlying factors in crop fields, including weather variability and climate fluctuations, soil type, and field management. For all these reasons, review reports on the practical application of HS in fields are scarce (Rose et al., 2014; Canellas et al., 2015b). The main focus of the present work is to (1) describe the mechanisms of the HS effect on plant growth, and (2) to illustrate the HS utilization under open-field and greenhouse conditions.

KEY BENEFITS OF HS ON PLANT GROWTH

One of the major impacts of HS on plant growth is the reinforcement in nutrient uptake and the elongation of the lateral root growth, often recognized as "auxin-like effect," which is a result of the induction of ATPase activity in the plasma membrane (Maggioni et al., 1987; Nardi et al., 1991; Pinton et al., 1992; Canellas et al., 2002; Quaggiotti et al., 2004; Zandonadi et al., 2007). The underlying mechanisms are generating a wider electrochemical gradient by ATPase induction and accelerating the nutrient uptake rate, which can also be confirmed by the overexpression of the transporter genes (Jindo et al., 2016; Zanin et al., 2018; Nunes et al., 2019). The availability of micronutrients such as iron can be improved with HSs, not only by chelation but also by promoting the root capability to uptake nutrients from the soil solution (Aguirre et al., 2009; Zanin et al., 2019).

Understanding the underlying mechanisms of plant response is a noteworthy keystone for the HS use in the field, and the first step would be a better understanding of the effect of HS on carbon and nitrogen cycles, which is related to primary metabolism (Canellas and Olivares, 2014; Olk et al., 2018; Canellas et al., 2019). HS also interferes with secondary metabolism by altering gene expression and changing the content of chemical compounds in plant cells, such as those related to the Krebs cycle, metabolism of nitrate and phosphorus, glycolysis, and photosynthesis (Roomi et al., 2018; Lotfi et al., 2018).

Historically, from the 1980s until the end of the 1990s, studies on the effect of HS on photosynthesis and ATP production were the major topics of research. A critical view of these works can be found in the previous papers of Nardi et al. (2002, 2009). Trevisan et al. (2011) found a high level of transcription of genes involved in primary metabolism in *Arabidopsis thaliana* and supported previous studies about the physiological effects of HS on plant metabolic pathways. Nardi et al. (2007) evaluated the impact of different HS on the enzymatic activities involved in glycolytic and respiratory processes of maize seedlings including glucokinase, phosphoglucose isomerase, PPi-dependent phosphofructokinase, and pyruvate kinase, as well as the activity of citrate synthase, malate dehydrogenase, and isocitrate $NADP^+$ -isocitrate dehydrogenase. In the proteomic analysis conducted by Nunes et al. (2019), differences were detected in the maize seedling root proteins related to energy metabolism, cytoskeleton, cellular transport, conformation and degradation of proteins, and DNA replication. Thirty-four proteins were significantly more abundant in the seedlings treated with HA, whereas only nine proteins were abundant in the control. The main effect of HA was protective, mainly associated with increased expression of 2-cys peroxidase, putative VHS/GAT, and glutathione proteins (Nunes et al., 2019).

The transcriptome and proteome are more abundantly reported than metabolomics studies. The plant metabolome is the entirety of the small molecules present in the plant and can be regarded as the ultimate expression of its genotype in response to environmental changes (Fiehn, 2002). Aguiar et al. (2018) observed that the application of HA on sugarcane significantly decreased the concentration of 15 metabolites, which generally included amino acids. HA increased the levels of 40 compounds, which are associated with the stress response (shikimic, caffeic, hydroxycinnamic acids, putrescine, behenic acid, quinoline xylulose, galactose, lactose proline, oxyproline, and valeric acid), and this is aligned with up-regulation of the protein involved in redox homeostasis (Roomi et al., 2018).

Plant secondary metabolism produces a large number of specialized compounds that do not directly aid in the growth and development of plants but are required for the plant to survive in its environment and under biotic and abiotic stress. Salinity and drought are the most frequent stresses studied in fields and under greenhouse conditions (Ali et al., 2020). Several reports have been published on the impact of HS on the growth of pepper, common beans, rice, tomato, corn, sorghum, and cucumber under these stress conditions (Demir et al., 1999; García et al., 2012; Berbara and García, 2014; Rose et al., 2014; Prado et al., 2016; Van Oosten et al., 2017; Bulgari et al., 2019; Pinos et al., 2019; Ali et al., 2020). One of the underlying mechanisms of the impact of the HS is the interaction with auxin, jasmonic acid and abscisic acid by phytohormonal regulation in the root, which are well-known plant hormones for the stress of drought and salinity (De Hita et al., 2019; Ali et al., 2020). Another example is the synthesis of flavonoids, which are involved in the interception of ultraviolet (UV) as an adaptive mechanism preventing UV in plant physiology (Hollósy, 2002). HA could induce the activity of the first enzyme in the phenylpropanoid pathway at the level of gene expression, similarly to other studies in which phenylpropanoid synthesis has been enhanced by fungal elicitors and hormones (Schiavon et al., 2010; Lewis et al., 2011).

The increase in phenolic compounds is another typical plant response to HA treatment (Ertani et al., 2011). During the progress of the domestication of cultivated plants over 10,000 years, the bitter and astringent taste from phenolic compounds, which often produced in the phenylpropanoid pathway of the secondary metabolism, has been gradually eliminated, resulting in the reduction of the natural plant protection against stress (Wink, 1988). The foliar application of HA improves this ancient mechanism reducing plant infection (Olivares et al., 2015) as well as enhancing plant protection (Hernandez et al., 2014).

Finally, HS is involved in the enhancement of plant protection against infestation. Joshi et al. (2014) present the list of pathogens and pests controlled through vermicompost application, highlighting that the main chemical components of the vermicompost belong to HS. There are four approaches by which HS can contribute to the plant defense mechanisms under field and greenhouse conditions: (1) enhancing the soil microbial activities that play as biological control agents, such as *Trichoderma* (McLean et al., 2012; Motta and Santana, 2013; Mohamadi et al., 2017); (2) direct interaction with plant pathogen (e.g., Nematodes, Late blight) (Zaller, 2006; Seenivasan and Senthilnathan, 2018; D'Addabbo et al., 2019; Liu et al., 2019); (3) physical protection for beneficial microbes, such as UV protection (Bitton et al., 1972; Muela et al., 2008; Kaiser et al., 2019); (4) enhancing plant antioxidant defense system against pathogen by modulating chemical compounds (e.g., phenols) and enzymes (e.g., phenylalanine ammonia-lyase) (Kesba and El-Beltagi, 2012; Olivares et al., 2015).

MODE OF APPLICATION IN FIELDS

The functions of HS for the enhancement of plant growth widely differ depending on the application mode, plant stage, and its rate, which will be discussed in the subsequent sections. Basically, there exist five application types of HS in the field (Erro et al., 2016; Ekin, 2019).

Direct Application in Soil (Liquid Status)

Researches and farmers adopt the direct use of HSs as an aqueous suspension. The effect of the direct application of liquid status has been demonstrated on the growth of different crops such as Lettuce (*Lactuva sativa*) and Grape rootstocks (*Vitis vinifera* L.) (**Supplementary Table S1**). Comparative advantages of liquid formulation include the possibility to combine with other inputs such as chemical fertilizer or beneficial microorganisms and adaptability to agricultural machinery for the implementation. Application time, depending on the plant development stage, must be considered.

Direct Application in Soil (Solid Status)

The solid-state application of HSs has been less explored for implementation in the field when compared with liquid formulations. The main agricultural applications of HSs in the form of powder or granules are soil amendments and organo-mineral fertilizers that require the highest dose per plot (**Supplementary Table S1**). The solid application brings a problem of uniform distribution of aqueous dispersion after dissolution on rhizosphere, gradient concentration, and re-sedimentation of HA on soil solution. Despite the difficulty of obtaining uniform HS aqueous suspension at the optimal doses, different rates of solid HS application had shown a direct positive effect on plant stimulation or soil physicochemical properties (**Supplementary Table S1**). Powder HA applied to soil at a rate of 75 g m^{-2} increased yield of thyme (*Thymus vulgaris* L.) and quality of essential oil (Noroozisharaf and Kaviani, 2018). In the same study, the highest dose of HA powder (100 g m^{-2}) improved nutrient content in leaves via positive modulation of nutrient transport through the chelation and stimulation of microbial activity by HS interaction. Undoubtedly, solid forms as powder or granules will be suitable in the future since the transport operation can be economically prohibitive for liquid HSs. However, a high volume of HS products is required for large-scale farming. Future research on the technology of on-farm solubilization of solid forms as stable final products will be demanded.

Foliar Application

Since the 1940s and 1950s, scientific research on the beneficial impact of foliar application has been explored (Tanou et al., 2017). There exist two theories to explain how exogenous inputs via foliar application are delivered to plant cell tissue, once they reached leaf surface: (1) transfer into leaf tissues via transcuticular penetration (Smilkova et al., 2019); or (2) penetration through leaf stomata (Tejada et al., 2016). Many authors report that micronutrient contents are increased by HS rather than macronutrient in field level (Fernández-Escobar et al., 1996; Çelik et al., 2011; Fatma et al., 2015; Balmori et al., 2019). After foliar treatment, nutritional parameters of polyphenol content and antioxidant activity to determine the quality of fruit are improved (Tarantino et al., 2018). In practice, liquid compost extracts, fully enriched with HS, represent a cost-effective tool to conduct foliar application (Zandonadi et al., 2013; Berbara and García, 2014). A wide range of plants have been tested with HS application under open-field conditions, such as garlic (Balmori et al., 2019), common beans (Kaya et al., 2005; Souri and Aslani, 2018), wheat (Zhang et al., 2016; Ahmad et al., 2018; Bezuglova et al., 2019), fenugreek (Ibrahim, 2019), tomato (Olivares et al., 2017), asparagus (Tejada and Gonzalez, 2003), maize (Canellas et al., 2015a) and citrus tree (Hameed et al., 2018). Foliar application is frequently reported in calcareous soil conditions where nutrient uptake, especially iron, is limited due to precipitation (Çelik et al., 2011; Souri and Aslani, 2018; Bezuglova et al., 2019). Foliar spray application is limited to suitable climate conditions, since high temperature and windy and rainy days are not recommended. High application rates provoke leaf burning as water evaporates and salts remain on the leaves, especially at high temperature (Fageria et al., 2009). The developing stage has to be considered since foliar application cannot be conducted after flowering in rice production, which could cause spikelet discoloration. Crop responses to foliar application are unlikely positive when there is nutrient deficiency in the soil (Fageria et al., 2009). Taking all together, the impact

of foliar-applied HS is less consistent than those observed when applied on the root, where HS is exposed to a more stable condition (De Hita et al., 2019).

Fertigation

Fertigation is extensively expanding over the world, especially in semi-arid and arid regions where water scarcity is an issue (Fallahi et al., 2017). García-Gaytán et al. (2018) widely describe the potential of different biostimulant materials used in fertigation. After the concentration of HS in rhizosphere increases by the irrigation, two contributions of HS to plant growth are presumably proposed: (1) straightening out soil fertility, which makes nutrient more available; (2) directly reaching out plant cell walls on the root surface so that plant can take up nutrients (Olaetxea et al., 2018). Regarding agronomic outcome in practice, Suman et al. (2016, 2017) showed the impact of the combined application of chemical fertilizer and HA in fertigation on productivity on capsicum and tomato under open-field conditions, concluding that HA could replace up to 20% of fertilizer. Selladurai and Purakayastha (2016) used a similar combination of liquid fertilizer by using the pedal-operated sprayer in soil in the open field, and they improved N, P, and K use efficiencies by 16.4, 9.3, and 18.3%, respectively. Water use also can be saved by the humic application (Selim and Mosa, 2012; Alenazi et al., 2016). The mode of fertigation has to be adjusted based on the type of crop. Selim et al. (2009) highlighted that subsurface drip irrigation method has a highly significant effect on potato tuber yield rather than surface drip irrigation, due to maintenance of optimum soil moisture content in the root zone in an Egyptian sandy soil. However, no effect was found in banana seedling with the drip irrigation with HS in tropical soil (de Melo et al., 2016), implying that crop and soil type have to be taken into account. A multiple-option of HS application, combining the use of solid HS at pre-sowing moment prior to fertigation with HS, can be useful to mitigate adverse environmental conditions (Smoleñ et al., 2017), or the use of wastewater for fertigation with HS incorporated into soil for saving water resources (Masciandaro et al., 2014).

Immersion

A limited number of works are reported on the seedling with the immersion method under field and greenhouse condition (Bettoni et al., 2016a,b; Gemin et al., 2019). This method is commonly used in hydroponic and growth chamber conditions (**Supplementary Table S1**).

BENEFITS AND LIMITATIONS OF HS APPLICATION IN THE FIELD

Proper implementation of HS in field conditions is an essential point for experiment design. Several works report a comparative study of different applications (**Supplementary Table S1**). Waqas et al. (2014) compared three application modes (foliar spray, soil application, and immersion) for mung bean. They concluded that no significant differences were observed across different applications. A similar result was reported by Karakurt et al. (2009) on pepper comparing between foliar spray and soil application. In contrast, other reports showed that foliar spray performed higher yield than soil application in tomato (Yildirim, 2007), maize (Tejada et al., 2016), almond (Saa et al., 2015), and sugarcane (Da Silva et al., 2017). An ideal implementation would be combined applications rather than a single application method, which was demonstrated in Bettoni et al. (2016b) with higher nutritional quality and yield of onion.

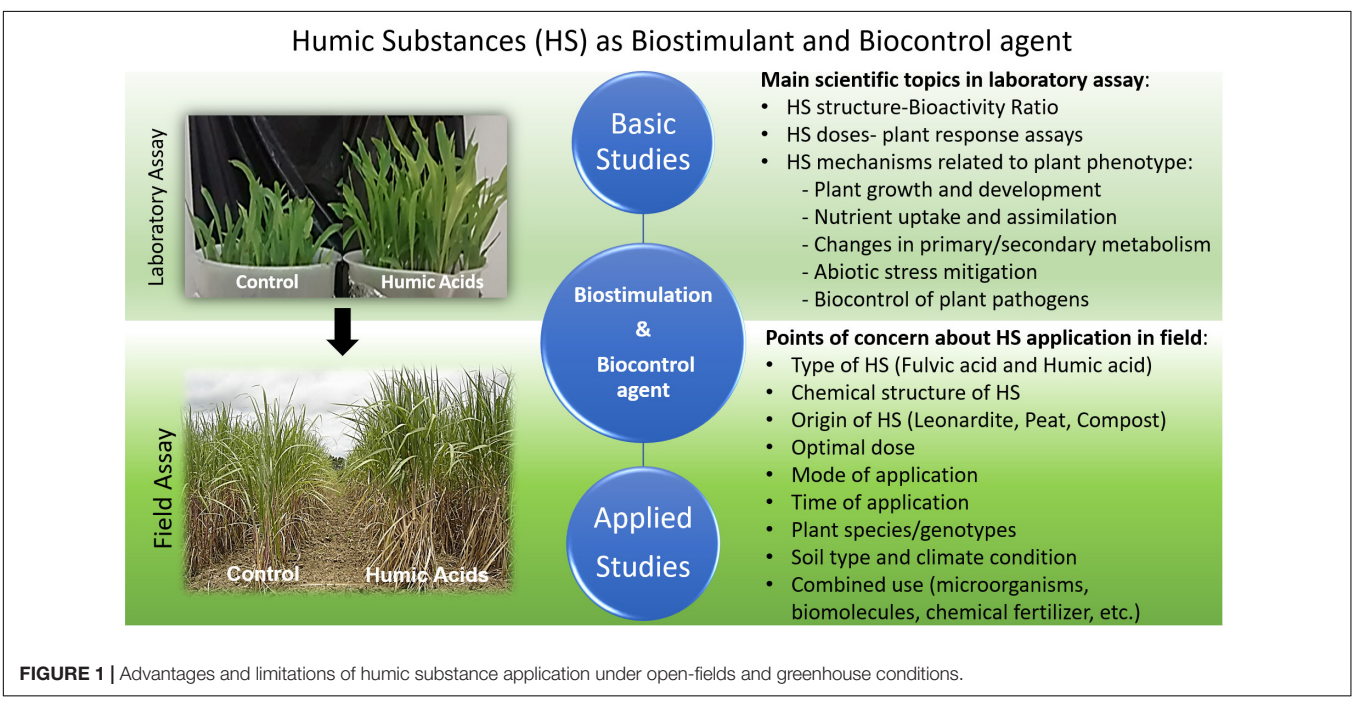


FIGURE 1 | Advantages and limitations of humic substance application under open-fields and greenhouse conditions.

It is noteworthy that the positive effect of HS application on plant growth is not always guaranteed. The points of concern about the HS application are listed in **Figure 1**. In particular, the chemical structure of HS, optimum application rate, and the mode of use play a crucial role in performing a visible outcome on the ground. At first, finding out an optimal dosage is an essential process, and this is changeable with application mode and plant type. Some specific plants such as lettuce (*L. sativa*) from *Asteraceae* family and *Arabdopsis thaliana* from *Brassicacea* family are more sensitive to the change in the concentration of HS and application mode (Rodda et al., 2006; Dobbss et al., 2007; Hernandez et al., 2013). Secondary, the type of HS is a vital point, which is related to the chemical structure and molecular size of HS. The interaction between the chemical composition of HSs and bioactivity was studied (Canellas et al., 2009; Aguiar et al., 2013; Martinez-Balmori et al., 2014) and the importance of hydrophobic/hydrophilic ratio is a key factor as a suitable indicator to predict bioactivity based on their chemical properties. This ratio is prominently high in HA rather than FA due to the enrichment of the aromatic carbon group. Also, similar or even better crop responses have been achieved by HSs derived from compost rather than from leonardite, peat, or other pedogenic stable organic matter reservoirs (Ayuso et al., 1996). Another factor is the chemical variation due to different extraction techniques and nutrient enrichment processes (Hartz and Bottoms, 2010). In line with this study, Chen et al. (2004) concluded that soil application of commercial humic products at typical rates (2 to 3 kg ha^{-1}) is ineffective in promoting significant agronomical response to different crops under an open-field condition.

Furthermore, Chen et al. (2004) highlighted that the recommended dose for commercial HS product is at least 10 times smaller than required to stimulate plant growth under laboratory and greenhouse assays (75 mg L^{-1}, equivalent to 50 kg ha^{-1}). Regarding soil types, Pylak et al. (2019) report that HSs are not particularly effective in reducing the solubility of heavy metals in acidic soils. Using commercial HA products in combination with liquid fertilizers, Hartz and Bottoms (2010) mentioned that a positive crop response was found only in soil with low organic matter content. Also, suitable application time is a concerned issue. While the use of HS at the early developing stage usually enhances the root elongation, sugar content, grain weight, and fruit size increase at a late vegetative stage (Canellas et al., 2015b).

CONCLUSION

HS application originally from wastes as a biostimulant for plant growth is a beneficial and eco-friendly approach, and it fits into the concept of circular economy focusing on the conversion to a new resource. Plant anatomical and biochemical changes in the root system by HS are the main factors responsible for increased nutrient uptake, although the increase in the nutrient availability through chelation is another HS contribution to plant growth. The hydrophobicity/hydrophilic ratio is a useful indicator to understand the chemical structure of HS and to estimate the effect on plant growth. Although different dose ranges of HS application in field and laboratory condition are suitable, it is recommendable to conduct a preliminary test to find an optimum rate considering crop type, soil properties, and application mode.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception, design, data collection, analyses, and manuscript preparation. KJ, FO, DM, LC, wrote the article. KJ, FO, MS-M, CK, and LC supervised and completed the writing. All authors read and approved the final manuscript.

FUNDING

KJ wishes to acknowledge financial support (3710473400-1). The authors FO, DM, and LC were supported by Fundação Carlos Chagas Filho de Amparo á Pesquisa do Estado do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Conselho Nacional de Desenvolvimento de Pesquisa e Tecnologia (CNPq), Newton Foundation and FINEP Pluricana Project.

ACKNOWLEDGMENTS

We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00426/full#supplementary-material

TABLE S1 | Effect of humic substance with different application modes on plant growth under different experimental conditions: Field trial (FD); Growth chamber (GC); Greenhouse (GH) Hydroponic (HP).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 25 May 2020
doi: 10.3389/fpls.2020.00648

Diving Into Reef Ecosystems for Land-Agriculture Solutions: Coral Microbiota Can Alleviate Salt Stress During Germination and Photosynthesis in Terrestrial Plants

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From their chemical nature to their ecological interactions, coral reef ecosystems have a lot in common with highly productive terrestrial ecosystems. While plants are responsible for primary production in the terrestrial sphere, the photosynthetic endosymbionts of corals are the key producers in reef communities. As in plants, coral microbiota have been suggested to stimulate the growth and physiological performance of the photosynthetic endosymbionts that provide energy sources to the coral. Among them, actinobacteria are some of the most probable candidates. To explore the potential of coral actinobacteria as plant biostimulants, we have analyzed the activity of *Salinispora* strains isolated from the corals *Porites lobata* and *Porites panamensis,* which were identified as *Salinispora arenicola* by 16S rRNA sequencing. We evaluated the effects of this microorganism on the germination, plant growth, and photosynthetic response of wild tobacco (*Nicotiana attenuata*) under a saline regime. We identified protective activity of this actinobacteria on seed germination and photosynthetic performance under natural light conditions. Further insights into the possible mechanism showed an endophytic-like symbiosis between *N. attenuata* roots and *S. arenicola* and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity by *S. arenicola.* We discuss these findings in the context of relevant ecological and physiological responses and biotechnological potential. Overall, our results will contribute to the development of novel biotechnologies to cope with plant growth under saline stress. Our study highlights the importance of understanding marine ecological interactions for the development of novel, strategic, and sustainable agricultural solutions.

Keywords: coral-bacteria interactions, actinobacteria, plant biostimulants, *Salinispora* symbiont, agricultural solutions, plant growth, saline stress

OPEN ACCESS

Edited by:
Andrés Calderín García, Universidade Federal Rural do Rio de Janeiro, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *31 January 2020*
Accepted: *27 April 2020*
Published: *25 May 2020*

Citation:
Ocampo-Alvarez H, Meza-Canales ID, Mateos-Salmón C, Rios-Jara E, Rodríguez-Zaragoza FA, Robles-Murguía C, Muñoz-Urias A, Hernández-Herrera RM, Choix-Ley FJ and Becerril-Espinosa A (2020) Diving Into Reef Ecosystems for Land-Agriculture Solutions: Coral Microbiota Can Alleviate Salt Stress During Germination and Photosynthesis in Terrestrial Plants. Front. Plant Sci. 11:648. doi: 10.3389/fpls.2020.00648

INTRODUCTION

With the expectation that future changes in demographic and climatic scenarios will put additional pressure on the environment and agricultural production worldwide, the need to develop sustainable high-quality and high-yield crop alternatives is more important now than ever before (Yamaguchi and Blumwald, 2005). Projections of future environmental changes have created agricultural challenges characterized by increased biotic and abiotic stress (Meena et al., 2017). In particular, soil salinity has become especially important in recent years and is considered to be one of the most pressing abiotic factors threatening agricultural production (Isayenkov and Maathuis, 2019). One promising initiative for a new model of environmentally friendly agriculture is based on converting the natural processes that occur in soil-plant systems into biotechnologies that enhance crop production (Sahu et al., 2018). In this context, a great deal of research has focused on understanding the relationships among plants and soil-borne symbiotic microorganisms that increase the ecological success of plants in their natural habitats (Compant et al., 2019; Kumar and Meena, 2019). Given the ecological advantages offered by these microorganisms and their bioactive compounds, they have been employed as biostimulants to improve growth and yield for multiple plant species (Yakhin et al., 2017).

The most common living biostimulants are microorganisms that either colonize the rhizosphere or live within plant tissues (i.e., endophytic microorganisms) and form close mutualistic relationships. Plants benefit from these microorganisms through multiple mechanisms, such as the provision of growth phytohormones, increased mineral solubilization through pH regulation, molecular nitrogen fixation, and the induction of defense and resistance responses to both biotic and abiotic pressures (Smith et al., 2015; Lata et al., 2018). For instance, saline environmental resistance has been shown to occur due to the activity of the plant-associated bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This deaminase prevents ethylene biosynthesis and thus prevents stress-related ethylene-induced responses from affecting plant growth (Singh et al., 2015). Reciprocally, plants share photosynthetically fixed carbon to maintain a community of beneficial microorganisms. As plants are a secure source of photosynthates, any non-photosynthetic organism capable of accessing plant energy in a stable mutualistic interaction is thus conferred an advantage by its associated plant (Smith et al., 2015).

In a sense, reef ecosystems have a lot in common with highly productive terrestrial ecosystems. Reef corals present a high density and diversity of associated and beneficial microorganisms. The best-known microorganism that forms a very close relationship with corals is the symbiotic photosynthetic dinoflagellate *Symbiodinium* spp., which produces most of the food for the coral through photosynthesis (Bourne et al., 2016). However, to fulfill the high demand of photosynthates required by corals in a highly erratic environment, we hypothesize that other related mutualists, such as bacteria, may serve as biostimulants that enhance the capabilities of *Symbiodinium*. There is evidence that aquatic phototrophs, such as microalgae, can establish mutualistic interactions with terrestrial bacteria. Microalgae-bacteria interactions have been artificially induced with soil-borne bacteria strains that possess plant-growth-promoting (PGP) activity in terrestrial environments. For example, the interaction of the soil-borne bacteria *Azospirillum brasilense* with the microalgae *Chlorella sorokiniana* was found to increase photosynthetic activity, population density, and accumulation of cellular compounds in the microalgae (Palacios et al., 2016; Amavizca et al., 2017). This biostimulant activity of *A. brasilense* was further shown to be due to a constant provision of the phytohormone indoleacetic acid (IAA) by the bacteria (De-Bashan et al., 2008; Choix et al., 2014; Palacios et al., 2016).

In a recent study, Ainsworth et al. (2015) reported that actinobacteria share a niche habitat with *Symbiodinium* spp. in coral-gastro-dermal cells. It is also known that actinobacteria found in corals, such as members of the order Frankiales, are capable of fixing nitrogen (Sellstedt and Richau, 2013). Moreover, genomic analysis of marine Actinobacteria genera, such as *Streptomyces* and *Salinispora,* have shown the presence of genes related to the metabolic routes for phytohormone and siderophore production (Penn et al., 2009; Qin et al., 2017), which are properties of interest for terrestrially cultured plants. Actinobacteria is an important microorganism phylum whose members have several biotechnological applications. These bacteria are well recognized for possessing an arsenal of biosynthetic pathways for different secondary metabolites (Kasanah and Triyanto, 2019), including known and putative plant biostimulants (Palaniyandi et al., 2013; Olanrewaju and Babalola, 2019).

Although studies of actinobacteria and biostimulant activity have been carried out mainly with strains isolated from terrestrial sources, such as the rhizosphere of crop plants (El-Tarabily et al., 2009; Anwar et al., 2016; Chen et al., 2018; AbdElgawad et al., 2019), the search for novel actinobacteria functions has recently expanded to other less-explored habitats. Studies have been carried out with organisms isolated from seawater, sediments (i.e., *Saccharopolyspora* and *Streptomyces* strains; Rashad et al., 2015; Nafis et al., 2019), and the rhizospheres of both marsh and mangrove plants (Suksaard et al., 2017; Gong et al., 2018). Like their terrestrial counterparts, some of the marine strains have been found to possess the ability to produce phytohormones, fix nitrogen, solubilize phosphate, produce siderophores, and decrease ethylene overproduction via the enzyme ACC deaminase (Rashad et al., 2015). Furthermore, other marine actinobacteria, such as *Streptomyces*, *Isoptericola,* and *Arthrobacter*, have been found to enhance the germination of *Limonium sinensis* plants in soil with different salinities (Qin et al., 2014). However, to the best of our knowledge, the activity of coral-associated actinobacteria has not yet been explored.

In this study, we hypothesized that marine actinobacteria associated with coral reefs would support activities that are beneficial for terrestrial plants by enhancing their ability to tolerate abiotic stress, such as increased soil salinity. We tested this hypothesis by isolating actinobacteria from two coral species (*Porites lobata* and *Porites panamensis*) from the tropical central Pacific. We evaluated their ability to act as biostimulants during

the germination and early growth of *Nicotiana attenuata* as well as their ability to increase the resistance of this plant to saline stress (**Figure 1**). *N. attenuata* is a member of the Solanaceae family and is closely related to important crop plants, such as the tomato, whose natural history and responses to environmental stress have been extensively studied (Baldwin et al., 1994).

MATERIALS AND METHODS

Biological Samples

Salinispora strains were isolated from two coral species, *P. lobata* and *P. panamensis*. Coral samples were collected by scuba diving in two coral reefs from the tropical central Pacific (19° 5′ 55.21″ N, 104° 23′ 24.47″ W and 19° 3′ 28.87″ N, 104° 15′ 40.25″ W; **Supplementary Figure S1**). Coral branches (2 cm) were collected in triplicate from healthy individuals of each species. The collected branch surface was washed several times with sterile seawater to eliminate the mucus layer and epibiont microorganisms. The coral tissue was obtained by airbrushing (80 psi) with 10 mL of sterile seawater. The tissue samples were dried in a laminar flow hood for 72 h and stamped in 10% A1 culture medium (Mincer et al., 2002) supplemented with 100 $\mu g\ mL^{-1}$ cycloheximide and 5 $\mu g\ mL^{-1}$ gentamicin (Sigma-Aldrich, St. Louis, MO, United States). Strain identification was first carried out using the morphological characteristics of the cultures as well as the seawater requirements for growth and was further confirmed by 16S rRNA gene sequencing (Mincer et al., 2002; Gontang et al., 2007). *Salinispora* strains were preserved on plates with A1 medium until further use. Cultures for seed treatment were grown on 50 mL liquid A1 medium at 210 rpm and 28°C for 8 days.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Genomic actinobacteria DNA was extracted using a DNeasy® Blood and Tissue kit (Cat. No. 69506; Qiagen Corp., Germany) according to the methods described by Gontang et al. (2007). For the amplification of the 16S rRNA gene, the primers FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) were selected, and a Dream Taq Green Master Mix (2X) kit was used following the protocols of the manufacturer (#K1081, Thermo Scientific, Vilnius, Lithuania). The PCR products were purified with a Wizard® SV Gel kit and PCR Clean-Up System (Promega, Madison, United States) and sent to the DNA Synthesis and Sequencing Facility of the Institute of Biotechnology of UNAM in Mexico for sequencing. The forward and reverse 16S rRNA sequences were assembled and deposited in the GenBank[1] database under the accession numbers MT002753 (X29) and MT002754 (F51).

Sequences of endophytic actinobacteria, including the reported *S. arenicola* strains (phylotype A and type strain ATCC_BAA-917), were obtained from the NCBI database[2]. Representative microorganisms were selected based on a nearest reported sequence (neighbor) determined by a BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) analysis with the MT002753 and MT002754 sequences. Multiple sequence alignments were generated using Clustal X (Larkin et al., 2007). The phylogenetic relationships were analyzed using the neighbor-joining method, as implemented in MEGA7 with evolutionary distances computed using the p-distance method (Nei and Kumar, 2000; Kumar et al., 2016). We used 1000 bootstrap replicates for tree support and *Blastococcus litoris* and *Propionibacterium damnosum* as outgroups.

Azospirillum brasilense (ATCC 29710), a known terrestrial bacteria with plant growth promotion activity, was preserved on TYG-agar medium (Bashan et al., 2011). For seed-treatment cultures, *A. brasilense* was inoculated into 50 mL of TYG-broth medium and grown at 210 rpm and 28°C for 12 h prior to being introduced into *N. attenuata* seeds.

All plant experiments were conducted with a 31st inbred generation of *Nicotiana attenuata* Torr. ex S. Wats. (a.k.a. wild tobacco; seeds collected from DI Ranch, Motoqua, United States; Baldwin, 1998).

Germination Assays

Nicotiana attenuata germination is known to follow burn-soil and gibberellic acid (GA_3) signals (Baldwin and Morse, 1994), which allowed us to coordinate the onset of germination using smoke-cues and GA_3 to arrest seed dormancy. *N. attenuata* seeds were sterilized for 5–7 min in a 1 mL hypochlorite (2%) aqueous solution supplemented with 10 μL of 0.5% (v/v) Tween-20 (Sigma-Aldrich, St. Louis, MO, United States). Seeds were washed three times with sterile distilled water and incubated for 1 h in 1 mL of sterile 50-fold diluted liquid smoke (House of Herbs, Inc., United States) supplemented with 100 μL of 0.01 M GA_3 (Biogib, Arysta LifeScience, Mexico). Then, seeds were triple rinsed in sterile distilled water and treated for 30 min with 1 mL of the bacterial suspensions of *S. arenicola* X29, F51, *A. brasilense* (cultured as described in the "Biological Samples" section), or A1 growth media (control). After which, the seeds were sown in plates with solid plant growth media with or without 100 mM NaCl (Sigma-Aldrich). Solid plant growth media contained 0.6% (w/v) phytagel (Sigma-Aldrich, St. Louis, MO, United States) supplemented with Gamborg's B-5 Basal Medium with Minimal Organics (Sigma-Aldrich). The plates were maintained in a growth chamber (Thermo Scientific) at 28°C with a 16:8 h light/dark photoperiod (155–300 $\mu m/s/m^2$). Germination was evaluated for the next 18 days after sowing.

The experimental units were arranged in a two-level factorial fixed design with the first and second factors being the salinity and bacteria treatments, respectively. A total of eight treatments were used, from which two served as the negative controls. A total of 100 seeds distributed on four plates were tested for each treatment. Contaminated seeds were not included in the sample size. Statistical differences among treatments and conditions were determined by a two-way permutational ANOVA ($\alpha = 0.05$; 10,000 residual permutations) under a reduced model based on a Euclidean distance matrix in the PRIMER + PERMANOVA software (v.7; PRIMER-e, Plymouth, United Kingdom).

[1] http://www.ncbi.nlm.nih.gov/genbank/index.html

[2] http://www.ncbi.nlm.nih.gov/

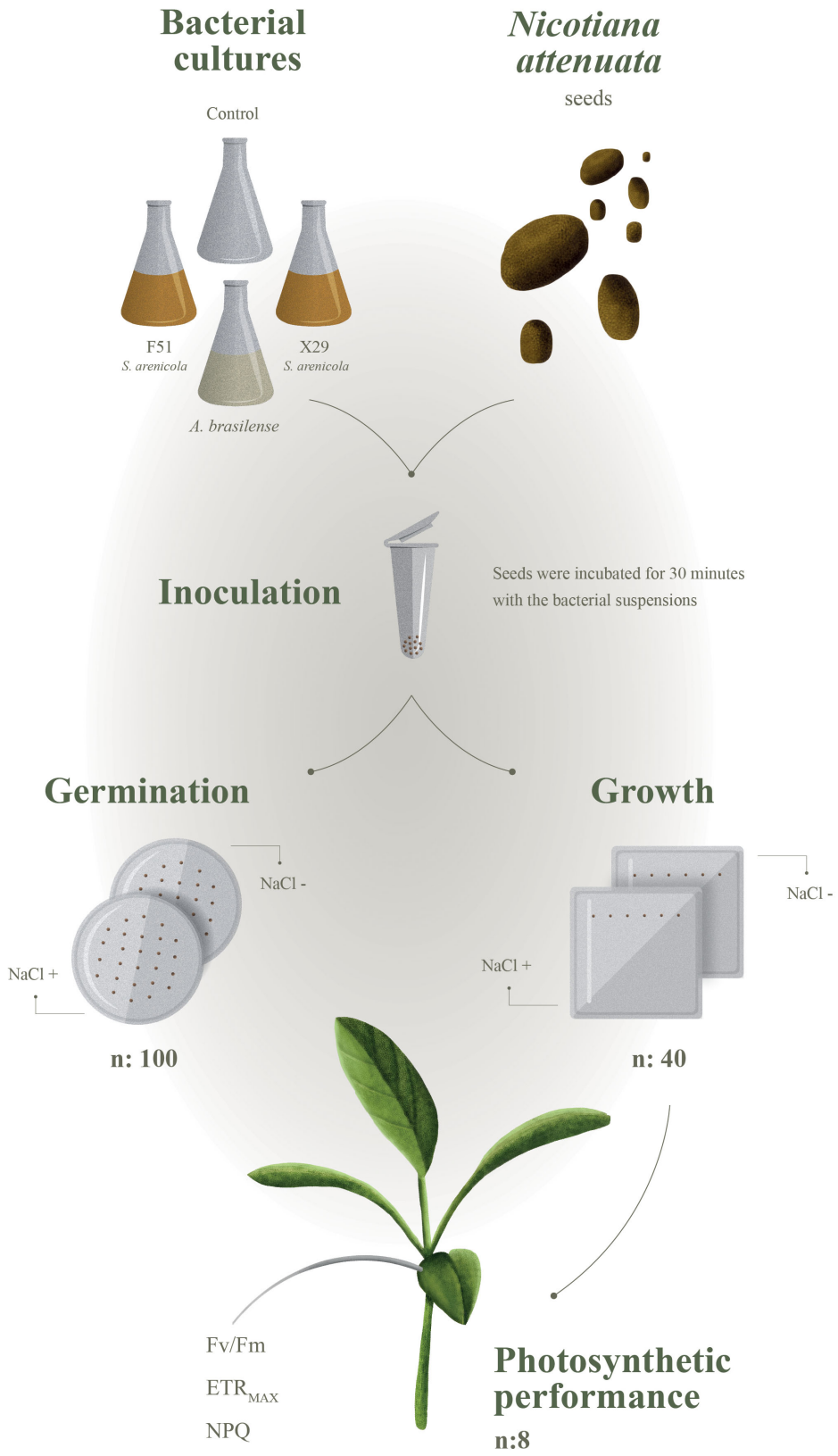


FIGURE 1 | Experimental design to evaluate if *Salinispora* strains can benefit terrestrial plants (*Nicotiana attenuata*) under saline stress.

Plant Growth Analysis

The growth-promoting effect was evaluated by treating the seeds as described (see section "Germination Assay") with *S. arenicola strains* (X29 and F51), *A. brasilense,* or A1-growth media (negative control). Eight seeds were sown on one side of a square cell culture plate containing solid plant growth media. A total of 40 seeds were used for each treatment. The plates were placed on a vertical plane with the seeds on top. Pictures were taken of the cultured seedlings every day after germination with a scale in frame, and growth was measured using the image processing and analysis software ImageJ v. 1.51 (National Institutes of Health). Significant differences were analyzed by two-way ANOVA ($\alpha = 0.05$) followed by a Tukey HSD test between the treatments and control.

Photosynthetic Performance of *Nicotiana attenuata* Plants

The photosynthetic performance of the plant *N. attenuata* was evaluated by non-intrusive pulse-amplitude modulated chlorophyll fluorometry (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) according to Schreiber (2004). To gain an overview of photosynthetic performance, we conducted rapid light-curve experiments that allowed us to determine the maximum electron transfer rate (ETR_{MAX}) as a proxy of the photosynthetic rate, the maximum photochemical quantum efficiency of photosystem II (PSII; F_V/F_M) as a measure of the health of the photosynthetic apparatus, and the non-photochemical quenching of PSII Chlorophyll *a* fluorescence (NPQ) as a proxy to estimate photoprotective capacity. Experiments were conducted on the same set of *N. attenuata* plants (22 DAG) used in the plant growth analysis, with at least eight plants per treatment analyzed. Plants were submitted to two light regimens: artificial low light (PAR 300 μmol photon m^{-2} s^{-1}) and natural high light conditions (∼2,000 μmol photon m^{-2} s^{-1}). Plants were exposed for at least 3 h to the respective light conditions and dark acclimated for 30 min prior to the analysis. Statistical differences were determined by two-way ANOVA and pair-wise differences were determined by a Tukey HSD test.

Microscopic Analysis of Roots

Roots of *N. attenuata* were harvested 22 days after sowing. Roots were heat-fixed, clarified in 70% (v/v) EtOH-KOH for 24 h, and gram stained to visualize root-associated bacteria. Stained bacteria were then observed under a Primo Star compound light microscope (Carl Zeiss, Göttingen, Germany).

Isolation of *Salinispora* From *N. attenuata* Roots

At the end of the experiments, *N. attenuata* roots were harvested under sterile conditions, homogenized softly with liquid media using a sterile mortar and pestle, and plated on A1 medium to confirm the presence of *Salinispora* strains in the *N. attenuata* roots. *Salinispora* strains were identified by their morphological features.

Statistical Analysis

Normality and homogeneity of variance were evaluated for all data sets. If the assumptions were fulfilled, the data was analyzed by a one or two-way ANOVA and Tukey HSD tests to compare means of the different treatments and conditions. If assumptions were not fulfilled, permutational ANOVAs were used. Statistical analysis was conducted in Sigma Plot v. 12 (Systat Software, Inc.), R studio (Version 1.2.5033, with R version 3.16, © 2009–2019 RStudio, Inc.) and PRIMER + PERMANOVA software (Version 7.0.13; PRIMER-e, Plymouth, United Kingdom).

RESULTS

Isolation and Identification of *Salinispora* spp. as Coral-Associated Bacteria

Corals form associations with several microorganisms, including Actinobacteria species, known to reside outside and around the mucus layer as well as inside of coral tissues (e.g., on the gastrodermal layer of polyps). By carefully removing the mucus layer and harvesting the clean tissue (see the "Marine Biological Samples and Microorganism Culture" subsection in the "Materials and Methods" section) of *P. lobata* and *P. panamensis* corals, we were able to isolate actinobacteria from coral gastrodermal tissues. These actinobacteria were grown in plates with and without seawater, isolated, and characterized morphologically. Among the actinobacteria that grew strictly in seawater, we were able to identify two putative *Salinispora* strains, X29 from *P. lobata* and F51 from *P. panamensis*. The identity of X29 and F51 was further confirmed by 16S rRNA sequencing. The NJ-phylogenetic tree shows the 16S rRNA sequence similarity of the actinobacterias strains isolated in this work with the previously reported *S. arenicola* phylotype A (100%) and the type strain ATCC_BAA-917 (99.93%) (Jensen and Mafnas, 2006; Becerril-Espinosa et al., 2013; Millan-Aguinaga et al., 2017) (**Figure 2**). Within this analysis, we also observed a close relationship with other plant-endophytic bacteria from other terrestrial and marine genera (**Figure 2**).

Salinispora arenicola is considered a strict marine bacterium, whose ecological role and relevance is still largely unknown. However, *S. arenicola* has recently gathered considerable attention due to its high proportion of specialized metabolite biosynthetic genes with promising biotechnological potential (Amos et al., 2017; Bauermeister et al., 2018).

Inoculation of *N. attenuata* Seeds With *S. arenicola* Strains Enhanced Plant Germination Under Saline Conditions

Since *Salinispora* is a strict marine bacterium, we hypothesized that it would possess biostimulant properties to cope with salt stress. Therefore, we tested *S. arenicola* activity during the germination of *N. attenuata* seeds *in vitro* under normal and saline conditions (100 mM NaCl). Seeds were treated with either one of the two varieties of *S. arenicola* (i.e., X29 or F51), a known biostimulant bacterium *A. brasilense*, or with sterile liquid growth media as a negative control. Treated seeds were sown in



FIGURE 2 | Phylogeny relationship between the coral-isolated strains, *Salinispora*, *Micromonospora*, and *Verrucosispora*. The phylogenetic tree generated from 16S rRNA gene sequences for the neighbor-joining analysis with 1,000 bootstraps. *Blastococcus litoris* and *Propionibacterium damnosum* were used as outgroups. The tree is drawn to scale, with branch lengths measured in the number of base differences per site (strain, accession numbers in parentheses). T, type strain. ***, strains observed in this study. Isolation source: PP, *Porites panamensis*; PL, *Porites lobate*; SC, Sea of Cortez; CR, Costa Rica, ▲ = root mangrove, ■ = marine sediment, ● = root land.

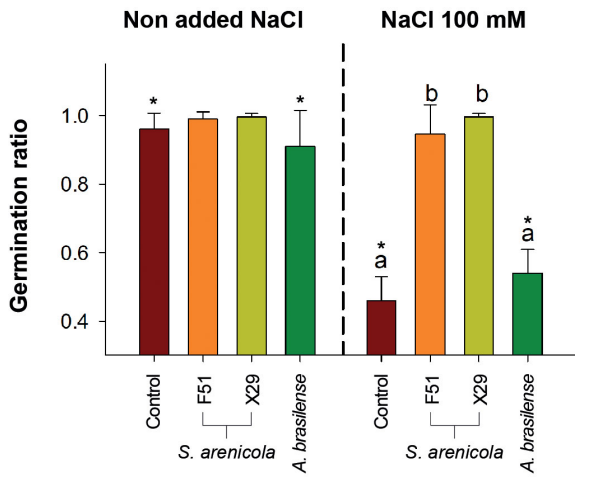


FIGURE 3 | Germination ratio of *N. attenuata* treated with the coral bacteria *S. arenicola* under saline stress. Seeds were treated with either *S. arenicola* strain X29 (yellow), F51 (orange), *Azospirillum brasilense* (green), or growth media (Control; red) and sown in plates with or without with 100 mM NaCl added. Results are the average of at least three independent experiments (±SD). Differences among germination ratios were analyzed by a permutational ANOVA [$\alpha = 0.05$, $p < 0.001$] followed by pairwise test between the treatments and Control. Lowercase letters to denote statistical differences among treatments in each condition. The symbol (*) denotes statistical differences of the interactions between Treatments × conditions ($p < 0.001$).

culture plates with and without NaCl (see the "Plant Material, Germination, and Growth Assay" subsection in "Materials and Methods" section), and germination was recorded. Under non-saline conditions, differences in germination were barely observed between the *S. arenicola* strains and that of the control (**Figure 3**), with only a slight but not significant increase observed in the germination of seeds treated with X29 between days 7 and 10. However, under conditions of increased salinity, we observed significant differences in the germination ratio among seeds treated with either the X29 or F51 strains and those of the control or *A. brasilense*-treated seeds ($P < 0.001$; **Figure 2**). Under conditions of increased salinity, the germination success at the end of the experiment (day 18) of seeds treated with *S. arenicola* was at least twofold higher with F51 (germination rate, GR: 0.95 ± 0.09) and X29 (GR: 1 ± 0.0) than *A. brasilense* (GR: 0.54 ± 0.07) or the growth-media control (GR: 0.46 ± 0.07). Interestingly, seed treatment with the broadly used biostimulant *A. brasilense* had no effect on germination under any conditions (**Figure 3**). We further tested the growth-promoting activity of these treatments under the same conditions and found no significant increase in the growth rates among the different treatments (**Supplementary Figure S2**).

Overall, these results highlight the potential capacity of the X29 and F51 strains of the marine actinobacteria *S. arenicola* to induce resilience in seed germination in saline environments. Even so, the observed responses remain to be tested under field conditions and in different plant systems.

Treatment of Plants With Marine Bacteria Improves Photosynthetic Responses Under Saline and High Light Conditions

We further explored if the induced resilience to salinity by *S. arenicola* might be observed in other plant physiological

responses besides germination. Photosynthetic fluorescence has been suggested to be a hallmark of stress responses (Guidi et al., 2019). We first evaluated photosynthetic responses under culture conditions with relatively low light levels (c.a. 300 μmol m^{-2} s^{-1}). We observed no significant differences in the photosynthetic descriptors of F_V/F_M and ETR_{MAX} in the plants among all treatments after 30 min of dark acclimation when analyzed under low light conditions (**Figures 4A,B**), suggesting a non-positive effect of these photosynthetic responses.

As it has been observed before (Külheim et al., 2002), it is likely that differences in photosynthetic performance are not always evident under low light conditions. Hence, we tested photosynthetic performance under harsher light conditions. We compared both the photosynthetic performance of *S. arenicola*-treated and untreated plants after exposing them to natural sunlight (1,500–2,000 μmol m^{-2} s^{-1}) for c.a. 3 h to induce moderate light stress. Under these relatively high light conditions (**Figure 4**), we observed significant differences in all photosynthetic parameters among the treatments. We found a clear indication of photoprotection in the *S. arenicola*-treated plants. The PSII quantum efficiency of the plants treated with the *S. arenicola* strains (F_V/F_M 0.7 ± 0.05) outperformed that of the control plants by 25% (F_V/F_M c. 0.5 ± 0.06) and that of the *A. brasilense*-treated plants by 15% (F_V/F_M c. 0.6 ± 0.05; **Figure 4A**).

Changes in ETR_{MAX} followed a similar pattern as those of the PSII quantum efficiency. *Salinispora* strain F51- and X29-treated plants presented 88 and 68% higher ETR_{MAX} values than those of the control plants, respectively, which was also surprisingly true for *A. brasilense* (48 and 66%, respectively; **Figure 4B**). These findings suggest that the photosynthetic rate was greater in the marine bacteria-treated plants than in the plants of the other treatments. Higher photosynthetic rates are often correlated to increased growth rates. However, no differences were observed in plant growth *in vitro* (**Supplementary Figure S2**), which might be due to the smooth low light culture conditions, as suggested by the lack of differential photosynthetic responses in **Figure 4**. The observed changes under high light conditions suggest that differences in growth may be observed under conditions of natural sunlight and salinity; however, this remains to be tested.

The thermal dissipation of energy measured as NPQ is the principal photoprotective mechanism in plants used to avoid the deleterious effects of excessive light. Interestingly, under low light conditions, no significant differences in NPQ values for the F51, X29, and *A. brasilense*-treated plants were observed. However, under high light conditions, we found that the NPQ values for all *Salinispora*-treated plants were twice that of the control plants, implying a primed capacity of *S. arenicola*-treated plants to overcome excessive light conditions (**Figure 4C**). Interestingly, NPQ values between *Salinispora*- and *A. brasilense*-treated plants were not significantly different (**Figure 4C**), which further suggests that the associations between symbiont bacteria and plant roots may prime stress responses that allow the plant to better cope with high light.

Together, these results suggest a photoprotective effect of the marine actinobacteria *S. arenicola* in plants under natural light and saline conditions. This effect was even higher than in the well-known biostimulant and terrestrial bacterium *A. brasilense* under the experimental conditions.

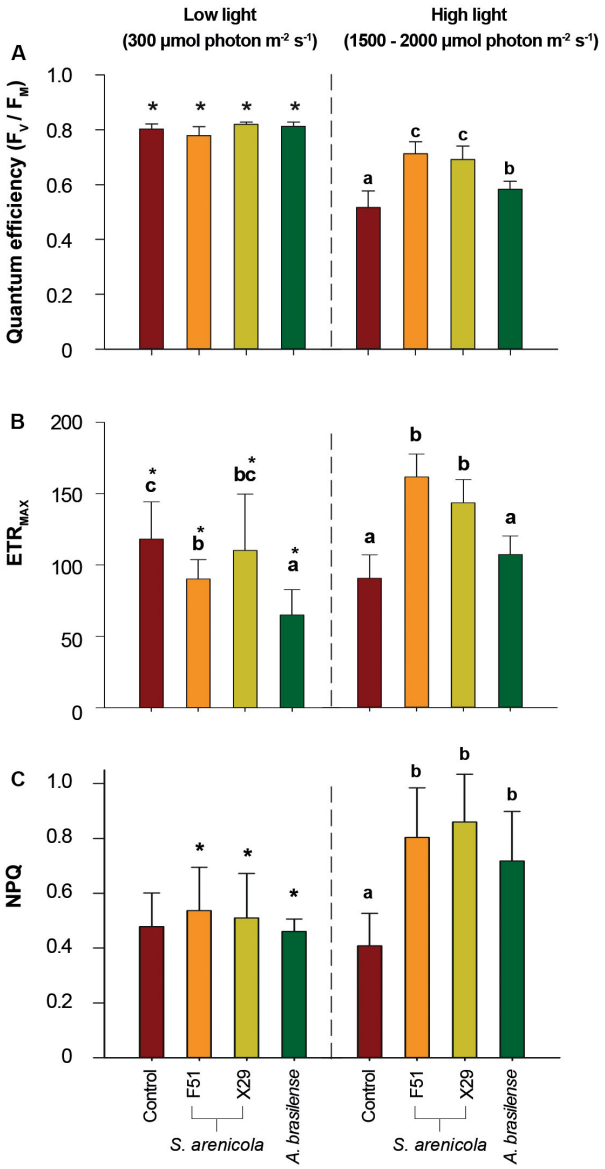


FIGURE 4 | Photosynthetic performance of *N. attenuata* plants grown under saline stress and different light conditions treated with *S. arenicola* bacteria. Plant seeds were treated with bacterial suspensions (*S. arenicola* strain F51, orange; *S. arenicola* strain X29, yellow; *A. brasilense*, green; and growth media Control, red), and the photosynthetic parameters were measured for two light conditions (artificial low light and natural high light). **(A)** F_V/F_M, PSII maximum quantum yield of photosystem II; **(B)** ETR_{MAX}, maximum electron transport rate; **(C)** NPQ, non-photochemical quenching of Chlorophyll *a* fluorescence. Results represent the average of eight independent plant measurements. Error bars are standard deviations. Differences were analyzed using a two-way ANOVA ($\alpha = 0.05$, $p < 0.001$). * indicates differences between conditions among treatments ($\alpha = 0.05$, $p < 0.001$). Inbox letters highlight differences between treatments for each condition analyzed by Tukey HSD test.

Salinispora arenicola Formed an Endosymbiotic-Like Relationship With Plant Roots

Biostimulant bacteria are known to interact in different ways with plant roots. Microorganisms grow outside and around the plant epithelium (exophytic) or inside the tissues (endophytic), signaling and providing nutrients. In order to observe the interactions between *N. attenuata* roots and *S. arenicola*, we collected the treated roots, gram-stained them, and analyzed the roots under a microscope. We found that the *S. arenicola* hyphae were highly interwound with *N. attenuata* root apical meristems (**Figure 5A**), with no bacteria present outside the roots. In contrast, *A. brasilense,* which is known to act as an exophytic symbiont, was mainly observed outside, surrounding the root epithelium (**Supplementary Figure S3**). The *N. attenuata* seeds treated with media, which served as a negative control, showed no bacteria addition at all (**Figure 5A**).

These observations suggest an endophytic-like interaction between *N. attenuata* roots and the actinobacteria *S. arenicola*. Moreover, to confirm this observation, we harvested and cultured small cuts of the *N. attenuata* roots on bacteria media under sterile conditions. We were able to re-isolate the *Salinispora* strains (**Supplementary Figure S4**), which further implies an endophytic growth of the marine bacteria on *N. attenuata* roots.

Salinispora arenicola A Possesses ACC Deaminase Activity

Among the different reported methods that bacteria use to enhance salinity resistance, one of the most studied and recognized is that of ACC deaminase activity by symbiont bacteria. We tested for ACC deaminase activity using the *S. arenicola* strains. Bacteria were inoculated in a medium with only ACC as a nitrogen source and allowed to grow. *S. arenicola* strains were able to grow in nitrogen-restricted media (only ACC), following similar kinetics to those of the nitrogen-rich media, suggesting that *S. arenicola* possesses ACC deaminase activity (**Figure 5B**). Nonetheless, growth in either media was found to be very slow. From this result, we inferred that at least one mechanism for the observed induced resistance to salinity by *S. arenicola* was present, which might have been either a decrease

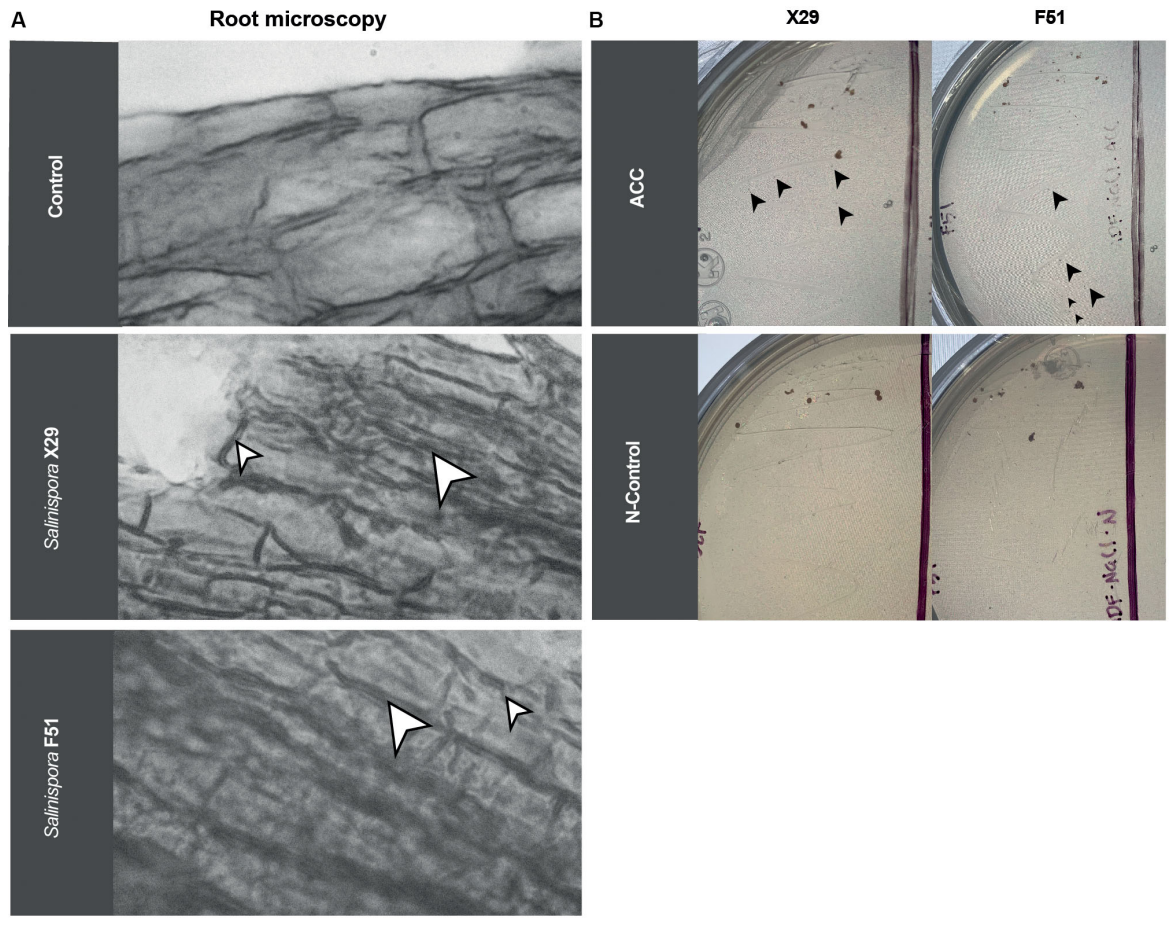


FIGURE 5 | Root endophytic-like interactions and ACC deaminase activity of *S. arenicola* strains on *N. attenuata*. **(A)** Microscopic photography of *N. attenuata* roots grown after seed treatment with *Salinispora* (X29 and F51) strains or sterile growth media (Control). White arrows point to bacteria. To improve the contrast, the images were converted to black and white. **(B)** *S. arenicola* cultured under growth media with nitrogen as the control (N-control) and ACC as the only nitrogen source (ACC). Black arrows point to new colonies.

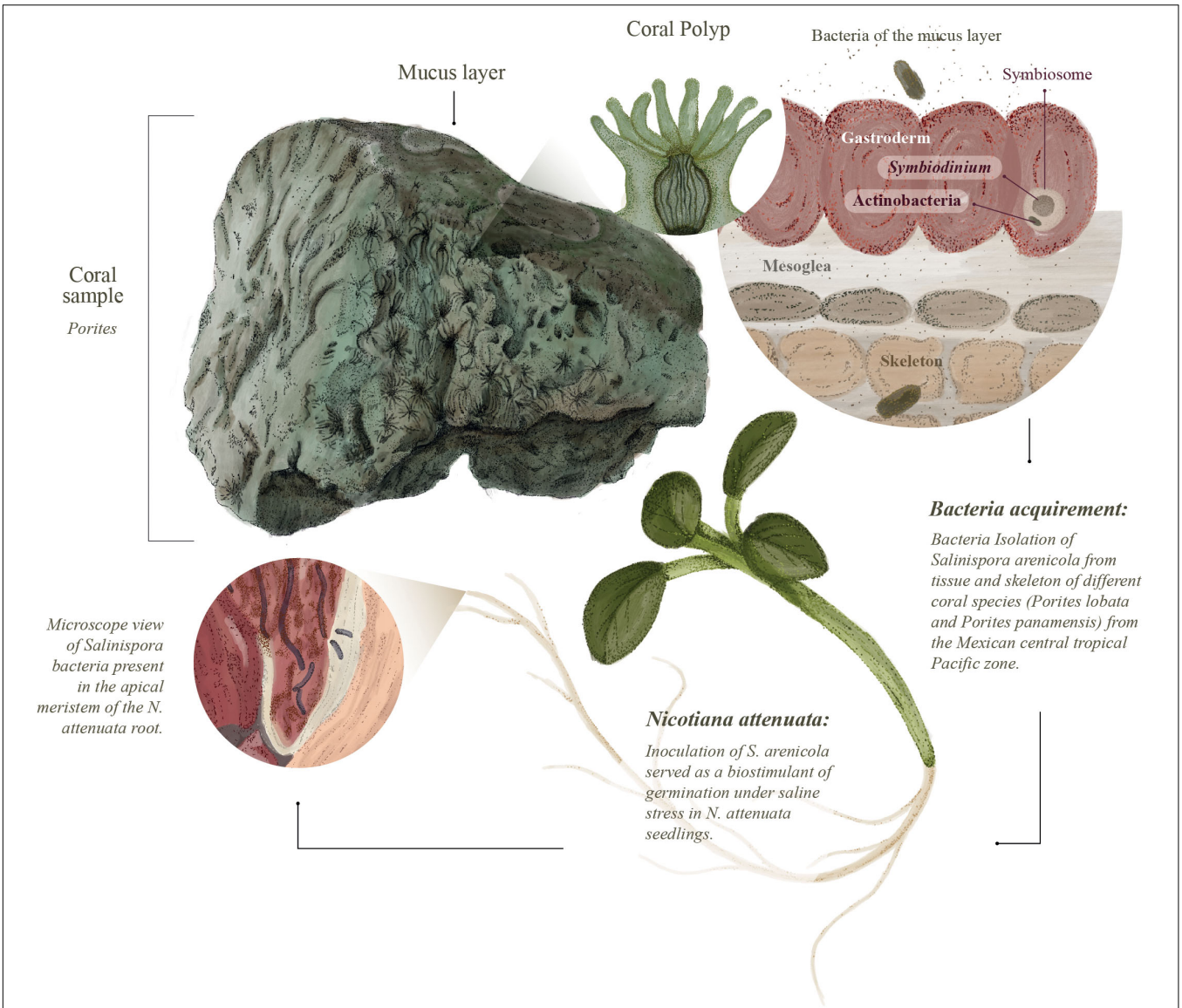


FIGURE 6 | Discovery of coral microbiota with biostimulant activity of land plants. *Salinispora* actinobacteria were isolated from coral polyp tissues of *Porites* spp. harvested from the Mexican tropical central Pacific coast. These Actinobacteria may form associations with other symbionts, such as *Symbiodinium*. We tested the biostimulant activity of the isolated *Salinispora* strains and found a protective effect on *Nicotiana attenuata* plants germinated and grown in saline environments. We also found that the isolated *Salinispora* actinobacteria form endophytic-like interactions with *N. attenuata* roots and likely ACC activity, which may contribute to the observed biostimulant effect.

in stress-related signaling induced by ethylene production (its biosynthesis is prevented by the deamination of ACC) or by the provision of an alternative nitrogen source to the plant.

DISCUSSION

Here, we address the biostimulant activity of the coral symbiont actinobacteria *S. arenicola* in the solanaceous plant *N. attenuata* (**Figure 6**). For the first time, we found and isolated *S. arenicola* phylotype A from the associated microbiota of the coral tissues of *P. lobata* and *P. panamensis* (**Figures 1**, **6**). We also observed that *S. arenicola* alleviated salt stress during germination and photosynthesis in *N. attenuata* plants. Our results revealed a positive effect of the actinobacteria in the germination of seeds under salt stress (**Figure 3**), which was likely through the activity of the ACC deaminase found in *S. arenicola* (**Figure 5B**). Interestingly, no changes in growth were found among the treatments, possibly because of the low light conditions of the culture (**Supplementary Figure S2**), as suggested by the photosynthetic responses (**Figure 4**). Photosynthetic responses were found only to change under high light (sunlight) conditions and not under culture light conditions. Under high light conditions, we observed a photoprotective effect as evidenced

by an increase in PSII quantum efficiency (NPQ and ETR_{MAX}) in plants treated with *S. arenicola*. Overall, we have shown the potential use of marine resources for the development of alternative sustainable agricultural tools to overcome current and upcoming environmental challenges.

Salinispora are strict saline actinobacteria with promising biotechnological potential, which is supported by their high proportion of biosynthetic genes (c.a. 10% of their genome) for specialized metabolites, some with unique chemical structures (Mincer et al., 2002; Udwary et al., 2007; Penn et al., 2009; Prieto-Davó et al., 2016; Amos et al., 2017). Like many other coral-associated bacteria, the specific ecological functions of *Salinispora* in the coral are unknown. It has been proposed that actinobacteria may influence bacterial community structure and protect corals from pathogens by releasing antibiotics (Mahmoud and Kalendar, 2016). Recently, it was shown that *Salinispora* produces Staurosporine, a potent antibiotic, in their native sediments, actively influencing the microbial community assemblage (Patin et al., 2017; Tuttle et al., 2019). Therefore, in the corals *P. panamensis* and *P. lobata,* the isolated *Salinispora* strains may exert a defense-like function against pathogens. However, due to the great extent of specialized secondary metabolites with unknown functions, *Salinispora* probably exerts some other ecological functions in corals.

There is some evidence that habitat-adapted symbiotic bacteria isolated from halophytes enter into symbiosis with many other terrestrial plants, including crops, and confer the capacity to grow under salt stress conditions (Rodriguez et al., 2008; Redman et al., 2011; Albdaiwi et al., 2019). The mechanisms associated with salt stress reduction in plants induced by these halophilic bacteria are varied (i.e., production of ACC deaminase, activation of antioxidant enzymes to eliminate reactive oxygen species, improvement of plant nutrition, production of phytohormones, accumulation of osmolytes, changes in the root architecture and hydraulic conductance, and the increased synthesis of chlorophyll and pigments to preserve photosynthetic activity; Etesami and Beattie, 2018). Recently, it has been reported that actinobacteria isolated from the marine environment enhanced germination in plants grown under high salinity conditions. This result was further linked to the activity of ACC deaminase (Qin et al., 2009, 2014; Gong et al., 2018). It is believed that ACC deaminase redirects ACC metabolism away from ethylene production, avoiding ethylene root growth inhibition and instead providing nitrogen (Van de Poel and Van Der Straeten, 2014). Our results (**Figure 5B**) suggest that ACC deaminase-like enzymes could be present in the symbiotic *Salinispora* strains assayed since we found the growth of *Salinispora* in culture medium with ACC as the only source of nitrogen. Therefore, ACC deaminase could be one of the factors that exerts a positive influence on germination and photosynthesis in plants under salt stress conditions. However, there are no reports of *Salinispora* owning an ACC deaminase gene nor did we find a reported putative homolog among the different genome databases (Integrated Microbial Genomes and Microbiomes[3]; NCBI[4]). We did find a PALP domain-containing protein[5] with similarities to D-cysteine desulfhydrase and ACC deaminase from related actinobacterial taxa (*Nonomuraea wenchangensis* and *Streptomyces thermoautotroph,* 54.5 and 48.1% similarity, respectively[6]). Future research is needed to reveal the specific ACC deaminase-like enzyme or the other fundamental molecular mechanisms behind the observed responses.

In contrast to terrestrial actinobacteria, *Salinispora* acquired genes during evolution that conferred the ability to adapt to the marine environment, highlighting light electron transport, sodium and ABC transporters, and channels and pores (Penn et al., 2009; Penn and Jensen, 2012). Possibly, mechanisms for stress alleviation exerted by *Salinispora* may be related to the more than 50 acquired genes for marine adaptation, like genes for specific ion transporters that contribute to decreasing salinity stress and the release of many osmolytes.

We also found that the interaction between *N. attenuata* and *S. arenicola* resembled that of plant-endophytic symbiosis (**Figure 5A**). Other actinobacteria (i.e., *Micromonospora* and *Verrucosispora*) from the same family of the coral symbiont *Salinispora* have been isolated from the root nodules of terrestrial and marine plants where they presented endophytic interactions and conferred advantages to their plant hosts (**Figure 2**; Li et al., 2013; Ngaemthao et al., 2017; Kuncharoen et al., 2018). Bioinformatic analysis suggests that *Salinispora* spp. have evolved from this terrestrial *Micromonospora*, with many of the metabolic genes being conserved (Trujillo et al., 2014). Likely, *Salinispora* conserves the capacity to establish plant-endophytic relations and some of their plant-biostimulant properties. Interestingly, the plant-biostimulant activity observed by *S. arenicola* was different from that reported for *A. brasilense.* The bioactivity of *A. brasilense* is based on the production of the phytohormone IAA. Screening of *S. arenicola* extracts revealed no traces of IAA (**Supplementary Figure S5**). Moreover, *A. brasilense* did not affect germination and only exerted a mild effect on photosynthetic performance under saline stress (**Figure 4**).

Improvement in photosynthetic performance in plants under saline stress has been associated with the overproduction of chlorophyll and carotenoid pigments as well as an increase in antioxidant enzymes. For example, in *Maiz* seeds, biopriming with the halotolerant *Pseudomonas geniculata* was found to increase chlorophyll and carotenoid pigment content (c.a. 50%) as well as antioxidant enzyme activity (c.a. 150%; Singh et al., 2020). Recently, IAA has been shown to specifically up-regulate chlorophyll content via Auxin response factor 6A (Yuan et al., 2019), which may account for the mild positive effect on the photosynthetic performance of *N. attenuata* plants treated with *A. brasilense* under conditions of saline stress. However, the photosynthetic performance induced by *A. brasilense* was easily surpassed by the effect of *S. arenicola* in plants (**Figure 4**).

Similarly, increased pigment content may be expected in *Nicotiana* plants when treated with *Salinispora*, although

[3]https://img.jgi.doe.gov/

[4]https://www.ncbi.nlm.nih.gov/

[5]https://www.uniprot.org/uniprot/A0A2W2CP37

[6]https://www.uniprot.org/blast

through a different mechanism. Saline stress activates ethylene biosynthesis and signaling, which is also known to reduce photosynthesis in young leaves while promoting plant senescence (Tholen et al., 2008; Ceusters and Van de Poel, 2018). The discussed ACC deaminase activity by *S. arenicola* may also explain the enhanced photosynthetic responses under saline stress, by either decreasing ethylene stress-related photosynthetic signaling or by providing an alternative nitrogen source and thus boosting photosynthetic performance. Experiments with the direct supplementation of ethylene and impaired ethylene signaling in plants are required to resolve the mechanism behind this response.

Despite the c.a. 400 million years of independent evolution and contrasting habitats between marine and terrestrial ecosystems, research has shown essential similarities in the chemical cues that regulate ecological interactions (Rasher et al., 2015). This is further supported by the surprisingly well conserved metabolic-related ortholog genes observed in *S. arenicola* and their terrestrial counterparts (Trujillo et al., 2014). In this study, we observed the capacity of this marine actinobacteria to establish an endosymbiotic-like interaction with terrestrial plants (**Figure 5**). However, there are important differences among the organisms of these two ecosystems, which were highlighted by the addition of a vast number of metabolic genes in *S. arenicola* that are different from those of their terrestrial counterparts (Penn et al., 2009; Penn and Jensen, 2012; Trujillo et al., 2014). These differences may provide alternative biotechnological solutions, such as the one reported here.

Plant- and seed-borne endophytes provide beneficial attributes to plants by reciprocally allowing a closer exchange of resources and signals between organisms (Truyens et al., 2015). While plants are responsible for primary production in the terrestrial sphere, the photosynthetic endosymbionts of corals are the key producers in reef communities. Both plants and algae respond to similar chemical and biochemical cues of a related nature, such as phytohormone IAA and possibly ACC deaminase. As much more is known about the chemical ecology of terrestrial ecosystems, here we posit the need to better understand marine ecological interactions as an important alternative source for future discoveries to overcome current environmental and agricultural challenges.

The present report shows the use of *S. arenicola* as a terrestrial plant biostimulant with the potential to alleviate germination and photosynthesis under saline stress, which is a great potential benefit for crop plants. However, further research on the capacity of *S. arenicola* to enhance crop yield and plant growth should be conducted to uncover the full potential benefits of this coral bacteria. Furthermore, although endosymbiosis and the biostimulant activity of plant growth promoting bacteria are known to occur in many related species, our research was focused on one terrestrial plant, which may or may not be reproducible in other crop-plant systems. Future experiments should evaluate this activity in related crop plant species, such as in tomato plants. Moreover, studies of the innocuity of the bacteria and ecological safety are required as well as of the efficiency of formulations. In addition, an economic cost-benefit analysis will be required. Therefore, the pursuit to discover the potential uses of marine organisms for land-agriculture solutions is both promising and extensive.

CONCLUSION

Soil salinity is one of the most damaging environmental stressors worldwide that is responsible for significant reductions in croplands, crop productivity, and crop quality. Our results provide evidence of the use of the marine actinobacteria *S. arenicola* as an alternative to counteract the adverse effects of soil salinity on the germination and photosynthetic performance in plants. Although environmental issues and field conditions remain to be analyzed, the reported plant biostimulant activity of *S. arenicola* can easily be scaled up for crop use, as it has been done for other organisms, such as *A. brasilense*.

Historically, actinobacteria have distinguished themselves as organisms with great biotechnological potential for the production of antibiotics and other compounds. However, there is still much to explore in this phylum and in other marine microorganisms. We do not rule out whatsoever that the properties of *S. arenicola* that are described here are only a small part of their biotechnological potential, such as the production of novel specialized metabolites. Certainly, a better understanding of the ecological interactions of marine microorganisms, such as those in coral reefs, will provide novel tools to meet current and future environmental and agricultural challenges. A hidden marine treasure awaits discovery.

DATA AVAILABILITY STATEMENT

Salinispora arenicola strains X29 and F51 sequences can be found in the NCBI database under the accession numbers MT002753 and MT002754, respectively.

AUTHOR CONTRIBUTIONS

HO-A, IM-C, and AB-E conceived and designed the experiments. HO-A, IM-C, CM-S, AM-U, FC-L, and AB-E performed the experiments and interpreted the results. HO-A, IM-C, FR-Z, and AB-E analyzed the data. CM-S designed and elaborate the figures with contributions of HO-A, IM-C, RH-H, and AB-E. HO-A, IM-C, and AB-E wrote the text with contributions of ER-J, FR-Z, CR-M, AM-U, RH-H, and FC-L. All authors approved the final version of the manuscript.

FUNDING

This work was supported by the project number UDG-PTC-1460 of PRODEP to HO-A; Universidad de Guadalajara by PROSNI-2017 and P3E to HO-A, PROSNI-2018 to IM-C,

PROSNI-2017-18 to AB-E, and the Project number 257987 CB2015 of CONACyT to FR-Z.

ACKNOWLEDGMENTS

We are thankful to MSc. Eduardo Juarez Carrillo, MSc. Martha Alicia Lara Gonzalez, Andrés de Jesús López Gervacio, Jaime Gómez Villela, Rodrigo Perez Ramirez, Cristina Lopez Beltran, and many other undergrad interns of the Molecular Ecology, Microbiology, and Taxonomy Laboratory (LEMITAX) as well as the Marine Ecosystem and Aquaculture Laboratory (LEMA) for their help with the plant growth and photosynthetic experiments.

We thank the journal reviewers for their valuable comments and suggestions. We thank Andrea Liévana MacTavish for her valuable suggestions and English language review. AB-E and FC-L acknowledge CONACYT for its support under the Program-projects 2196 and 2517 Cátedras CONACYT, respectively.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00648/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 May 2020
doi: 10.3389/fpls.2020.00647

Biostimulant Potential of Acetic Acid Under Drought Stress Is Confounded by pH-Dependent Root Growth Inhibition

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Recent reports of acetic acid-induced drought tolerance and avoidance across a diverse range of plant species encourage consideration of this low-cost commodity organic acid as a biostimulant. These results are surprising as they contrast with earlier studies showing pH-dependent root growth inhibition at similar concentrations. We test the hypothesis that the concentration of the membrane permeable undissociated form of acetic acid (CH_3COOH) selectively inhibits maize root growth, and subsequently evaluate its impact on seedling water use and growth under deficit irrigation. We demonstrate conclusively for the first time that when germinating maize on filter paper, low pH exacerbates, and high pH mitigates, this inhibition of root growth in a predictable manner based on the dissociation constant of acetic acid. The buffering capacity of potting media can reduce this root damage through keeping the acetic acid primarily in the membrane impermeable dissociated form (CH_3COO^-) at near neutral pH, but peat substrates appear to offer some protection, even at low pH. While both deficit irrigation and acetic acid reduced water use and growth of maize seedlings outdoors, there was no significant interaction between the treatments. Twenty nine millimolar total acetic acid (CH_3COOH + CH_3COO^-) reduced transpiration, compared to lower and higher concentrations, but this did not specifically improve performance under reduced water availability, with parallel declines in shoot biomass leading to relatively consistent water use efficiency. Any acetic acid biostimulant claims under water stress should characterize its dissociation level, and exclude root damage as a primary cause.

Keywords: drought, *Zea mays*, roots, maize, acetate, acetic acid, water use efficiency

OPEN ACCESS

Edited by:
Carlos Alberto Silva, Universidade Federal de Lavras, Brazil

Reviewed by:
Michael Bitterlich, Leibniz Institute of Vegetable and Ornamental Crops, Germany
Andrew Merchant, University of Sydney, Australia

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *23 January 2020*
Accepted: *27 April 2020*
Published: *25 May 2020*

Citation:
Allen MM and Allen DJ (2020) Biostimulant Potential of Acetic Acid Under Drought Stress Is Confounded by pH-Dependent Root Growth Inhibition. Front. Plant Sci. 11:647. doi: 10.3389/fpls.2020.00647

INTRODUCTION

A low dose (<50 mM) of acetic acid has recently been proposed as a biostimulant under drought stress for major crops as diverse as maize (*Zea mays* L.) (Kim et al., 2017), cassava (*Manihot esculenta* Crantz) (Utsumi et al., 2019), and mung bean (*Vigna radiata* L.) (Rahman et al., 2019). Acetic acid could bypass many of the barriers to commercialization of new biostimulants (Yakhin et al., 2017), as a well-studied compound with low cost (1 US$ per kL of 50 mM, based on 330 US$ per t; ICIS Chemical News, 2020) scaled-up production (12 million t/year; Le Berre et al., 2014)

through industrial and food use, with known toxicology (Le Berre et al., 2014) and even regulatory approval as an organic herbicide at higher doses (20%; US EPA, 2019). The biostimulation is surprising as both drought and acetic acid have been ubiquitous throughout plant evolution and crop domestication, and so the key supportive prior observations are summarized in the next paragraph, but note they were all conducted in controlled environments.

Root growth in 5-day-old barley (*Hordeum vulgare* L.) seedlings was increased when germinated in 0.1–1 mM acetic acid solutions at a pH of 6.5 (Lynch, 1977). In greenhouse-grown maize, 10-day-old seedlings were treated with 0–50 mM of acetic acid through the growth media for 4 days, water was withheld for 6 days and then re-watered for 5 days, with survival significantly higher after application of 30 mM acetic acid compared to the control (Kim et al., 2017). A significant increase in above ground biomass, leaf area, leaf total chlorophyll content, instantaneous leaf-level Water Use Efficiency (WUE), leaf temperature and shoot Relative Water Content (RWC) was observed in response to foliar application of 20 mM acetic acid to 16-day-old potted mung beans every 2 days for 2 weeks, with a significant reduction in leaf transpiration rate (Rahman et al., 2019). When this foliar acetic acid application was combined with saline growth media irrigation, in addition to all these impacts, acetic acid also significantly increased root biomass, root length and leaf carbon assimilation rate, compared to the foliar water sprayed controls with the same saline growth media irrigation (Rahman et al., 2019). In greenhouse-grown cassava, treatment with 10 mM acetic acid for 1 week enhanced drought avoidance during water withholding for the subsequent 2 weeks, as demonstrated by reduced wilting, higher leaf RWC, higher chlorophyll content, higher leaf temperature, and lower transpiration, compared to droughted controls (Utsumi et al., 2019).

Alongside these positive impacts, the prior studies identified dosing complexities which need to be addressed before acetic acid can be applied as a biostimulant to commercial maize production. The improvements observed with 30 mM acetic acid in maize were not significant at either lower (10 and 20 mM) or higher (50 mM) doses (Kim et al., 2017). In contrast to the protection from drought-induced wilting seen with 10 mM, 20–50 mM acetic acid applications to cassava actually induced wilting in the absence of drought (Utsumi et al., 2019). The barley seedling root growth stimulation seen at 0.1–1 mM acetic acid at a pH of 6.5 was eliminated for these same concentrations at a pH of 3.5 and a substantial root inhibition was observed at higher acetic acid concentrations (10 and 50 mM) in barley at a pH of 6.5, but not in maize (17 mM at a pH of 6.4) (Lynch, 1977). Root growth in reed (*Phragmites australis*) was reduced at 0.3 mM, and entirely inhibited at 1.7 mM acetic acid (Armstrong et al., 1996).

As an important food preservative, the mode of action of acetic acid stress on micro-organisms has long been studied. As a weak acid, the concentration of the undissociated ([CH_3COOH], hereafter referred to as [HAc], Lawford and Rousseau, 1993) form of acetic acid approaches a pH-dependent equilibrium with the concentration of the dissociated form ([CH_3COO^-], hereafter referred to as [Ac^-]) in aqueous solutions. HAc is lipid soluble and therefore can pass across cell membranes, in contrast to the hydrophilic Ac^- (Lawford and Rousseau, 1993). Under acidic extracellular conditions, HAc crossing the plasma membrane will have the potential to dissociate to H^+ and Ac^- ions inside the cell as a near neutral intracellular pH is often maintained (Russell, 1992; Warnecke and Gill, 2005). While there has been debate about the relative toxicity of these species inside the cell (Russell, 1992), it's clear that extracellular HAc is more toxic than extracellular Ac^-, due to its ability to get into the cells of yeast (Noda et al., 1982) and bacteria (Diez-Gonzalez and Russell, 1997; Warnecke and Gill, 2005), and this mechanism has been proposed to also apply to plants (Armstrong et al., 1996). The equilibrium of [HAc] and [Ac^-] is shifted significantly within agronomically-relevant pH ranges, and the potential for an interaction between total acetic acid concentration (defined as [HAc] + [Ac^-]) and pH is mostly absent from the plant literature discussed above, with pH almost never actively controlled and typically not even reported.

Is it possible that a dose- and pH-dependent impact on root growth is the primary response to growth media acetic acid application and that is subsequently responsible for all the phenotypes reported? Here we tested for the first time the hypothesis that it is [HAc], rather than [HAc] + [Ac^-] that impacts seedling root growth, predicting that for a given applied [HAc] + [Ac^-] the detrimental effect on roots is alleviated by raising the pH and exacerbated by lowering the pH in a predictable manner based on the known dissociation constant in aqueous solutions. Secondly, we evaluated whether these observations on seedling root growth at different pHs in aqueous solutions translate to germination in growth media with pH buffering capacity. Finally, we applied acetic acid to maize growing in pots outdoors for the first time, imposing a fully factorial [HAc] + [Ac^-] by deficit irrigation design, and examined impacts on growth and water use, as a necessary step toward the evaluation of acetic acid as a commercial biostimulant in maize.

MATERIALS AND METHODS

Acetic Acid and pH Impacts on Seedlings

All experiments were undertaken in a domestic environment, therefore chemicals were selected based on local availability and regulatory approval for consumer use. Acetic acid treatments (0, 0.01, 0.1, 1, 10, 20, 29, 38, 47, and 100 mM of [HAc] + [Ac^-]) were prepared from food-grade acetic acid (Heinz All Natural Distilled White Vinegar 5% acidity, Kraft Heinz Foods, Pittsburgh, PA, United States), diluted with municipal drinking water. This 5.23% acetic acid (weight%) was fermented from distilled maize ethanol, and also contained 0.20% residual ethanol, 0.02% ethyl acetate with $< 0.01\%$ other organic acids as determined by ^1H quantitative NMR (John Edwards, Pers. Comm.). The simplicity of this industrial vinegar (Gerbi et al., 1997; Sáiz-Abajo et al., 2005; Mas et al., 2016; Schneider et al., 2018) strongly contrasts with the diversity of composition of fruit- and wine-derived traditional vinegars (Caligiania et al., 2007; Edwards, 2018). 31% HCl (Crown® Muriatic Acid, Packaging Service, Pearland, TX) and 18–28% NaOH with 0–1% KOH (Instant Power® Hair Clog Remover, Scotch, Dallas, TX) were used to develop 10 aliquots

of each of the 10 [HAc] + [Ac^-] spanning a broad range of pH (>2 and <13), measured before and after seedling growth with a LAQUAtwin pH-33 (Horiba, Irvine, CA, United States) sensor. [HAc] was estimated based on acetic acid stock, measured pH value and an acid dissociation constant (K_a) of 1.8×10^{-5} at 25°C (Lawford and Rousseau, 1993). Creped seed germination paper (0.25 × 0.38 m, SB39211, Nasco, Fort Atkinson, WI, United States) was moistened with the pH-adjusted acetic acid solution and then 6 fungicide-treated maize seeds (Hybrid 1156, Steyer Seeds, Tiffin, OH, United States) were spaced evenly 5 cm from the long edge of the paper, rolled tightly, secured with a rubber band below the seeds, placed in a 0.03 × 0.2 m (diameter × length) 110 ml glass test tube with the seed at the top of the tube and filled with additional pH-adjusted acetic acid solution. These 100 test tubes were completely randomized in racks and placed under plant growth lights (GLP24FS/19W/LED, Feit Electric, Pico Rivera, CA, United States) producing ∼200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at the top of the paper rolls, as measured with a quantum sensor (LGBQM, Hydrofarm, Petaluma, CA, United States), with a 16/8 photoperiod. After 4 days of growth at room temperature, the maximum root and shoot length of each seedling was recorded. While study of primary root and coleoptile length is an established high throughput technique to monitor impacts on growth (Pace et al., 2014), in this study we did not attempt to establish a correlation with seedling root and shoot biomass. Mean (±1 standard deviation) air temperature, relative humidity and vapor pressure deficit (VPD) was 26.4 ± 1.0°C, 54 ± 3% and 1.6 ± 0.2 kPa, respectively, as measured with an adjacent sensor (WH31B, Ambient Weather, Chandler, AZ) logged every 5 min by a weather station (WS-2000, Ambient Weather).

Acetic Acid and Growth Media Impacts on Seedlings

Seedling root and shoot growth were compared in a factorial experiment of 2 growth media × 6 acetic acid treatments, arranged in a randomized complete block design with 6 replicates. Translucent polypropylene pots (0.95L, S-22771, Uline, Pleasant Prairie, WI, United States) were filled with either higher-pH potting mix (Miracle-Gro Moisture Control Potting Mix, Scotts, Marysville, OH, United States) or lower-pH sphagnum peat moss (Miracle-Gro, Scotts). The peat media contained 99% peat, 0.14% wetting agent, 0.10% ammoniacal nitrogen, 0.09% nitrate nitrogen, 0.11% P_2O_5, and 0.15% K_2O. The potting mix was a proprietary blend of sphagnum peat moss, processed forest products, compost, coir, perlite, wetting agent, 0.11% ammoniacal nitrogen, 0.10% nitrate nitrogen, 0.11% P_2O_5, and 0.16% K_2O. Mean (± 1 standard deviation) electrical conductivity (HI98331 Gro line, Hanna Instruments Inc., Woonsocket, RI) was 1.27 ± 0.13 mS cm^{-1} for potting mix and 1.21 ± 0.05 mS cm^{-1} for peat, after saturation with distilled water. While these were selected due to their contrasting advertised pH (2.8–4.0 for peat and 6.5–7.5 for potting mix) and similar fertilizer contents and conductivities, we cannot exclude the possibility of other differences influencing seedling responses to acetic acid, such as hydraulic properties and microbial communities. Individual pots were brought to 100% growth media RWC by irrigating with 0, 10, 20, 29, 38, or 47 mM [HAc] + [Ac^-] until they reached the target weight on a calibrated balance (0.1 g precision, SPX2201, Ohaus, Parsippany, NJ, United States). Free-draining growth media after saturation, based on the average of representative pots, defined 100% growth media RWC. No pH adjustment was made to these acetic acid solutions. Single maize seeds were planted 3–4 cm deep on August 26, 2019 and kept at room temperature for 2–3 days until emergence to avoid potential germination inhibiting pot temperatures above 35°C (Andrade et al., 2018), and then they were moved to an outdoor location in Katy, TX (29°42′N 95°50′W) out of direct sunlight. During this experiment, mean (± 1 standard deviation) air temperature, relative humidity and VPD was 25.8 ± 0.2°C, 54 ± 1%, and 1.52 ± 0.03 kPa indoors and 29.3 ± 4.2°C, 72 ± 16%, and 0.53 ± 0.51 kPa outdoors, with no precipitation. Maximum solar radiation measured by the weather station in full sunlight during this period was 928 W m^{-2}. PAR was recorded in the shade at ∼2 h intervals throughout a day with a quantum sensor and correlated to solar radiation measured by the weather station, and this was used to estimate an approximate maximum PAR in the shade during the experiment of 230 μmol m^{-2} s^{-1}, or 12% of full-sun. Growth media pH was estimated by sampling ∼15 ml of media from all pots in 3 reps during seedling growth in a 150 ml polypropylene cup (B071D8S33H, Tashibox, ASF TASHI LLC, Pittsfield, MA), adding 2.5 times its weight in distilled water, incubated for 30 mins on an orbital shaker (COZOORKJBDUS, Amazon, Seattle, WA) at 100 rpm before measuring the pH of the solution. Root and shoot length was measured 4 days after planting. As in the prior experiment we did not attempt to establish a correlation with seedling root and shoot biomass. Statistix version 9.0 (Analytical Software, Tallahassee, FL) was used for a blocked factorial analysis of variance, followed by a Tukey all pairwise comparison ($\alpha = 0.05$).

Acetic Acid and Drought Impacts on WUE

A factorial experiment of 4 water × 6 acetic acid treatments was used, arranged in a randomized complete block design with 6 replicates. Pots and acetic acid solutions were prepared as in section Acetic Acid and Growth Media Impacts on Seedlings, except only the higher pH potting mix was used. Planting occurred on August 19, 2019, pots were covered with a low density polyethylene lid with a 2.9 cm diameter hole drilled in the middle, and pots were maintained at room temperature for 3–4 days. With observed tray temperatures in August in southern TX (**Figure 1B**) beyond the 35–40°C permissible limit for maize germination (Andrade et al., 2018), this indoor-to-outdoor seedling transfer was required. Four water treatments (25, 50, 75, and 100%) were imposed between the extremes of 0 and 100% potting mix RWC, with the former defined after drying representative substrate to a constant weight at ∼77°C in a fan-assisted oven (JKP30SP2SS, General Electric, Louisville, KY, United States). All pots were started at 100% RWC, and then every 1–3 days, depending on the rate of weight loss, each pot was weighed and, as needed, returned to the target weight

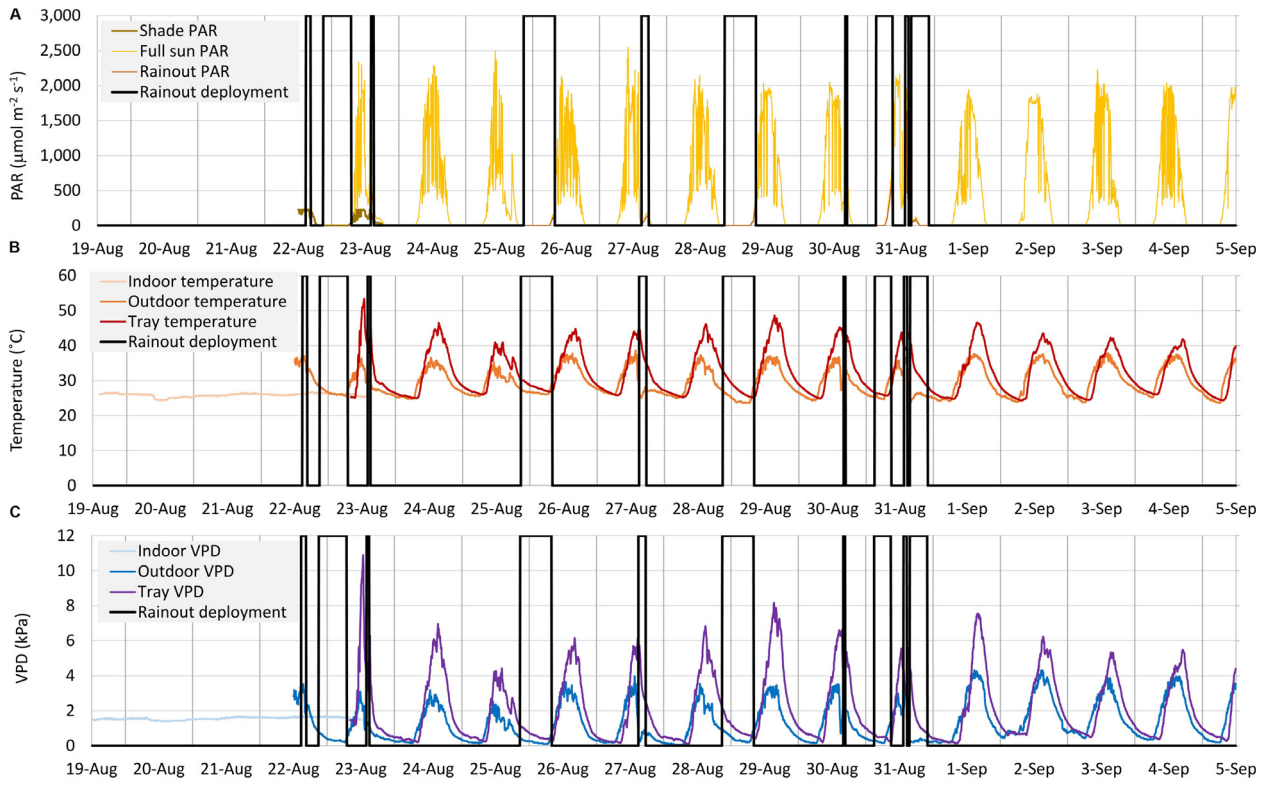


FIGURE 1 | Time course for Photosynthetically Active Radiation (PAR) **(A)**, Temperature **(B)** and Vapor Pressure Deficit (VPD) **(C)** under indoor, shade (acclimation), full-sun and during rainout shelter deployment phases of the outdoor Water Use Efficiency (WUE) assay. Tray temperature and VPD reflect the average of three sensors adjacent to the plant pots.

by watering with the appropriate acetic acid concentration. Pot location within a 24 plant block was re-randomized after each weighing. Similar pots with drilled lids but without seed, and 3 pots which failed to germinate, were used as evaporation controls. Precipitation was excluded by manual deployment of transparent 150 µm polyethylene rainout shelters (3 × 0.6 × 0.5 m, Haxnicks 50–5000, Tierra-Derco, Jasper, IN, United States) when rainfall was forecast. PAR was measured under the rainout shelter at ∼2 h intervals throughout a day with the quantum sensor and correlated to solar radiation measured by the weather station. The rainout shelters, which reduced PAR by about 15%, were deployed for 13% of the outside phase duration of the WUE assay, with more than 60% of this deployment time in the dark (**Figure 1A**). Due to uncertainty in weather forecasting, not all deployments coincided with measurable rainfall (e.g., night of August 28–29). Midday air temperatures were around 35°C, with tray temperatures, adjacent to the plant pots, above 40°C (**Figure 1B**). While daytime deployment increased the difference between outside air and tray temperatures (e.g., August 27), this was primarily driven by delaying the precipitation-associated outside air cooling, rather than a greenhouse effect, and peak tray temperatures did not coincide with deployment times (**Figure 1B**). The observed divergence in VPD between outside air and the trays at these times was associated with higher humidity, as well as these lower temperatures, outside the shelters during the precipitation event, and extreme tray VPD also did not coincide with deployment times (**Figure 1C**).

Zeta-cypermethrin insecticide (GardenTech Sevin Insect Killer Concentrate, TechPac, Atlanta, GA, United States) was applied at labeled maize rate on August 27. Non-destructive chlorophyll contents were estimated 13 days after planting from absorbance with the atLEAF STD (FT Green, Wilmington, DE) (Zhu et al., 2012) approximately at the midpoint of the youngest leaf with ligule emergence, avoiding the mid vein. Plant mature leaf area 14 days after planting was estimated non-destructively from the width (digital calipers) and length (ruler) of each leaf with ligule emergence, assuming rectangular leaf geometry. Stem (culm) volume 15 days after planting was calculated from stem diameter and stem height, assuming cylindrical geometry. Stem diameter was measured as the maximum width at lid height with digital calipers. Stem height was read with a ruler from the lid to the youngest visible ligule. Vegetative developmental stage was based on the number of leaves with an emerged ligule at harvest 16–17 days after planting. The fresh weight of the biomass above the lid was recorded on the calibrated balance (0.1 g precision), manually cut into ∼2 cm pieces and water content determined on a ∼1.5 g subsample utilizing pre-dried mini cupcake paper cases, oven and a calibrated balance (1 mg precision, USS-DBS15-3, U.S. Solid, Cleveland, OH, United States), to enable estimation of above-ground dry weight.

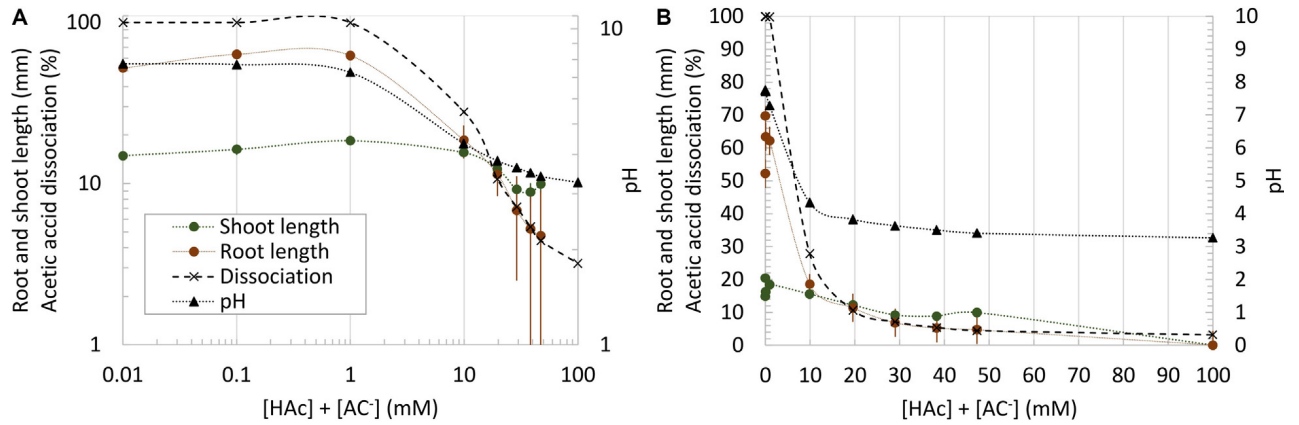


FIGURE 2 | Total (undissociated and dissociated) acetic acid concentration ([HAc] + [Ac$^-$]) impacts on seedling growth in germination paper rolls, along with pH and acetic acid dissociation. Mean root and shoot data (±1 standard error; n = 6) are plotted both on a logarithmic **(A)** and linear **(B)** scale.

After harvest the area that roots were visible on the bottom of each pot were estimated from images captured by a flatbed scanner (H625cdw, Dell, Round Rock, TX, United States). A grid was pasted over each pot base image in Microsoft Powerpoint and a root was manually scored as present for each of the 314 squares if it covered ≥50% of the square. While root observations at transparent interfaces with growth media are well established techniques (Huck and Taylor, 1982; Smit et al., 2000), in this system we did not relate this non-destructive root area measurement with more physiologically-relevant root phenotypes, such as biomass.

Evapotranspiration rate was calculated as the sum of the loss of weight between irrigations. The hole in the lid will result in some direct evaporation from the growth media that will vary due to both the remaining available water in the pot and the weather conditions during that period. A sigmoidal curve was fit to the relationship between evaporation rate from the unplanted/ungerminated controls and their weight (pot + lid + media + water), separately for each watering interval, using the equation below implemented in the eeFIT (v1.05) Microsoft Excel Add-In (Vivaudou, 2019):

$$Evaporation\ rate = Max\frac{W^h}{W + K^h}$$

Where:

Evaporation rate = grams of water loss per day per pot.

Max = Maximum evaporation rate; iteratively fit, initiated at 1 g day^{-1}.

W = Measured weight of pot before re-watering (g).

h = Maximum slope; iteratively fit, initiated at 1.

K = Weight of pot at 50% of *Max*; iteratively fit, initiated at 1 g.

While W included the pot, lid and growth media weight, these were small and relatively consistent compared to the variation in water content between control pots. This estimate of evaporation rate for each day and pot weight was then subtracted from the measured evapotranspiration rate for each plant to estimate daily transpiration rate and this was summed over the experiment and used with above ground biomass to estimate WUE.

RESULTS

Acetic Acid and pH Impacts on Seedlings

Maize root and shoot growth in unbuffered water-soaked germination paper rolls were significantly ($p < 0.05$) inhibited by acetic acid at doses as low as 10 mM, compared to 0 mM controls, however, this parallels similar drops in pH and acetic acid dissociation over this range (**Figure 2**). In contrast to **Figure 2**, when root and shoot growth were examined in rolled germination paper across 100 incubations where pH and acetic acid concentration were uncoupled, the robust trend between [HAc] + [Ac$^-$] and seedling growth was lost (**Figures 3A,B**). However, the root and shoot inhibition was also not simply predicted by pH (**Figures 3C,D**) or [H$^+$] (**Figures 3E,F**). Rather, shoot and root length was responding tightly to [HAc] (**Figures 3G,H**). These results in **Figures 3C–H** are based on pH measured at the end of the experiment, but similar results were obtained when the initial pH was used (data not shown). An estimated [HAc] above 10 mM inhibited root growth by >90%, while shoot growth inhibition didn't reach this level until >45 mM [HAc]. No robust stimulation of seedling root or shoot growth were observed over 4 orders of magnitude of [HAc] + [Ac$^-$] (**Figures 3A,B**).

Acetic Acid and Growth Media Impacts on Seedlings

The peat and potting mix substrates had markedly different pH values, and in contrast to the irrigation solution, increasing acetic acid concentrations did not reduce the pH. **Figure 4** demonstrates that with pH values below 4.5 for the irrigation solutions and peat, the acetic acid was almost entirely undissociated. In contrast, the buffering capacity of the potting mix maintained the pH above 6 where the acetic acid was mostly dissociated. Significant acetic acid impacts on seedling shoot

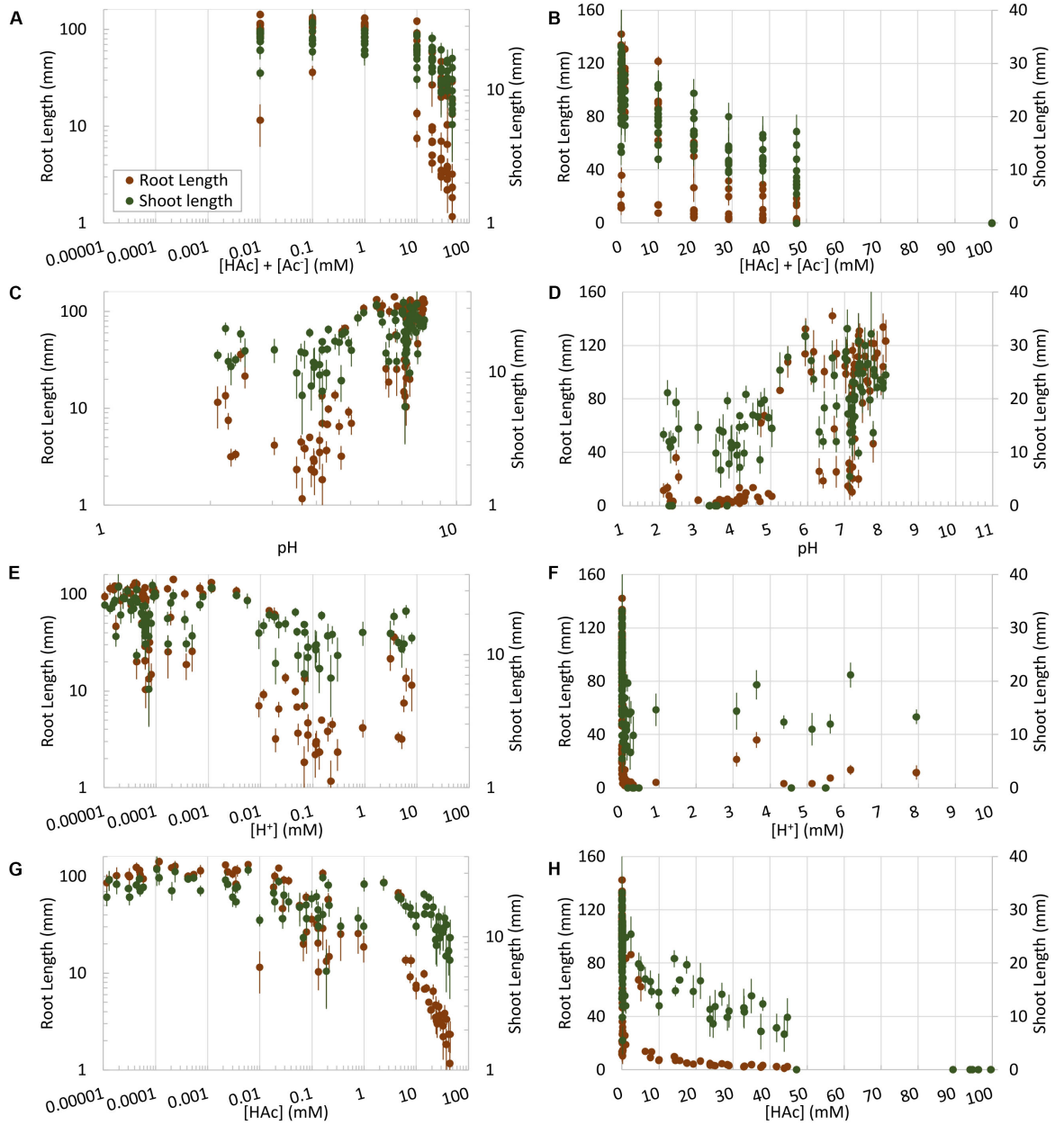


FIGURE 3 | Undissociated acetic acid ([HAc]) **(G,H)** impacts on root and shoot growth on germination paper, along with total acetic acid ([HAc] + [Ac$^-$]) **(A,B)** or acidity expressed as [H$^+$] **(E,F)** or pH **(C,D)**. Mean root and shoot data (±1 standard error; n = 6) are plotted both on logarithmic **(A,C,E,G)** and linear **(B,D,F,H)** scales.

and root growth were observed ($p < 0.05$), but not related to the substrate type ($p > 0.1$). Growth media type × acetic acid was weakly significant (p = 0.05–0.1), specifically with 38 mM [HAc] + [Ac$^-$] significantly ($p < 0.05$) reducing both root and shoot length in peat, compared to the 0 mM controls, whereas in the potting mix 47 mM was required to significantly stunt even the root growth (**Figures 5A,C**). When expressed on an undissociated basis (**Figures 5B,D**), significant root and shoot inhibition was detected at 1.2 mM [HAc] in potting mix, but only above 29 mM in peat, and none of these treatments on peat produced the >90% reduction in root length seen on germination paper at these [HAc] (**Figures 3G,H**). No significant stimulation of seedling root or shoot growth was observed at any acetic acid dose or substrate (**Figures 5A,C**).

Acetic Acid and Drought Impacts on WUE

Water and acetic acid treatments impacted seedling growth, but no significant interactions were observed ($p > 0.1$), therefore

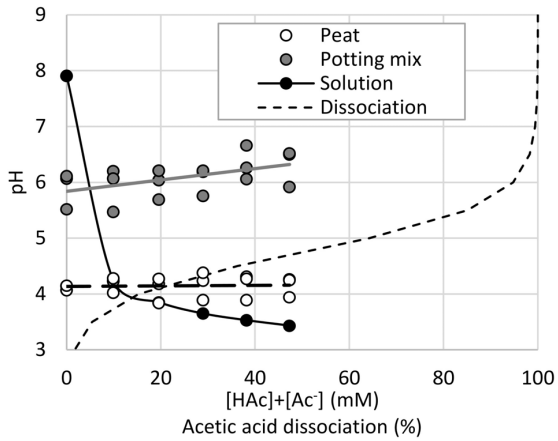


FIGURE 4 | pH of a range of total (undissociated and dissociated) acetic acid ([HAc] + [Ac$^-$]) irrigation solutions and the resulting pH of treated seedling peat and potting mix growth media (n = 3), fitted with a linear regression, along with the expected dissociation of acetic acid at 25°C.

only main water and acetic acid effects are shown in **Figure 6**. Clear dose responses to water availability were observed in many of these phenotypes, including shoot weight (**Figure 6A**), stem volume (**Figure 6I**), transpiration (**Figure 6C**), and shoot water content (**Figure 6G**). Whole plant WUE (**Figure 6E**) and root area (**Figure 6O**) exhibited significantly higher values at intermediate water availability. Leaf area (**Figure 6K**), leaf chlorophyll content (**Figure 6M**) and developmental stage (**Figure 6Q**) were not significantly impacted by water treatment.

In contrast to water availability, none of these phenotypes exhibited a straight-forward acetic acid dose response, demonstrated by no significant differences between the lowest (0 mM) and highest (47 mM) [HAc] + [Ac$^-$] for any measurement (**Figure 6**). This is despite 10, 20, and/or 29 mM [HAc] + [Ac$^-$] having significantly lower stem weight, stem volume, transpiration, chlorophyll content and root area, than 0 mM (**Figure 6**). As with the water treatments, no significant acetic acid impacts were observed on leaf area (**Figure 6L**) or developmental stage (**Figure 6R**). No growth stimulation was observed at any acetic acid dose (**Figure 6**).

DISCUSSION

In contrast to prior observations in barley (0.1–1 mM acetic acid at a pH of 6.5) (Lynch, 1977), no treatment increased root growth on germination paper in maize, despite application of 4 orders of magnitude in acetic acid concentration (**Figure 3**) and 12 pH units. Rather we confirmed our prediction that lowering the pH (e.g., <3) of low acetic acid doses (e.g., 10 mM) increased damage to roots, and raising the pH (e.g., >5) of

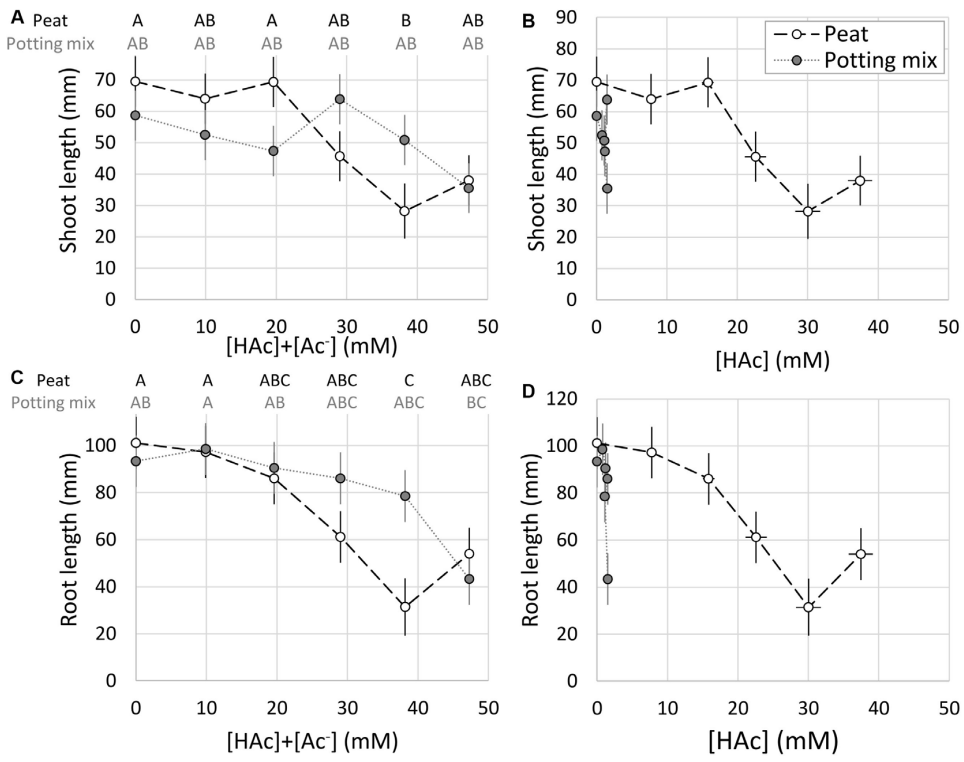


FIGURE 5 | Total ([HAc] + [Ac$^-$]) **(A**,**C)** and undissociated ([HAc]) **(B,D)** acetic acid concentration impacts on seedling root **(C,D)** and shoot **(A,B)** growth (mean ± 1 standard error of the mean) in peat and potting mix growth media with contrasting pH. Significant differences between treatments ($p < 0.05$) are marked with contrasting letters (**A**,**C**; blocked factorial ANOVA; Tukey pairwise comparison; n = 6).

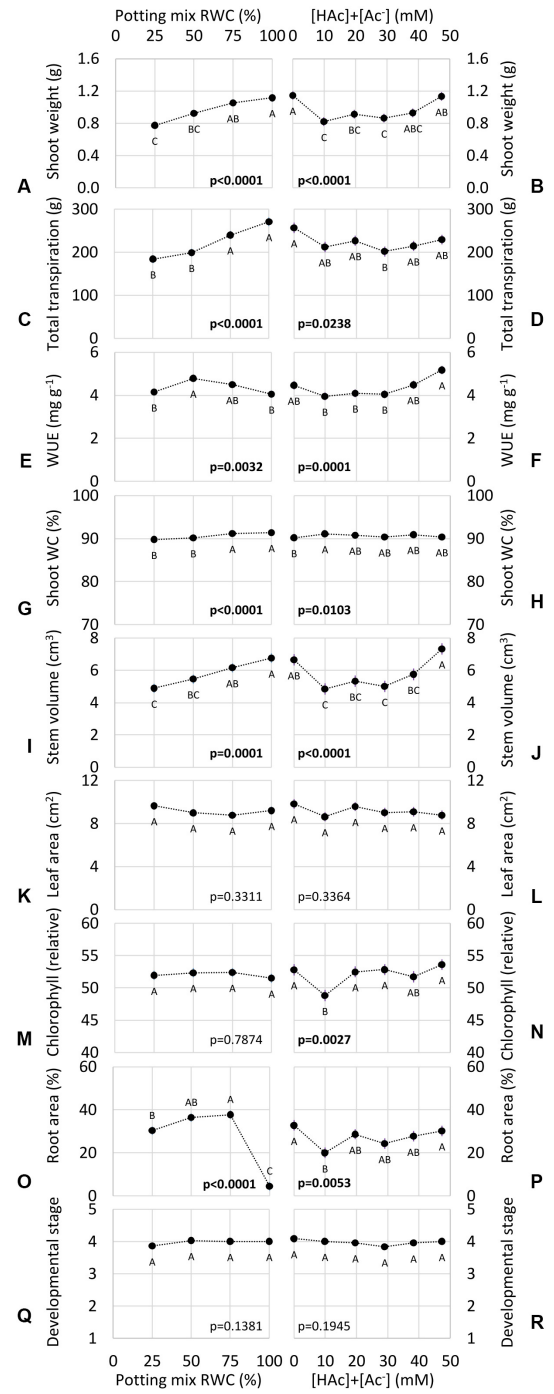


FIGURE 6 | Potting mix Relative Water Content (RWC) and total (undissociated and dissociated) acetic acid ([HAc] + [Ac$^-$]) treatment impacts in the outdoor Water Use Efficiency (WUE) assay on a range of seedling growth phenotypes (means ± 1 standard error of the mean), including shoot weight **(A,B)** and Water Content (WC) **(G,H)**, leaf area **(K,L)** and chlorophyll content **(M,N)**, stem volume **(I,J)**, root area **(O,P)** and plant transpiration **(C,D)**, WUE **(E,F)** and developmental stage **(Q,R)**. None of the water by acetic acid interactions were significant ($p > 0.1$), so only the main effects are presented. Contrasting letters denote significant differences ($p < 0.05$) within water **(A,C,E,G,I,K,M,O,Q)** or acetic acid **(B,D,F,H,J,L,N,P,R)** treatments (blocked factorial ANOVA; Tukey pairwise comparison; $n = 6$).

high doses of acetic acid (e.g., 20 mM) protected roots growing on poorly buffered germination paper, in a predictable manner based on the known dissociation constant of this weak acid. This supports our hypothesis that [HAc] is causing seedling growth inhibition responses on germination paper, rather than [HAc] + [Ac$^-$], with greater sensitivity of root than shoot growth (**Figure 3**). High pH protection of high acetic acid concentrations is not complete, suggesting additional detrimental factors are at play under these conditions. This root growth sensitivity complicates interpretation of the recently reported water stress related phenotypes induced by acetic acid (Kim et al., 2017; Isaji et al., 2018; Rahman et al., 2019; Utsumi et al., 2019), where [HAc] was undefined. The presence of low concentrations of residual ethanol in the treatment vinegar has the potential to confound these acetic acid effects. While this cannot be entirely discounted from the available data, significant treatment impacts were observed at 10 mM acetic acid, where ethanol concentrations would be <0.4 mM, that is more than two orders of magnitude below the ethanol treatment with observed impacts on abiotic stress tolerance in *Arabidopsis* and rice (*Oryza sativa* L.) (Kato-Noguchi, 2008; Nguyen et al., 2017). In contrast to weak acids like acetic, there is also not an obvious mechanism for the observed interaction between ethanol concentration and pH.

The organic components of the potting media, especially the peat present in both, contribute significant buffering capacity (Blok et al., 2017) which eliminated any reduction in pH from acetic acid addition, uncoupling acetic acid concentration and expected dissociation, in contrast to unbuffered solutions (**Figure 4**). **Figure 5** is consistent with the prior observation that high acetic acid stunts seedling growth (**Figure 3**), but this is mostly ameliorated by the high pH (∼6) and buffering capacity of the potting mix. While this inhibition was apparent at lower acetic acid concentrations when grown in the lower pH (∼4) peat, this was not as large an impact as expected, considering almost all the acetic acid is expected to be undissociated at this pH. This suggests that peat provides additional protection of roots from high [HAc] damage, compared to rolled germination paper, such as through increased macronutrient availability. Intriguingly, with peat-derived (Billard et al., 2014) humic substances also identified as biostimulants in maize (Savy et al., 2020), there is the potential for an indirect impact of acetic acid on seedlings through production and release of more active humic compounds from the growth media. With acetic acid a known substrate of soil bacteria and fungi, particularly under aerobic conditions (Sigren et al., 1997; Chauhan and Ogram, 2006; Ostendorf et al., 2007; Herron et al., 2009), microbial community and oxygen availability are also potential interacting factors for additional study, along with testing in media only differing in buffering capacity and more diverse and agronomically-relevant soils. While these seedling experiments were helpful in understanding the interaction between root growth, acetic acid and pH, they were all conducted under well hydrated conditions, and so may not relate to the reported drought-tolerance phenotypes.

The robustness and sensitivity of the WUE assay was demonstrated in **Figure 6**, which shows expected drought-intensity response phenotypes, with declining above-ground biomass, transpiration and water content at lower water levels.

In contrast, there is no clear dose response to acetic acid concentration, but rather plants watered with 10–29 mM [HAc] + [Ac^-] in potting mix had reduced growth and water use, compared to both lowest (0 mM) and highest (47 mM) treatments. Intriguingly, a reduction in transpiration rate in response to 29 mM [HAc] + [Ac^-] (**Figure 6**) aligned closely with improved desiccation survival in maize treated with 30 mM previously observed (Kim et al., 2017), which could be expected from such anti-transpirant behavior. However, this reduction in water use was not associated with any phenotypes associated with improved performance under drought, such as WUE or biomass (**Figure 6**). Furthermore, as with Kim et al. (2017) these phenotypes disappeared at both lower and higher acetic acid doses (**Figure 6**). Precise concentration control in crops would be agronomically challenging for such a substrate for microbial growth (Sigren et al., 1997) with high solubility. Field experiments in soil under agronomically-relevant limited water availability are required to determine, first, whether acetic acid-induced reductions in water use and root growth are reproducible, and second, if this is associated with an increase in yield.

CONCLUSION

The concentration of the membrane permeable undissociated form of acetic acid was demonstrated to drive maize seedling root inhibition under unbuffered conditions. This was confirmed in potting media, although peat provided partial protection from high [HAc]. A reduction in transpiration was observed with 29 mM [HAc] + [Ac^-], but this did not lead to an increase in growth or interaction with deficit irrigation. Field trials are necessary to determine the biostimulant potential of this reduction in water use, under agronomically-relevant water-limited conditions. Furthermore, future studies on acetic acid impacts on drought tolerance need to characterize the treatment in terms of [HAc], and root growth inhibition impacts on transpiration should be excluded before claims of improved performance with reduced water availability are warranted.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

MA and DA designed the study. MA completed the experiments. DA conceived the study, performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

ACKNOWLEDGMENTS

We are most grateful to John C. Edwards of Process NMR Associates, LLC, for sharing the unpublished ^{1}H q-NMR data on the vinegar. We would also like to thank Alison Allen for sharing the workspace and for alerting us to the risk of high temperature germination inhibition.

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Conflict of Interest: DA was an employee of Shell International Exploration and Production Inc.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

PERSPECTIVE
published: 25 May 2020
doi: 10.3389/fpls.2020.00634

Phytomicrobiome Coordination Signals Hold Potential for Climate Change-Resilient Agriculture

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A plant growing under natural conditions is always associated with a substantial, diverse, and well-orchestrated community of microbes—the phytomicrobiome. The phytomicrobiome genome is larger and more fluid than that of the plant. The microbes of the phytomicrobiome assist the plant in nutrient uptake, pathogen control, stress management, and overall growth and development. At least some of this is facilitated by the production of signal compounds, both plant-to-microbe and microbe back to the plant. This is best characterized in the legume nitrogen fixing and mycorrhizal symbioses. More recently lipo-chitooligosaccharide (LCO) and thuricin 17, two microbe-to-plant signals, have been shown to regulate stress responses in a wide range of plant species. While thuricin 17 production is constitutive, LCO signals are only produced in response to a signal from the plant. We discuss how some signal compounds will only be discovered when root-associated microbes are exposed to appropriate plant-to-microbe signals (positive regulation), and this might only happen under specific conditions, such as abiotic stress, while others may only be produced in the absence of a particular plant-to-microbe signal molecule (negative regulation). Some phytomicrobiome members only elicit effects in a specific crop species (specialists), while other phytomicrobiome members elicit effects in a wide range of crop species (generalists). We propose that some specialists could exhibit generalist activity when exposed to signals from the correct plant species. The use of microbe-to-plant signals can enhance crop stress tolerance and could result in more climate change resilient agricultural systems.

Keywords: phytomicrobiom, stress resiliance, biostimulants, crop, signal compounds

OPEN ACCESS

Edited by:
Andrés Calderín García, Universidade Federal Rural do Rio de Janeiro, Brazil

Reviewed by:
Joelle Sasse Schlaepfer, University of Zurich, Switzerland
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 January 2020*
Accepted: *24 April 2020*
Published: *25 May 2020*

Citation:
Lyu D, Backer R, Subramanian S and Smith DL (2020) Phytomicrobiome Coordination Signals Hold Potential for Climate Change-Resilient Agriculture. Front. Plant Sci. 11:634. doi: 10.3389/fpls.2020.00634

INTRODUCTION

Plants in nature are always in relationships (Raina et al., 2018) with a microbial community (the phytomicrobiome); some members of the soil microbial community assist plant growth and development (Prithiviraj et al., 2003; Smith et al., 2015a,b). The phytomicrobiome plus the plant constitute the holobiont—the holobiont is the entity that evolution acts upon, and that produces crop yield (Smith et al., 2017; Cordovez et al., 2019). When adaptation to environmental stressors is needed, the plant: (1) alters its own gene expression and resulting physiology, and also (2) adjusts the diversity, composition, and activity of its phytomicrobiome (Smith et al., 2015b; Gopal and Gupta, 2016). The latter allows for very short-term adjustments, including evolution of the phytomicrobiome; the plant genome evolves much more slowly (Mueller and Sachs, 2015). The genome of the phytomicrobiome (much larger than the plant genome) plus the plant

genome comprises the hologenome or the pan-genome (the host plus the microbial metagenome) (Berendsen et al., 2012; Guerrero et al., 2013; Turner et al., 2013; Bordenstein and Theis, 2015).

It seems that evolution of more complex eukaryotic cells (Phylum Lokiarchaeota—Turner et al., 2013; Spang et al., 2015) from simpler prokaryotes, allowed development of the holobiont (Embley and Martin, 2006; Douglas, 2014; Koonin and Yutin, 2014; Graham et al., 2018). Beneficial relationships between terrestrial plants and microbes have existed since plants moved into the terrestrial environment, almost half a billion years ago (Knack et al., 2015). For about a billion years prior to this, algae had relationships with compatible microbial species, sometimes leading to new organisms. For example, *Ascophyllum nodosum* appears to be a fusion of a macroalga and a fungus (Deckert and Garbary, 2005).

The phytomicrobiome is tissue-specific and relationships vary in intimacy all the way to complete incorporation/fusion, as is the case with mitochondria and chloroplasts (Backer et al., 2018). The most abundant and diverse element of the phytomicrobiome is the rhizomicrobiome where microbes live around or within the root tissues, often in the spaces between cells of the cortex (the root is the niche space of these microbes), and use root exudates as a source of energy/reduced carbon (Schlaeppi and Bulgarelli, 2015). Rhizomicrobiome members can stimulate root growth and so improve plant water and nutrient uptake.

SIGNAL EXCHANGE BETWEEN PLANTS AND MICROBES

The activity, diversity, and composition of the phytomicrobiome are often regulated by signal exchange between plants and microbes. This is best understood for the legume-rhizobia nitrogen fixation symbiosis; an isoflavonoid signal released from the plant is recognized by appropriate rhizobia that move up the concentration gradient toward the plant root. The isoflavonoid also triggers expression of nodulation and nitrogen fixation-related genes within appropriate rhizobia, some of which cause production of lipo-chitooligosaccharide (LCO) signals back to the plant (Lian et al., 2002). Detection of LCOs by the plant leads to nodule formation (Buhian and Bensmihen, 2018) and nitrogen fixation, once rhizobia have entered the nodule (Smith et al., 2015a,b; Bender et al., 2016). In some legumes, exposure to appropriate LCOs, in the absence of rhizobia, is sufficient to cause nodule formation; however, the nodules do not fix nitrogen. In another example, mycorrhizal fungi establish relationships with a wide range of plants (MacLean et al., 2017), mainly to facilitate uptake of soil phosphorus and, in some cases, contributing to the parasitism of other plants (e.g., some epiphytic orchids) (Latef et al., 2016). The plant produces strigolactones as a signal to the appropriate fungus and the fungus produces LCOs or similar compounds as return signals (MacLean et al., 2017). *Parasponia*, the only non-legume fixing nitrogen in symbiosis with rhizobia uses signals similar to the legume symbiosis (Behm et al., 2020) and the signals have been determined to be involved in establishment of the *Frankia* symbioses, although the exact identities are still unknown (Cissoko et al., 2018).

Research has demonstrated that molecular signaling between plants and members of the phytomicrobiome is involved in a large range of plant–microbe interactions. For example, our laboratory has shown that LCOs and thuricin 17 (a microbe-to-plant signal produced by *Bacillus thuringensis* NEB17) regulate plant growth and related activities, including abiotic stress responses (Smith et al., 2015a,b, 2017). Application of these signals cause expanded leaf area and increased photosynthetic rates (Almaraz et al., 2007; Khan et al., 2008); initial responses to these signals alter plant hormone profiles (Prudent et al., 2016). While specific LCO-crop species pairs exist in the context of legume nitrogen-fixing symbioses, there is evidence that the ability of LCOs to enhance plant stress tolerance is non-specific to crop species (Prudent et al., 2015; Smith et al., 2015a,b). This suggests that the role of LCOs in altering plant stress resilience is an older function than the signaling role in nitrogen fixation. We should anticipate this kind of two-way signal exchange in a reasonable proportion of beneficial plant–microbe relationships.

When a microbe-to-plant signal is required, perhaps due to abiotic/biotic stress conditions, the plant may produce a signal that triggers the release of a return signal by a microbe—this is **positive control** (**Figure 1**) (MacLean et al., 2017; Buhian and Bensmihen, 2018). Many microbes do not release microbe-to-plant signal molecules in culture, in the absence of the plant. However, addition of root exudates, which contain compounds that serve as plant-to-microbe signals, may induce the production of microbe-to-plant signal compounds. **Negative control** would occur when a plant-to-microbe signal compound inhibits microbial signal production; in the absence of the plant-to-microbe signal compound, the microbe produces a microbe-to-plant signal. This behavior might explain why *Bacillus thuringiensis* NEB17 produces large amounts of thuricin 17 when in culture (Subramanian and Smith, 2015), which is metabolically expensive.

Members of the phytomicrobiome can be categorized as generalists or specialists depending on the range of plant species, they elicit effects from: **specialists** affect a narrow range of plant species whereas **generalists** affect a wide range of plant species (**Figure 2**). For example, rhizobia produce LCO signals that are extremely plant species-specific during establishment of the nitrogen-fixation symbiosis (Poustini et al., 2007; Clúa et al., 2018). This constitutes a specialist effect. In contrast, when LCOs promote stress resilience across a wide range of plant species, this constitutes a generalist effect (Smith et al., 2015a,b). Specialists may only exert their effects in the presence of a plant-to-microbe signal compound which is, perhaps, only excreted by plant roots under specific conditions (e.g., nodulation, abiotic or biotic stress). This induces production and release of the microbe-to-plant signal compound into the rhizosphere. An example of a specialist microbe-to-plant signal is lumichrome which is produced by the degradation of riboflavin by specific microbes, such as *Pseudomonas* (Yanagita and Foster, 1956) and *Sinorhizobium meliloti* (Phillips et al., 1999), and promotes the growth of certain crops (Rovira and Harris, 1961; Sierra et al., 1999; Dakora, 2015). It may also be possible for a

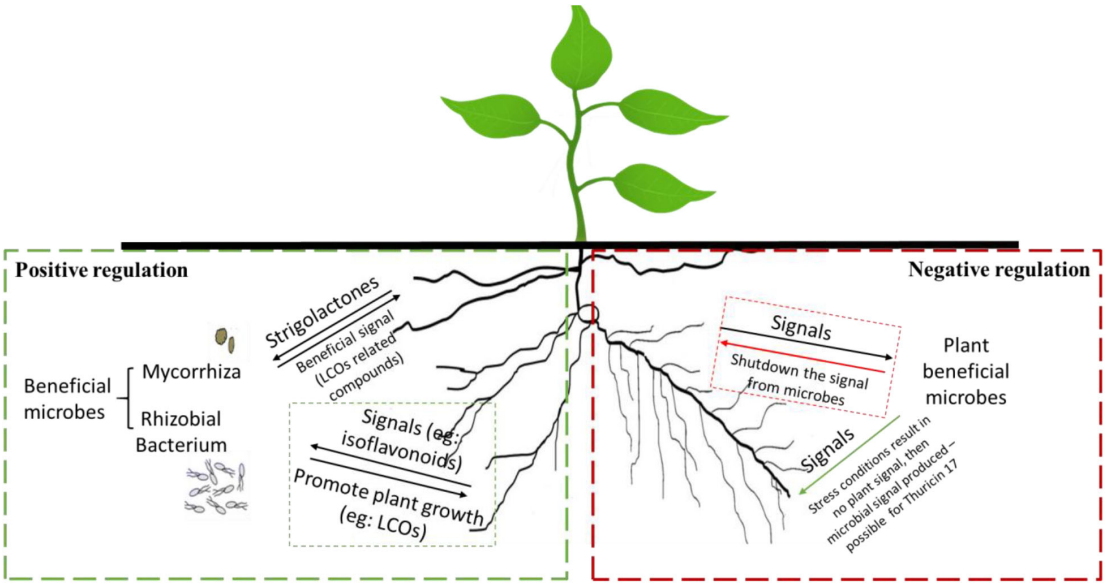


FIGURE 1 | Examples of positive (left side) and negative (right side) regulation of microbe-to-plant signal production by plant-produced signals. **Positive regulation** (left): the plant root secretes a plant-to-microbe signal compound that activates microbe-to-plant signal compound production. **Negative regulation** (right): the plant constitutively produces a plant-to-microbe signal compound that inhibits production of a microbe-to-plant signal compound. When expression of the plant-to-microbe signal compound is downregulated, production of the microbe-to-plant signal compound occurs.

microbe or its signal to be switched from specialist to generalist. For example, LCO is usually only produced by rhizobia in response to a microbe-to-plant signal molecule. However, when exogenous genistein is added to the rhizobial culture, the bacteria produce LCO even in the absence of living rhizobia. This LCO can then stimulate plant growth in a range of crop species (Smith et al., 2015a,b).

EVOLVED BENEFITS OF PLANT–MICROBE INTERACTIONS

When a microbe provides a strong benefit to a plant, the microbe and the plant have probably coevolved for a long time (Wallenstein, 2017) and the microbe may provide multiple benefits to the plant. For instance, microbes that help with abiotic or biotic stress resistance (through priming of stress response pathways, competition or antagonism against plant pathogens) may also assist in plant nutrient acquisition (N fixation, production of siderophores, P solubilization) (Fan et al., 2018). Members of the phytomicrobiome may coordinate activities to increase plant root exudation which benefits the whole microbial community; simultaneously, members of the phytomicrobiome compete for resources, including the niche space provided by plant roots. The microbes may produce compounds that inhibit microbial activity, such as antibiotics. For instance, bacteriocins are proteins synthesized by bacteria; bacteriocins kill closely-related strains, thereby minimizing competition from strains with the greatest metabolic similarity (Gray et al., 2006a,b). Thuricin 17 is both a bacteriocin and a microbe-to-plant signal compound that improves plant stress resilience and is thus a dual-function protein (Subramanian and Smith, 2015). This imposes constraints on evolution—a process that is always pragmatic, random, relentless, and ruthless—to maintain thuricin 17 production due to its multiple biological activities that benefit the microbe. The genome of *B. thuringiensis* strain NEB17 contains three tandem repeats of the gene that produces thuricin 17 and the copies have evolved differences. However, all the nucleotide differences are found in the third codon position and code for amino acid redundancies. So, while the nucleotide sequences vary among the gene copies, the amino acid sequence of the proteins does not (Gray et al., 2006a,b), illustrating the evolutionary conservatism resulting from the dual function nature of the protein encoded by the gene.

Plants have also evolved to recognize and respond to signals exchanged between members of the phytomicrobiome. For example, lactones, which are used as inter-microbial signals in quorum sensing, are monitored and responded to by plants (Ortiz-Castro and López-Bucio, 2019), possibly because biofilms, potentially produced as a result of quorum sensing, can provide benefits for plant growth (Hartmann and Rothballer, 2017; Ricci et al., 2019). In addition, plants and phytomicrobiome members communicate through many volatile organic compounds (VOCs; Lee et al., 2016; Kashyap et al., 2017; Liu and Brettell, 2019). For example, an immobile bacterium that lives in the phyllosphere produces a volatile signal to call over a mobile bacterium, to carry the immobile one along (Hagai et al., 2014).

Some members of the phytomicrobiome deter microorganisms that damage plants, or compete for resources (Droby et al., 2016; Ab Rahman et al., 2018). With increasing concern around environmental impacts of chemical pesticides (Bender et al., 2016), members of the phytomicrobiome

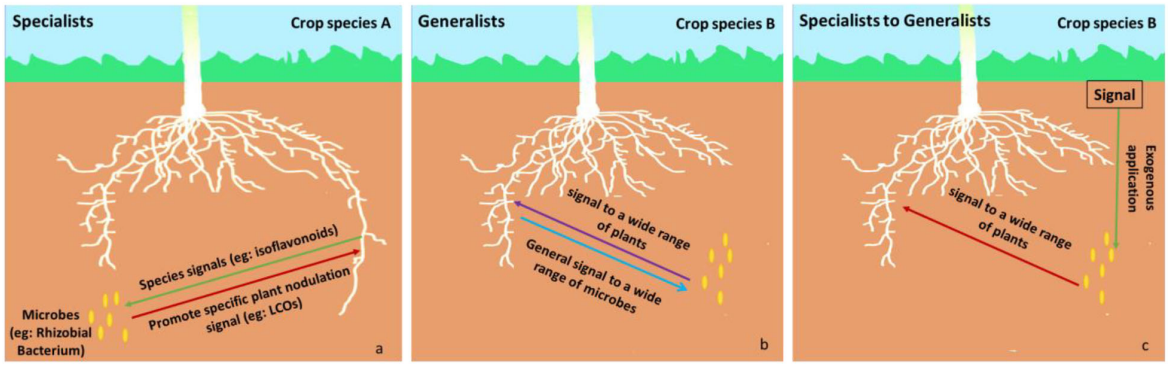


FIGURE 2 | Examples of **specialist** (left side) and **generalist** (middle)**. Specialist (a):** a specific signal (red arrow, e.g., LCOs) is only expressed in the presence of a specific plant-to-microbe signal compound (green arrow, e.g., isoflavonoids) produced by specific *crop species A* (e.g., a specific legume). **Generalist (b):** a general signal (purple arrow) is expressed in the presence of a general plant-to-microbe signal compound (blue arrow) produced by a wider range of plants, such as *crop species B*. In some cases, **exogenous application (Specialist to Generalist c)** of a specific signal (green arrow) could result in the production of a microbe-to-plant signal by a microbe that usually functions as a specialist—the microbe-to-plant signal can be recognized by a wide range of plant species and the microbe is converted from a specialist into a generalist one (e.g., if the plant-to-microbe signal from *crop species* A is applied to a microbe in the presence of *crop species B*). For example, **exogenous application** of a specific plant-to-microbe signal (e.g., genistein, an isoflavonoid from soybean, in a legume nitrogen-fixing symbiosis) results in the production of the microbe-to-plant signal (e.g., LCO) in the rhizosphere of a wide range of plants, where the microbe-to-plant signal has an alternative function (e.g., regulation of plant stress responses).

that produce compounds bacteriostatic or bactericidal to plant-detrimental organisms are of commercial interest (Ab Rahman et al., 2018; Anderson and Kim, 2018). The Bt toxin, originally from a *B. thuringiensis* strain, was genetically engineered into a wide variety of crops because of its insect control activity. Work in our laboratory has shown that the *B. thuringiensis* strain producing thuricin 17 also produces the very effective insecticide beta-exotoxin. In addition, we have recently isolated a pair of compounds, produced by a plant growth-promoting rhizobacteria (PGPR), that are effective against a tomato pathogen (Takishita et al., 2018). These are examples of compounds produced by the phytomicrobiome that can be commercialized to improve crop productivity. In addition, biocontrol organisms can also produce compounds that trigger plant immune responses which represents an alternative mechanism for pathogen control in crops (Ab Rahman et al., 2018; Takishita et al., 2018).

Because evolution never sleeps, signal exchange systems between plants and beneficial microbes have been exploited by parasitic organisms. For example, spores of the pathogen *Phytophthora* can detect isoflavonoid signals from soybean roots and swim up the concentration gradient to find the roots (Hua et al., 2015; Zhang et al., 2019). In addition, seeds of the parasitic plant *Striga* germinate when they detect the plant-to-microbe strigolactone signals, indicating proximity to host-plant roots (Yoneyama et al., 2019). This is a serious problem for crop production in some areas of the world.

THE PHYTOMICROBIOME AND PLANT STRESS

Effects of PGPR on plants can be inconsistent (Nelson, 2004). One possible explanation is that plant growth responses to many PGPR interact with plant stress (Smith et al., 2015a,b; Ilangumaran and Smith, 2017; Vimal et al., 2017; Backer et al., 2018). It is possible that in some cases when PGPR were reported to have effects on plant growth, the plants were growing under stressful conditions and PGPR improved stress resilience. This could have occurred as a result of seemingly benign factors such as, the timing of watering during experiments (leading to intermittent drought stress) or spikes in greenhouse temperatures (leading to acute heat stress). Plant phosphate stress responses may also shape the root microbiome in turn affecting plant immunity (Castrillo et al., 2017). Likewise, salicylic acid, involved in plant stress responses, is also essential for endophytic root microbiome assembly (Lebeis et al., 2015).

Under stress, a plant can: (1) become resilient or (2) become dormant (close stomata, senesce tissues—e.g., leaves under severe drought) (Considine and Considine, 2016). The second option is often associated with elevated levels of plant hormones such as abscisic acid and ethylene. From the perspective of the bacteria, it is desirable that the plant remains resilient, the first option, photosynthesizing and continuing to produce root exudates which serve as a carbon source for PGPR (Backer et al., 2018). Thus, there can be **dynamic tension** between those dependent on plant productivity (phytomicrobiome members and agriculturalists) and the plant, when it faces stress. An example of this would be the regulation of ethylene production from 1-aminocyclopropane-1-carboxylic (ACC) acid by members of the phytomicrobiome. Plant-associated microbes produce ACC deaminase, which prevents the final production of ethylene from ACC (Glick, 2014). This maintains low ethylene levels in the plant, and the plant is less likely to become dormant (Backer et al., 2018). Thus, the phytomicrobiome diverts plant activity to best suit microbial requirements for growth by improving plant nutrient availability and eliciting plant stress responses.

Application of LCO, thuricin 17, jasmonic acid, and VOCs to plants growing under stressful conditions has been reported to improve plant resilience to stress (Subramanian and Smith, 2015; Prudent et al., 2016). When LCOs were sprayed onto leaves of stressed soybean plants (growing at 15°C), stress response genes were the largest class of known-function genes with altered expression levels (Wang et al., 2012). This suggests that the plant switches from one set of stress response genes to another, perhaps, from genes related to dormancy to those related to stress resilience. Subsequent research revealed that treatment with LCO and thuricin 17 (Subramanian and Smith, 2015) increased levels of stress-related and energy metabolism proteins (Subramanian et al., 2016a,b). However, this strategy can go too far as the application of higher concentrations of LCO and thuricin 17 to very stressed plants can result in plant mortality (unpublished data). Other compounds such as jasmonic acid, a hormone involved in plant stress responses, also trigger LCO production by rhizobia (Mabood and Smith, 2005; Mabood et al., 2006b; Smith et al., 2015a). In addition, there are reports of VOCs enhancing plant stress tolerance (Kashyap et al., 2017).

CONTRIBUTION OF THE PHYTOMICROBIOME TO GLOBAL FOOD SECURITY

The phytomicrobiome and the signal compounds exchanged between plants and microbes play a key role in determining crop yields, particularly in the presence of challenges such as a/biotic stresses (Mueller and Sachs, 2015; Wallenstein, 2017), including those associated with climate change (drought, high temperature, flooding, salinity) (Almaraz et al., 2008; Smith and Zhou, 2014; Kashyap et al., 2017; Vimal et al., 2017; Backer et al., 2018). At a time when we are concerned about the environmental impacts of pesticides (Busby et al., 2017) and extensive fertilizer application, PGPR and microbe-to-plant signal molecules offer alternative strategies for increasing, or at least maintaining, crop yields with reduced pesticide and fertilizer inputs while developing more climate change-resilient agricultural systems. There is enormous potential in our ability to manipulate the phytomicrobiome and its signals, as our understanding of this very complex system grows (Mabood et al., 2006a; Schlaeppi and Bulgarelli, 2015; Smith et al., 2015b; Gopal and Gupta, 2016; Głodowska et al., 2017; Lamont et al., 2017; Wallenstein, 2017). In addition, LCO and thuricin 17 are effective at very low concentrations (LCOs: 10^{-6}–10^{-8} M, thuricin 17: 10^{-9}–10^{-11} M; Smith et al., 2015a,b; Subramanian and Smith, 2015) and are inexpensive to produce. The LCO technology is already being applied to tens of millions of hectares of agricultural land each year. The phytomicrobiome can contribute to the effort for global food security.

THE FUTURE OF PLANT–MICROBE INTERACTION RESEARCH

To identify new beneficial strains from the phytomicrobiome, or microbe-to-plant signal compounds, one must have clear objectives, for example: (1) to reduce the impact of stress on crop yields, (2) to reduce fertilizer application, and/or (3) to reduce disease impacts. Rapid and effective screening methods to identify promising microbes and/or microbe-to-plant signals are required (Mueller and Sachs, 2015; Backer et al., 2018). Generalist strains could be isolated from a wide range of plant species; our laboratory has isolated agriculturally useful PGPR from undomesticated plant species. While the phytomicrobiome of domesticated plants is under-investigated, that of undomesticated plants remains very unexplored. Furthermore, exciting discoveries under laboratory conditions may not always prove effective under field conditions since we do not understand all of the nuances of this highly complex and regulated system (Backer et al., 2018). The various natural environments contain a large indigenous community of microbes, experience a wide range of environmental conditions, and vary in soil properties from site to site (Sessitsch et al., 2019) so that a wide range of potential plant-beneficial microbes probably occur in non-agricultural settings.

New methods will have profound effects on research related to phytomicrobiome signaling and plant growth. **Phenotyping** allows determination of subtle but key effects on plants/holobionts, providing the capacity to determine features like space occupancy, in relation to plant light interception (Walter et al., 2015; Lopes et al., 2018). Newer **CT scanning** applications allow for determination of space occupancy and fractal dimensions of undisturbed roots in soil (Costa et al., 2003; Dutilleul et al., 2005, 2008; Lontoc-Roy et al., 2005; Han et al., 2008; Subramanian and Smith, 2015).

One should not fall into the trap of assuming that the effect(s) of a novel growth-stimulating microbe must result from previously established mechanisms (Backer et al., 2018). There will be novel signals with new and surprising new modes of action (Hagai et al., 2014). At this time, we have narrow understanding of how a tiny fraction of plant–microbe interactions occur and coordinate the activity of the holobiont. There is a breathtaking amount to learn.

AUTHOR CONTRIBUTIONS

DS gathered literature and prepared the manuscript. DL contributed to the initial writing and structuring of the manuscript. RB and SS provided feedback and oversaw progression of the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work contained therein.

FUNDING

Funding for this work was provided by a Discovery grant (award number 06328), from the Natural Science and Engineering Council of Canada.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 May 2020
doi: 10.3389/fpls.2020.00583

Plant Defense Stimulator Mediated Defense Activation Is Affected by Nitrate Fertilization and Developmental Stage in *Arabidopsis thaliana*

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OPEN ACCESS

Edited by:
Paolo Carletti, University of Padua, Italy

Reviewed by:
Zhonglin Mou, University of Florida, United States
Dongdong Niu, Nanjing Agricultural University, China

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *18 October 2019*
Accepted: *17 April 2020*
Published: *26 May 2020*

Citation:
Verly C, Djoman ACR, Rigault M, Giraud F, Rajjou L, Saint-Macary M-E and Dellagi A (2020) Plant Defense Stimulator Mediated Defense Activation Is Affected by Nitrate Fertilization and Developmental Stage in Arabidopsis thaliana. Front. Plant Sci. 11:583. doi: 10.3389/fpls.2020.00583

Plant defense stimulators, used in crop protection, are an attractive option to reduce the use of conventional crop protection products and optimize biocontrol strategies. These products are able to activate plant defenses and thus limit infection by pathogens. However, the effectiveness of these plant defense stimulators remains erratic and is potentially dependent on many agronomic and environmental parameters still unknown or poorly controlled. The developmental stage of the plant as well as its fertilization, and essentially nitrogen nutrition, play major roles in defense establishment in the presence of pathogens or plant defense stimulators. The major nitrogen source used by plants is nitrate. In this study, we investigated the impact of *Arabidopsis thaliana* plant developmental stage and nitrate nutrition on its capacity to mount immune reactions in response to two plant defense stimulators triggering two major defense pathways, the salicylic acid and the jasmonic acid pathways. We show that optimal nitrate nutrition is needed for effective defense activation and protection against the pathogenic bacteria *Dickeya dadantii* and *Pseudomonas syringae* pv. *tomato*. Using an *npr1* defense signaling mutant, we showed that nitrate dependent protection against *D. dadantii* requires a functional *NPR1* gene. Our results indicate that the efficacy of plant defense stimulators is strongly affected by nitrate nutrition and the developmental stage. The nitrate dependent efficacy of plant defense stimulators is not only due to a metabolic effect but also invloves NPR1 mediated defense signaling. Plant defense stimulators may have opposite effects on plant resistance to a pathogen. Together, our results indicate that agronomic use of plant defense stimulators must be optimized according to nitrate fertilization and developmental stage.

Keywords: defense (induced), elicitor, *Dickeya dadantii*, nitrate, developmental stage, Bion, Methyl-jasmonate, *Pseudomonas syringae* pv. *tomato*

INTRODUCTION

As sessile organisms, plants are exposed to many biotic stresses such as pathogenic microorganisms and herbivores. They have developed the capacity to activate defenses in response to pathogen attacks thus leading to different degrees of resistance which may be effective at the site of infection or systemically (Jones and Dangl, 2006; Wirthmueller et al., 2013; Miller et al., 2017; Alhoraibi et al., 2019; Shine et al., 2019). Complex signaling networks are activated according to the type of invading organism (Bürger and Chory, 2019; Shine et al., 2019; Wang et al., 2020). Defense-related signaling responses involve phosphorylation events, ionic fluxes and accumulation of phytohormones leading to transcriptional activation of genes coding for the synthesis of antimicrobial compounds such as phytoalexins or pathogenesis related (PR) proteins (van Loon et al., 2006; Pieterse et al., 2014; Piasecka et al., 2015; Klessig et al., 2018; Wang et al., 2020).

Salicylic acid (SA) is one of the major hormones involved in plant immunity and was described as being mainly involved in plant protection against biotrophic or hemibiotrophic pathogens (Pieterse and Van Loon, 1999; Glazebrook, 2005; Zhang and Li, 2019). Defense genes activated by SA include *PR5*, encoding a thaumatin-like protein and *PR1*, encoding an antimicrobial protein with sterol binding and peptide signaling functions (Uknes et al., 1992; Chen et al., 2014; Gamir et al., 2017). Jasmonic acid (JA) and ethylene (ET) were reported to be involved in plant protection against necrotrophic pathogens (Pieterse and Van Loon, 1999; Glazebrook, 2005; Mengiste, 2012) and may be induced by non-pathogenic plant growth promoting rhizobacteria (PGPR) (Backer et al., 2018). JA/ET-dependent responses promote activities of peroxidases, polyphenol oxidases and lipoxygenases (Van Wees et al., 1999; Ruan et al., 2019). Genes encoding the defensin PDF1.2 and the lipoxygenase LOX2, are widely used as markers of the JA/ET defense pathway (Manners et al., 1998; Thomma et al., 1998; Yang et al., 2019). The JA pathway, with *LOX2* as a marker gene, is effective against insect pests (Glauser et al., 2009).

Several reports indicate the existence of cross-talks between those defense signaling pathways (Pieterse et al., 2011; Thaler et al., 2012; Vos et al., 2015; Bürger and Chory, 2019; Yang et al., 2019). An antagonism was generally described between SA dependent defenses and JA/ET dependent defenses (Koornneef and Pieterse, 2008; Leon-Reyes et al., 2010; Van der Does et al., 2013; Caarls et al., 2017; Li et al., 2019; Yang et al., 2019). The mechanisms underlying this antagonism imply transcriptional regulations involving transcription factors such as WRKY70 and ROXY19 (Li et al., 2004, 2019; Ndamukong et al., 2007; Caarls et al., 2015). Interestingly, synergism between SA and JA pathways was also described (Mur et al., 2006; Liu et al., 2016).

The NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 protein (NPR1) (Cao et al., 1997; Mou et al., 2003; Durrant and Dong, 2004) is a key defense regulator, known to be involved in both SA and JA/ET signaling pathways (Pieterse and Van Loon, 2004; Withers and Dong, 2017; Barker, 2018; Backer et al., 2019). Its role in SA defense signaling has been well-studied and described. NPR1 binds SA (Wu et al., 2012; Manohar et al., 2015) and is thought to be a co-receptor with two other proteins NPR3 and NPR4 which also bind SA and act as transcriptional repressors of the SA response (Kuai et al., 2015; Withers and Dong, 2016; Ding et al., 2018). Following SA perception, NPR1 binds to TGA transcription factors leading to the transcription of PR genes (Després et al., 2000; Kinkema et al., 2000; Zhou et al., 2000; Chern et al., 2001; Kuai et al., 2015). The mechanism by which NPR1 is involved in JA/ET defenses remains unclear. An *A. thaliana npr1* mutant plant fails to induce *PR* gene expression in response to SA, while *NPR1* overexpression leads to an up-regulation of the *PR* genes and enhanced disease resistance (Cao et al., 1998). NPR1 is also involved in the activation of JA/ET dependent defenses but probably via an alternate mechanism. An *A. thaliana npr1* mutant is unable to activate the JA/ET dependent defenses in response to PGPR (Koornneef and Pieterse, 2008; Nie et al., 2017). Overexpressing engineered forms of NPR1 retained in the plant cytosol results in the suppression of JA signaling (Spoel et al., 2003, 2007; Yuan et al., 2007) indicating that the antagonistic effect of SA over JA signaling requires cytosolic NPR1. Spoel et al. (2003) suggested a cytoplasmic role of NPR1 in the cross-talk between JA/ET and SA defense pathways.

Different environmental conditions may influence plant pathogen interactions such as the type of light (Kazan and Manners, 2011; De Wit et al., 2013; Janda et al., 2015; Mintoff et al., 2015) mineral nutrition (Poschenrieder et al., 2006; Walters et al., 2007; Fagard et al., 2014; Aznar et al., 2015) or water availability (Nejat and Mantri, 2017). The impact of fertilizers, in particular nitrogen fertilization, on plant-pathogen interactions is well-documented however, the underlying mechanisms remain unclear (Shaner and Finney, 1977; Eyles et al., 2007; Pato and Obeso, 2013; Veresoglou et al., 2013; Fagard et al., 2014; Mur et al., 2017; Sun et al., 2020).

Nitrogen is present in the form of NO_3^-, NH_4^+ or amino acids, the availability of which depends on physical factors such as pH and temperature. Plants adapted to acidic pH tend to take up NH_4^+ or amino-acids and plants adapted to higher pH and aerobic soils (which is the case of most arable lands) tend to prefer NO_3^- (Masclaux-Daubresse et al., 2010). Nitrate is taken up at the root level by two different types of transport systems (Krapp et al., 2014; Wang et al., 2018): (1) a high affinity system involving the NRT1/NPF (nitrate transporter 1/peptide transporter family) family of transporters, (2) a low affinity system involving the NRT2 family of transporters. Following uptake, NO_3^- is reduced to NO_2^- by a cytosolic nitrate reductase, and then NO_2^- is further reduced by a plastidial nitrite reductase into NH_4^+ (Masclaux-Daubresse et al., 2010). Ammonium is incorporated into amino acids in plastids via glutamate synthase (GS)/glutamine-2-oxoglutarate-aminotransferase (GOGAT) cycle (Masclaux-Daubresse et al., 2010; Krapp et al., 2014; Wang et al., 2018). Nitrogen fertilization has been a major factor in improving crop productivity in the last decades (Hirel et al., 2007; Wang et al., 2018) but may increase disease impact depending on the considered pathosystem (Fagard et al., 2014; Mur et al., 2017; Sun et al., 2020). A better understanding of the mechanisms by which nutrient elements

influence plant defenses may be useful to improve cultural practices in order to optimize fertilization and reduce pesticide use thus decreasing the environmental impact of agriculture.

Emerging new plant protection strategies based on the exploitation of the capacity of plants to mount efficient immune responses are widely explored and are expected to allow the reduction of pesticide use (Ramamoorthy et al., 2001; Heil and Bostock, 2002; Bektas and Eulgem, 2015). These strategies rely on the use of plant defense stimulators which trigger plant defenses before or upon pathogen attack. Such plant defense stimulators include Bion® which contains the bioactive molecule S-acibenzolar-S-Methyl activating the SA dependent defense pathway and is used in agriculture to protect tomato or apple against pathogens. However the effectiveness of plant defense stimulators in the field remains uncertain and may depend on different internal and/or external factors such as the plant developmental stage, temperature, drought, and/or mineral nutrition (Walters et al., 2007; Steimetz et al., 2012; Carella et al., 2015).

Our work addresses the combined impacts of the plant developmental stage and nitrogen nutrition on the efficiency of plant response to plant defense stimulators. We show that plant response to plant defense stimulators depends on both developmental stage and nitrogen nutrition with a stronger effect of nutrition.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* Col-0 WT accession were obtained from Versailles Arabidopsis Stock Center (INRA Versailles France) and seeds of *npr1-1* mutant (N3726) in Col-0 WT background (Cao et al., 1997) were obtained from NASC[1]. Seeds were sown in unfertilized soil with different nitrate fertilization conditions 2, 10, and 26 mM of nitrate (**Supplementary Table 1**), in a growth chamber under the following conditions; 18 h of light 21°C, 6 h of dark 19°C, 70% relative humidity). Plants were grown until four different stages: Stage 1: plantlet (2 weeks after sowing (A.S.), Stage 2: vegetative stage (3 weeks A.S.), Stage 3: floral induction (4 weeks A.S.), Stage 4: flowering time (5 weeks A.S.).

Nitrate Content Quantification

Leaves were harvested 48 h after treatment and immediately crushed in liquid nitrogen then stored at −80°C. Nitrate content is measured by a spectrophotometric method by comparison with a $NaNO_3$ scale (Miranda et al., 2001). Ten milligrammes of frozen leaf powder were incubated in 300 µL of sterile distilled water during 20 min on ice. Samples were centrifuged at 15,000 rpm at 4°C during 20 min and supernatant was harvest twice. Ten microliters of supernatant were mixed with 90 µL of water and 100 µL of Miranda reagent (0.5M HCl, 0.25% Vanadium chloride, 0.005% *N*-1-naphtyethylendiamin, 0.1% Sulfanilamide) and incubated during 2 h at 60°C. Concentration of nitrate was then calculated based on a standard curve obtained with $NaNO_3$ standard solutions by spectrophotometry at 540 nm. For each experiment 20 rosettes were used.

Amino Acid Quantification

Amino acid content was measured by a spectrophotometric method adapted from Rosen (1957). Amino acids were extracted by vortexing 150 mg of frozen leaf powder with 1 mL of 2% 5-sulfosalicylic acid (w/v in water). Samples were then centrifuged at 13,000 rpm during 10 min and supernatant was harvested for the following steps. Fifty microliters of samples were mixed with 150 µL of water, 100 µL of cyanide acetate solution (0.2 mL of 10 mM KCN, 9.8 mL of 2.65M sodium acetate, 8% acetic acid pH 5.35) and 100 µl of ninhydrine solution (3% ninhydride in Ethylene glycol monomethylether) then incubated during 15 min at 100°C under fume hood, before adding 1 mL of 50% isopropanol. Samples were placed on ice to decrease temperature to room temperature. Two hundred microliters of samples were used to quantify amino acid content by comparison with standard solutions of L-glutamine by spectrophotometry at 570 nm. For each experiment 20 rosettes were used.

RNA Extraction and cDNA Synthesis

RNA extraction and cDNA synthesis were performed as described in Aznar et al. (2014). For each experiment 6 to 10 plants were used.

Quantitative PCR

Quantitative PCR reactions were carried out in 10 µL, with 5 µL of SybrGreen® (Bio-Rad, Hercules, CA, United States) mix, 0.3 µM of each primer, and 2.5 µL of cDNA. Quantitative PCRs were carried out using a CFX-96 Real Time PCR system thermal cycler (Bio-Rad, Hercules, CA, United States). The raw data obtained were processed using the CFX manager software (Bio-Rad, Hercules, CA, United States). For each analysis, a cycle threshold (Ct) value was extracted and then transformed into Starting Quantity (SQ) values based on a standard curve equation. Consequently, for each condition, since PCRs were performed in triplicate, 3 SQ values were obtained for each sample and then averaged (geometric mean of SQ values). The geometric mean of the SQ values obtained for each gene of interest was then divided by the geometric mean of SQ values obtained for the reference gene. Normalized transcript level was then obtained and expressed as arbitrary units. Clathrin was used as a reference gene because it was stably expressed under the different stages and the different nutritional conditions. Experiments were performed three times with similar results. Representative data are shown.

Plant Treatment With Plant Defense Stimulators

In all experiments, plants were kept under cover 24 h before plant defense stimulator treatment. Then, plants were sprayed with

[1]http://www.arabidopsis.info

methyl-jasmonate (at 0.1 mM with 0.5% DMSO); the commercial plant defense stimulator Bion® (at 0.015% in water w/v), or water. The Bion® concentration was determined based on a calculation starting from the recommended dose for its agronomic use. The recommended Bion®, dose for tomato in the field (0.05 kg/ha) and was adapted in volume concentration (g/L) considering a field spray at 330 L/ha, this corresponds to a 0.015% in water w/v Bion® solution. Spraying of the different elcicitors was performed separately to avoid cross contamination. Plants were kept under cover and grown in the same growth chamber. For each plant defense stimulator treatment, they were kept in separate boxes. Plants were then harvested 24 or 48 h following treatment, frozen in liquid nitrogen in order to extract RNA and amplify genes by qRT-PCR. For protection assays, plants were inoculated with the pathogenic bacteria as indicated below 48 h following plant defense stimulator treatments.

Bacterial Strains and Inoculation Method

The *Dickeya dadantii* 3937 strain was obtained from our own collection. Bacteria were grown in Luria-Bertani medium. Forty-eight hours following water or plant defense stimulator treatment, bacterial inoculation was performed. For plant inoculation, a bacterial suspension at an OD600 of 0.1 (10^8 C.F.U./mL) made up in 50 mM potassium phosphate buffer (pH 7) was used. Plants were covered during the whole assay to obtain saturating humidity and facilitate infection. To inoculate plants, a small hole was made with a needle in the leaf, and then, 5 µL of a bacterial suspension was deposited on the hole. In each experiment, 16 plants were inoculated for each condition and two leaves per plant were inoculated.

Disease severity levels were then scored 48 h post-inoculation (p.i) identified as the best timing for comparing disease severity (Rigault et al., 2017). The proportion of macerated surface in each inoculated leaf was calculated. The surface of the maceretad lesion and the surface of the corresponding leaves were measured using the open source software ImageJ https://imagej.nih.gov/ij/. This allowed calculating an average of lesion surfaces (in cm^2) and an average of proportion of macerated surface for each leaf.

The *Pseudomonas syringae* pv. *tomato DC3000* bacterial strain was from our own stock. Forty-eight hours following plant defense stimulator treatment, plants were sprayed with a bacterial suspension at a concentration of 5×10^7 cfu mL^{-1} in sterile water containing 0.01% Silwett. In planta bacterial populations were monitored 48 h after inoculation. Leaves were harvested then bacterial numbering was performed by tissue grinding followed by serial dilutions plated on King B medium with 60 µg/mL Rifampicin (King et al., 1954).

For each experiment 6 to 8 plants were used and 3 to 4 leaves were harvested or scored. This allowed us to analyze at least 20 leaves for each experiment.

Statistical Analysis

All experiments were performed in three to four independent biological replicate. The size of all the samples is indicated in the figure legends. EXCEL-STAT plugin was used to perform statistical analysis on data.

RESULTS

Plant Growth Modifications Under Different Nitrate Fertilization Conditions

The objective of this work was to evaluate both the effect of nitrogen nutrition and the developmental stage on defense activation by plant defense stimulators. For this purpose, different growth conditions in terms of nitrate supply and plant age were considered. In order to study the effect of developmental stages on defense activation in Arabidopsis, four developmental stages were considered based on the physiological steps representing four key phases in *A. thaliana* life cycle described in Boyes et al. (2001). In order to study the effect of nitrate nutrition, we chose to study three different levels: (1) limitation, (2) optimal fertilization, and (3) over-fertilization. In order to determine the nitrate concentrations required for these three physiological conditions, the following criteria were considered. A previous work on Arabidopsis nitrogen metabolism showed a differential accumulation of nitrogen related metabolites as well as enzymatic activities in plants cultivated under 2 and 10 mM nitrate (Lemaître et al., 2008). Plants grown under 10 mM nitrate displayed better growth than those grown under 2 mM nitrate (Loudet et al., 2003; Lemaître et al., 2008). Based on these data, the nitrate limitation nutrition used in the present study, was 2 mM nitrate. For the optimal growth conditions, in the present study, we used the 10 mM nitrate. Although, under agronomic conditions over-fertilization occurs quite regularly, it is unclear how over fertilization can affect plant defenses. To address this question, we determined a nitrate level higher than 10 mM, resulting in a reduced plant growth without being lethal or affecting too much plant development. For this purpose, plants grown under 20, 26, and 50 mM nitrate were tested. The 26 mM nitrate concentration slightly affected Arabidopsis growth (**Figure 1**) without being lethal; while 20 mM did not significantly affect plant growth and 50 mM was toxic (data not shown). To confirm that the three nitrate fertilization conditions have different impacts on plant physiology and/or development under the three growth conditions, we determined the impact of nitrate nutrition on some physiological and/or metabolic traits (**Figure 1**). Nitrate and amino acid contents were monitored. Plant growth was quantified via the number of leaves per plant and the projected rosette surface. Plants grown under limiting nitrate levels (2 mM) displayed lower nitrate, amino acid and reduced leaf number and rosette surface at stage 2 to stage 4. This indicates that the lower nitrate 2 mM supply has an impact on plant nitrogen metabolism that can be observed starting from the stage 2. Although growing plants under 26 mM nitrate did not result in an increase in nitrate or amino acid content, the number of leaves (stage 4) and the projected rosette area (stage 3) were affected compared to those grown under 10 mM nitrate (**Figure 1**). This indicates that 26 mM nitrate supply has a negative impact on plant development which can be observed starting from stage 3.

Together these data indicate that the three nutritional regimes impacted differently the plant physiological status and nitrogen metabolism. The 2 mM nitrate nutritional condition is limiting

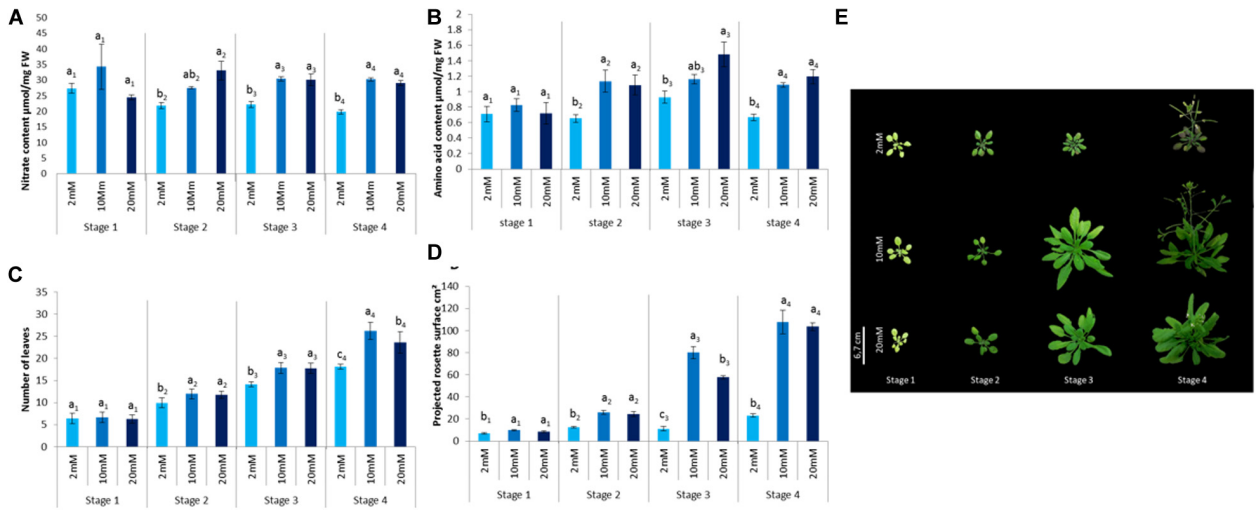


FIGURE 1 | Impact of different nitrate fertilization levels on *Arabidopsis thaliana* physiological traits. Plants were cultivated until the indicated developmental stages (four stages) under the indicated nutritional conditions (2, 10, 26 mM). **(A)** Total nitrate content was quantified in healthy plants. **(B)** Total amino acid content was quantified. **(C)** Number of leaves per plant. **(D)** Projected rosette surface. **(E)** Picture of representative plants cultivated under indicated levels of nitrate at indicated developmental stages. $N = 20$. Error bars represent standard deviation. Letters indicate similarities or differences based on a *t*-test performed to compare samples of the same stage ($p < 0.05$). Experiments were performed three times with similar results. Representative data are shown.

and 26 mM nitrate nutritional condition corresponds to an over-fertilization, while 10 mM nitrate corresponds to an optimal nitrate supply.

Plant Developmental Stage Affects the Capacity to Activate Defense Gene Expression in Response to Plant Defense Stimulators

During the different phases of plant development, important metabolic and transcriptomic changes occur which may affect basal defenses and activation of immune responses. These modifications could account for the variability of plant defense stimulator activity under agronomic conditions. To address this point, Arabidopsis plants were grown until the four developmental stages considered as key steps in Arabidopsis life cycle (Boyes et al., 2001). Three nitrate fertilization levels were applied (2, 10, and 26 mM). Plants were treated with either MeJA, which is known to activate the JA/ET defense pathway, or with Bion®, which is known to activate the SA pathway. Expression profiles of two marker genes of the SA pathway (*PR1* and *PR5*) and two marker genes of the JA/ET pathway (*LOX2* and *PDF1.2*) were monitored by qRT-PCR. To determine the time post-plant defense stimulator treatment the most relevant to monitor defense gene expression, plants were collected 24 and 48 h following Bion® treatment at the four developmental stages and under 2 and 10 mM nitrate nutritional conditions. These experiments showed that the highest level of gene expression was reached 48 h after treatment (**Supplementary Figure 1**). Thus, the rest of the experiments were performed by analyzing gene expression 48 h after plant defense stimulator treatment. In order to determine the effect of the plant developmental stage on defense gene expression, normalized transcript levels were compared under each treatment and each nitrate nutrition separately (**Figure 2**). **Figure 2** shows that plant developmental stage significantly affects most of the defense responses. Basal defenses are significantly affected by developmental stage as indicated by the expression profiles of the four marker genes in control pants (**Figures 2A–D**). The effect of developmental stage on defense activation by plant defense stimulators is significant under the three nutritional conditions. Interestingly, nitrate supply impacts the effect of stage on defense activation (**Figure 2** and **Supplementary Figures 2**, **3**). For example, at 2 mM nitrate supply, *PR1* and *PR5* transcript levels in response to Bion® are significantly reduced at stage 4 compared to other stages, while at 10 mM nitrate supply *PR1* and *PR5* transcript levels are globally high at all stages. Although the transcript level of *PR1* and *PR5* are low following MeJA treatment, they accumulate differentially depending on the developmental stage when plants are grown under 10 mM nitrate. There is no stage effect on *PR1* and *PR5* transcript levels in response to MeJA under limiting nitrate or over-fertilization. The transcript levels of the two JA/ET markers genes *PDF1.2* and *LOX2* are affected by the developmental stage whatever the nitrate nutrition. These two markers are more highly expressed at stage 2 following MeJA treatment under 2 and 10 mM nitrate, compared to the other stages. Together these data indicate that depending on the nutrition, developmental stage plays crucial role in the plant defense system.

Nitrate Fertilization Affects Plant Capacity to Activate Defense Gene Expression in Response to Plant Defense Stimulators

To determine whether nitrate fertilization affects defense activation, plants were treated with either MeJA or with Bion®

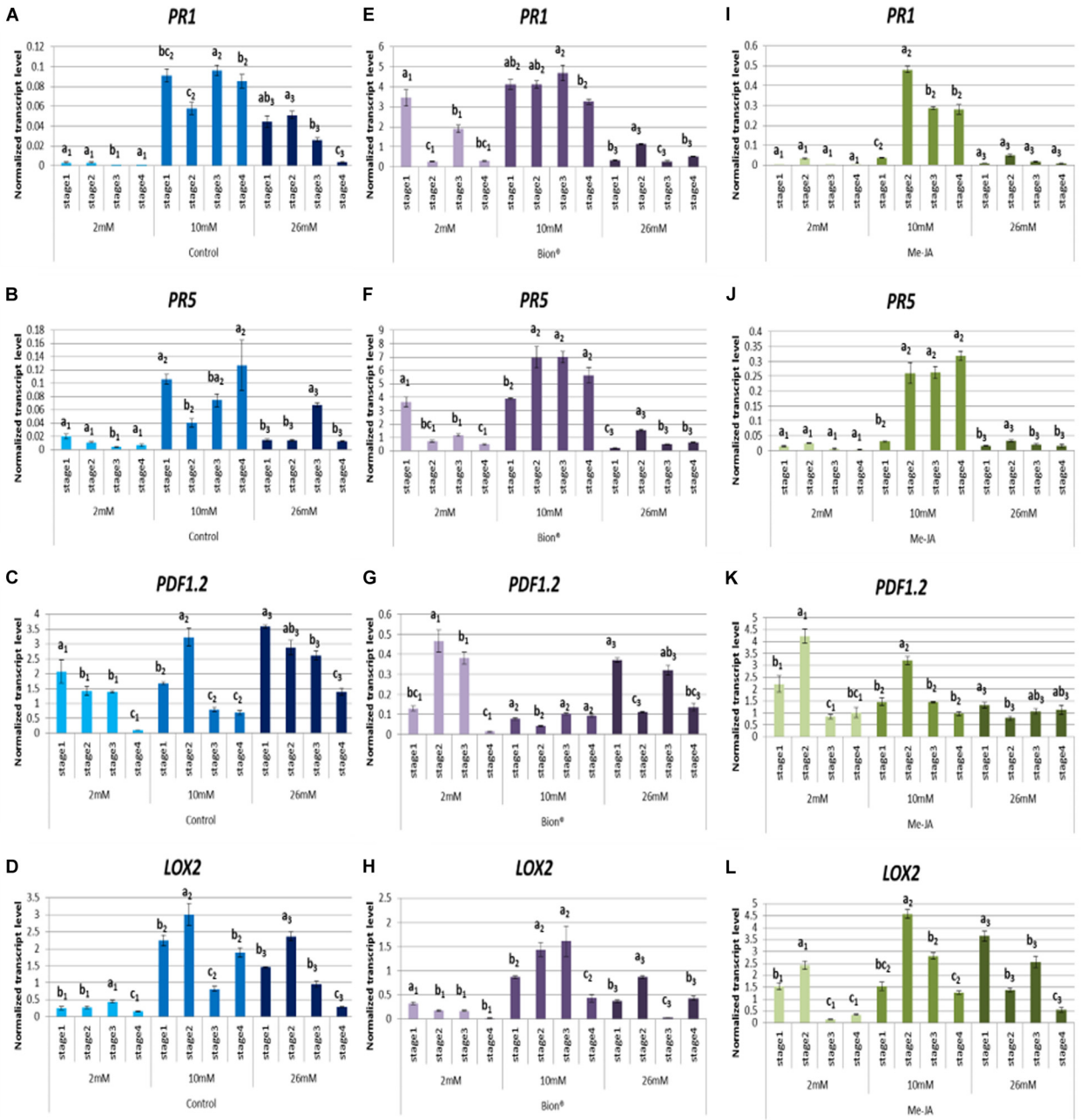


FIGURE 2 | Impact of developmental stage on defense gene expression 48 h after plant defense stimulator treatment. Plants were cultivated until the indicated developmental stages (four stages) under the indicated nutritional conditions (2, 10, 26 mM). They were treated with Bion®, MeJA or water as a control then harvested 48 h after treatment, 6 to 10 plants were harvested for each treatment in each experiment. Defense gene expression was monitored by q-RT-PCR. Data represent normalized transcript levels using Clathrin as a reference gene. **(A–D)** Indicate defense gene expression following water treatment, **(E–H)** Indicate defense gene expression following Bion® treatment; **(I–L)** Indicate defense gene expression following MeJA treatment. Experiments were performed 3 to 4 times with similar results. Representative data are shown. Error bars represent standard error. Different letters represent statistically significant differences between plant defense stimulator treatments under the same nutritional condition ($p < 0.05$ as calculated by *t*-test).

and expression profiles of two maker genes of the SA pathway (*PR1* and *PR5*) and two marker genes of the JA/ET pathway (*LOX2* and *PDF1.2*) were monitored by qRT-PCR 48 h after plant defense stimulator treatment (**Figure 3**). **Figure 3** shows that nitrate nutrition significantly affects most of the defense responses. Basal defenses are in most cases significantly affected by nitrate as indicated by the expression profiles of the four marker genes in control pants (**Figure 3**). At all developmental stages, nitrate supply significantly affected the expression of the SA markers following Bion® treatment and the highest expression

of these markers was obtained under 10 mM nitrate. Nitrate supply significantly affected the expression levels of the JA/ET defense markers following MeJA treatment. Interestingly, Bion® treatment results in the down regulation of PDF1.2 and LOX2 genes under all nitrate treatments (**Supplementary Figures 2, 3**).

Together these data indicate that plant defense stimulator mediated defense gene activation depends both on the stage and on nitrate nutritional condition.

Nitrate Supply Affects Plant Defense Stimulator Mediated Protection Against *Dickeya dadantii* and *Pseudomonas syringae* pv. *tomato*

The enterobacterium *D. dadantii* is a necrotrophic plant pathogen able to infect *A. thaliana* plants causing maceration symptoms as a results of the secretion of large amounts of plant cell degrading enzymes (Reverchon and Nasser, 2013). The model plant *A. thaliana*, in turn, activates different defenses to limit infection, such as the JA/ET defense pathway and the accumulation of reactive oxygen species (Fagard et al., 2007). The Gram negative bacterium *P. syringae* pv. *tomato* is a model plant pathogen (Xin and He, 2013). The SA signaling pathway is known to promote Arabidopsis defense against *P. syringae* pv. *tomato* and it is commonly used to monitor plant defense stimulator activities (McCann et al., 2012; Rufián et al., 2019). To know whether nitrate fertilization may influence the plant defense stimulators mediated protection, we decided to use plants at vegetative stage (stage 2). Indeed, at stage 2, differential expression profiles of defense genes were observed in response to Bion® and MeJA allowing a better interpretation of the putative connection between protection and defenses. In addition, this stage is commonly used in most studies, allowing a better interpretation of the data compared to the literature (Rufián et al., 2019). Two days after plant defense stimulator or water treatment, plants were inoculated with *P. syringae* pv. *tomato* or *D. dadantii*.

To know whether nitrate supply affects *A. thaliana* defenses against *P. syringae* pv. *tomato*, bacterial populations were monitored in control plants and compared with Bion® or MeJA treated plants under the three nitrate supply conditions. Symptoms caused by *P. syringae* pv. *tomato* can be seen in **Supplementary Figure 5**. Nitrate limitation (2 mM) resulted in reduced plant susceptibility to *P. syringae* pv. *tomato* in control plants, indicating that basal defenses against *P. syringae* pv. *tomato* are more efficient in nitrate starved plants than in optimally or over-fertilized plant (**Figure 4A**). Bion® treatment resulted in plant protection under 2 and 10 mM nitrate supply but was inefficient on over-fertilized plants. Interestingly, over-fertilization resulted in enhanced plant susceptibility to *P. syringae* pv. *tomato* following MeJA treatment (**Figure 4B**).

To know whether nitrate supply affects basal *A. thaliana* susceptibility to the *D. dadantii*, symptom severity on water treated control plants were compared under the three nitrate supply conditions. The level of nitrate fertilization had no effect on the proportion of macerated leaf surface in non-elicited plants (**Figure 5A**). Following MeJA treatment, the proportion of leaf macerated surface was decreased under optimal nitrate supply (10 mM), but no effect of MeJA was observed when plants were under-fertilized or over-fertilized (**Figures 3B,D** and **Supplementary Figure 4**). These data indicate that MeJA is efficient to protect *A. thaliana* against *D. dadantii* under optimal nitrate supply only. The proportion of leaf macerated surface was increased following Bion® treatment under 2 mM nitrate fertilization but unaffected by Bion® under 10 or 26 mM nitrate nutritional conditions.

These data indicate that the level of nitrate fertilization influences the capacity of the plant to activate efficient defenses following plant defense stimulator treatments against necrotrophic pathogens such as *D. dadantii* and hemibiotrophic pathogens such as *P. syringae* pv. *tomato*.

NPR1 Gene Is Involved in Nitrate Dependent Plant Defense Stimulator Mediated Defense Responses

As a key player in plant immunity, NPR1 was shown to be involved in both SA and JA/ET signaling pathways (Spoel et al., 2003; Pieterse and Van Loon, 2004; Mao et al., 2007). We investigated whether nitrate supply affected susceptibility of the *npr1-1* mutant to *D. dadantii*. For this purpose, symptom severity on Col-0 WT plants were compared to that of *npr1-1* mutant in water treated control plants under the three nitrate supply conditions. Over-fertilization and under-fertilization increased *npr1-1* mutant susceptibility compared to WT (**Figure 5A**) indicating that *NPR1* is required for the plant basal defense against *D. dadantii*. Plant defense stimulators activity was monitored in the *npr1-1* mutant background to know whether *NPR1* was involved in the plant response to plant defense stimulators under the different nitrate nutritional conditions. Interestingly, both plant defense stimulator treatments failed to show any effect on plant disease severity in the *npr1-1* mutant whatever the level of fertilization used (**Figure 5B**). Thus, while we observed on WT plants an impact of Bion® and MeJA on plant protection against *D. dadantii*, no effect was observed on the *npr1-1* mutant plants.

These data suggest that nitrate dependent defense activation by plant defense stimulators requires a functional plant defense signaling machinery which likely involves *NPR1*.

To investigate whether the role of *NPR1* in protection against *D. dadantii* following MeJA treatment and increased susceptibility to *D. dadantii* following Bion® treatment would involve the SA and/or the ET/JA, defense gene expression was monitored in the *npr1-1* mutant and compared to their expression in the Col-0 WT. For this purpose, *npr1-1* mutant plants were grown until stage 2 under 2, 10, or 26 mM, treated with water (control), Bion® or MeJA and transcript levels of defense genes were monitored by qRT-PCR. **Figures 6A,B** indicates that, as expected, the expression level of the two SA markers genes *PR1* and *PR5* is drastically reduced in the *npr1-1* mutant compared to Col-0 WT. The expression profiles of *LOX2* was similar in Col-0 WT and *npr1-1* under the nutrition conditions of 2 and 10 mM nitrate. Interestingly, the *LOX2* transcript level was globally lower in *npr1-1* plants under 26 mM

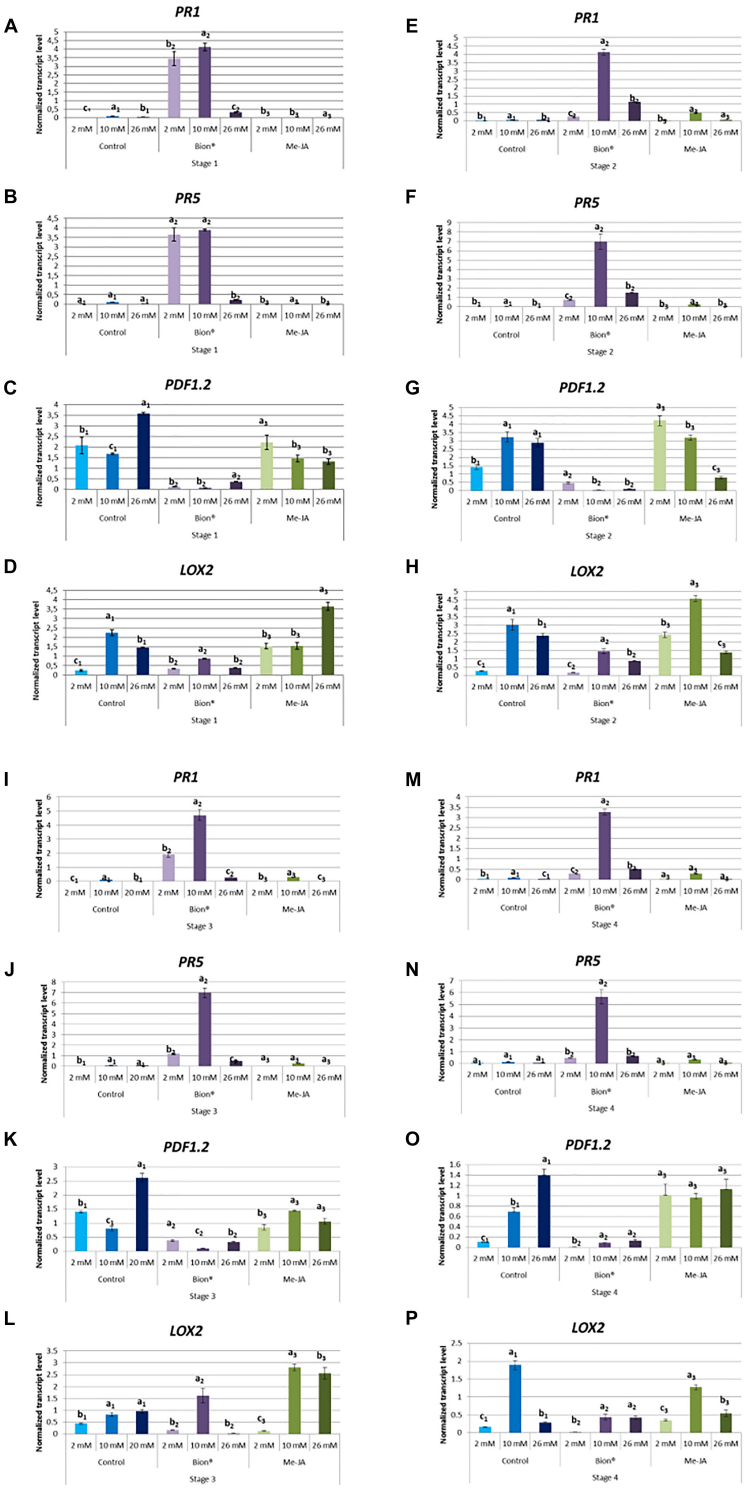


FIGURE 3 | Impact of nitrate nutrition on defense gene expression 48 h after plant defense stimulator treatment. Plants were cultivated until the indicated developmental stages (four stages) under the indicated nutritional conditions (2, 10, 26 mM). They were treated with Bion®, MeJA or water as a control then harvested 48 h after treatment, 6 to 10 plants were harvested for each treatment in each experiment. Defense gene expression was monitored by q-RT-PCR. Data represent normalized transcript levels using Clathrin as a reference gene. **(A–D)** Indicate defense gene expression at stage 1; **(E–H)** Indicate defense gene expression at stage 2; **(I–L)** Indicate defense gene expression at stage 3; **(M–P)** Indicate defense gene expression at stage 4. Experiments were performed 3 to 4 times with similar results. Representative data are shown. Error bars represent standard error. Different letters represent statistically significant differences between plant defense stimulator treatments under the same nutritional condition ($p < 0.05$ as calculated by *t*-test).

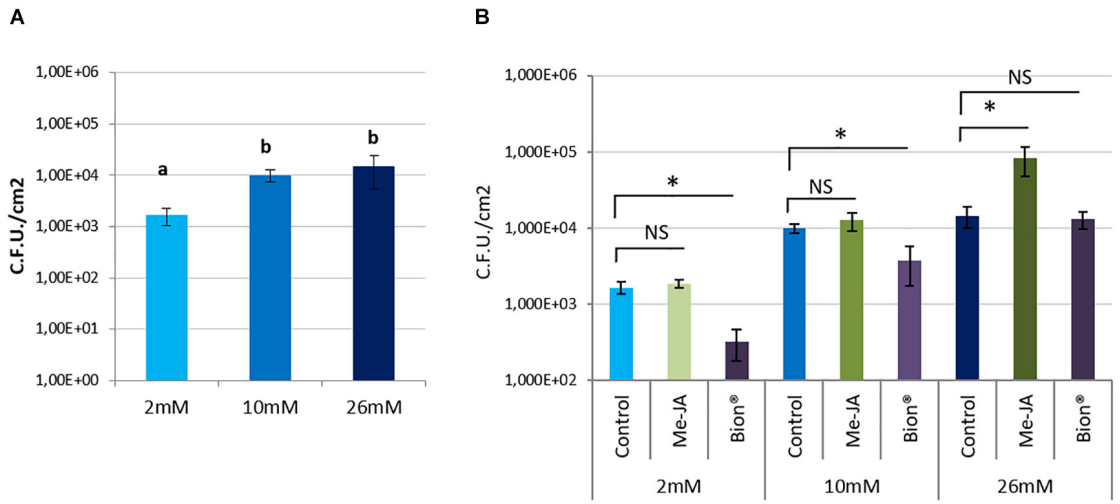


FIGURE 4 | Impact of nitrate fertilization on protection against the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* after plant defense stimulator application. Plants were cultivated until developmental stage 2 (rosette) under the indicated nitrate nutritional conditions (2, 10, 26 mM). They were treated with Bion®, MeJA or water as a control, then inoculated with *P. syringae* pv. *tomato* 48 h after plant defense stimulator treatment. Bacterial populations were monitored. Error bar represent standard deviation. *N* = 20 leaves. Different letters **(A)** or stars **(B)** represent statistically significant differences between control and plant defense stimulator treatments under the same nutritional conditions, NS represent "No Significant" differences ($p < 0.05$ as calculated by *t*-test). Experiments were performed three times with similar results. Representative data are shown.

nitrate compared to Col-0 WT plants (**Figure 6C**) which could explain the enhanced susceptibility of to *D. dadantii* in *npr1-1* naive plants compared to Col-0 WT naïve plants (**Figure 5A**). The increased susceptibility of Col-0 WT plants to *D. dadantii* under 2 mM nitrate following Bion® treatment (**Figure 5B**) is consistent with a down-regulation of *PDF1.2* and *LOX2* expression in Col-0 following Bion® treatment under 2 mM nitrate (**Figures 6C,D**). Although this increased susceptibility is abolished in the *npr1-1* mutant under these conditions, the down-regulation of *PDF1.2* and *LOX2* is still observed.

The protection of Col-0 WT plants agianst *D. dadantii* under 10 mM nitrate following MeJA treatment (**Figure 5B**) is consistent with an up-regulation of *LOX2* expression in Col-0 following MeJA treatment under 10 mM nitrate (**Figure 6C**). Although this protection is abolished in the *npr1-1* mutant, under these conditions, the up-regulation of *LOX2* is still observed.

Taken together, these data indicate that *NPR1* plays an important role in modulating Arabidopsis defenses depending on nitrate supply.

DISCUSSION

Most of the plant protection treatments directly target invading pathogens. In general, this kind of practices has proven to cause pathogen resistance toward pesticides, thus reducing their efficiency (Hahn, 2014). In addition, pesticide use has detrimental effects on animal health and environment. It is nowadays obvious that alternate and sustainable plant protection strategies are needed to avoid the detrimental effects of pesticide and reduce pathogen adaptation (Pretty, 2018). The use of plant defense stimulators is one of the proposed alternate crop protection strategies which is being investigated by scientists and farmers because they don't directly target the pathogen and they provide a wide protection range. However, plant defense stimulators efficiency is controversial. While they can protect plants from pathogen infections under controlled conditions, their efficiency in the field is often unstable (Bektas and Eulgem, 2015).

In this work, we investigated the possibility that plant responses to plant defense stimulators could be affected by the developmental stage and nitrogen nutrition. The objective was to determine whether by adjusting fertilization and targeting specific developmental stages, plant defense stimulators use could be optimized.

Plant intrinsic susceptibility to pathogens depends on plant developmental stage and nitrogen status. For instance, the bacterial fire blight causing pathogen *Erwinia amylovora* preferentially infects growing tissues and apple flowers (Malnoy et al., 2011). Conversely, senescence can be a factor which favors necrotrophic pathogen infection while it prevents biotrophic pathogen infections (Häffner et al., 2015). On the other hand, plant intrinsic susceptibility to pathogens can vary depending on the nitrogen fertilization. Complex interactions have been described concerning the connection between plant nitrogen status and tolerance to pathogens (Fagard et al., 2014; Mur et al., 2017; Sun et al., 2020). For instance, nitrate fertilization increases tomato tolerance to the fungal necrotroph *Botrytis cinerea* (Lecompte et al., 2010); while it increases the susceptibility of *A. thaliana* to this fungus (Fagard et al., 2014). Nitrogen fertilization has an impact on defense activation (Kruse et al., 2007; Kutyniok and Müller, 2013; Mur et al., 2017; Zarattini et al., 2017; Farjad et al., 2018) as well as on pathogen virulence factors (Van den Ackerveken et al., 1994; Snoeijers et al., 2000; Robert et al., 2004). Thus, the impact of nitrogen

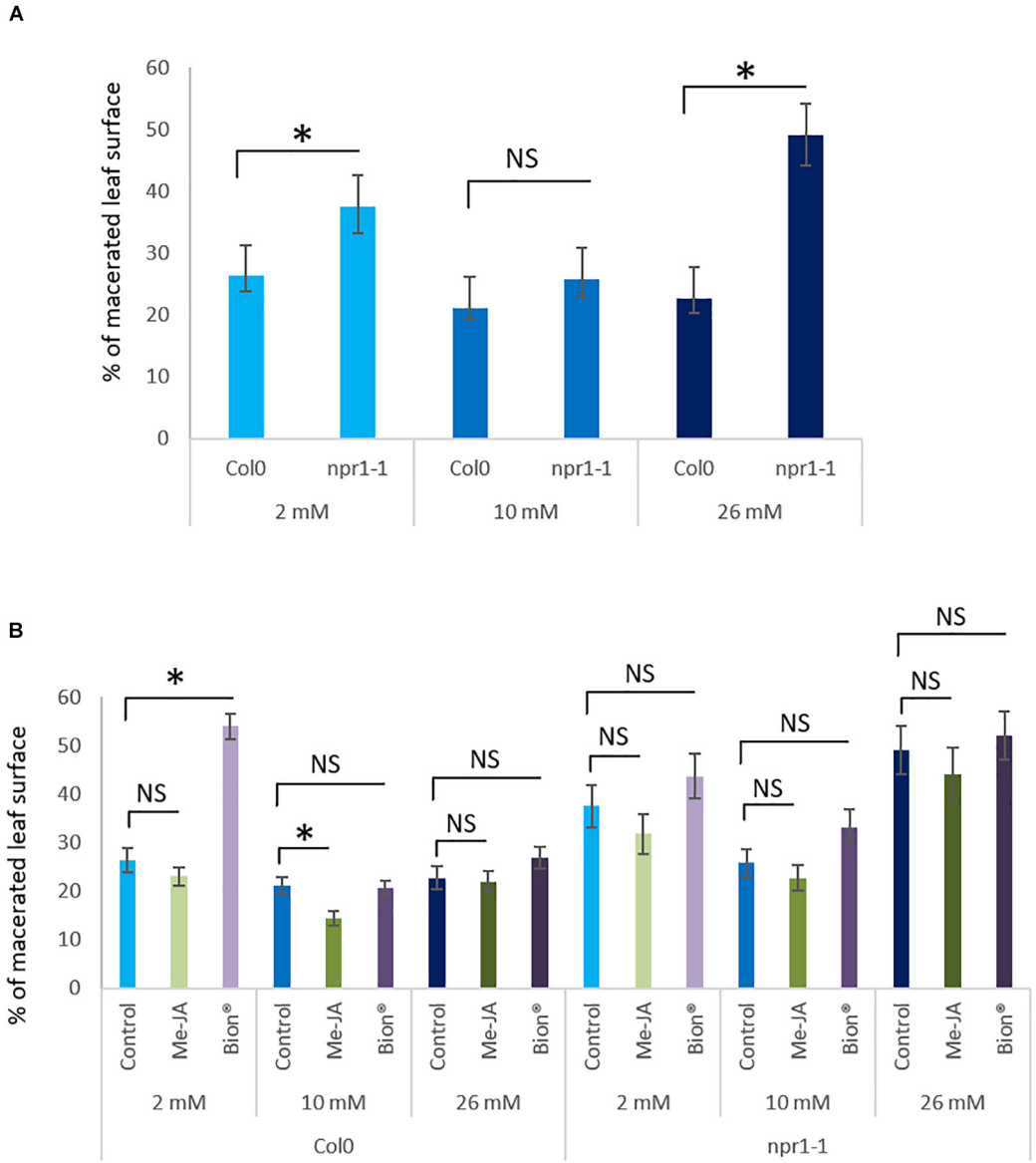


FIGURE 5 | Impact of nitrate fertilization on protection against the pathogenic bacterium *D. dadantii* after plant defense stimulator application on an *npr1* mutant. Plants were cultivated until developmental stage 2 (rosette) under the indicated nitrate nutritional conditions (2, 10, or 26 mM). They were treated with Bion®, MeJA or water as a control, then inoculated with *D. dadantii* 48 h after plant defense stimulator treatment. Symptoms were scored 48 h after inoculation. Percentage of macerated leaf surface are represented. Error bars represent standard error. *N* = 20 leaves. Stars represent statistically significant differences between *npr1-1* mutant plants and wild type Col-0 WT **(A)** and between control and MeJA and Bion®, treatment **(B)** for each level of fertilization. NS represent "No Significant" differences ($p < 0.05$ as calculated by *t*-test). Experiments were performed three times with similar results. Representative data are shown.

status on plant tolerance/susceptibility does not exclusively depend on nutritional availability to pathogen, but involves complex mechanisms.

The above-cited reports describe the impact of the developmental stage and nitrogen fertilization on plant intrinsic susceptibility to pathogens. However, very few data are available about the impact of the developmental stage and nitrogen fertilization on plant defense stimulator mediated defense activation. Our data show that the plant defense responses to two plant defense stimulators, which trigger two major defense signaling pathways are affected by both the developmental stage and nitrate nutrition in *A. thaliana*. Activation of SA pathway by Bion® was dependent of both the nitrate supply and the developmental stage (**Figures 2**, **3**), indicating that the fertilization and physiological stage parameters should be considered when using Bion® as an plant defense stimulator. Optimal nitrate nutritional conditions were the most favorable conditions for SA defense activation by Bion®. Dietrich et al. (2004) showed that nitrogen limitation resulted in reduced defense

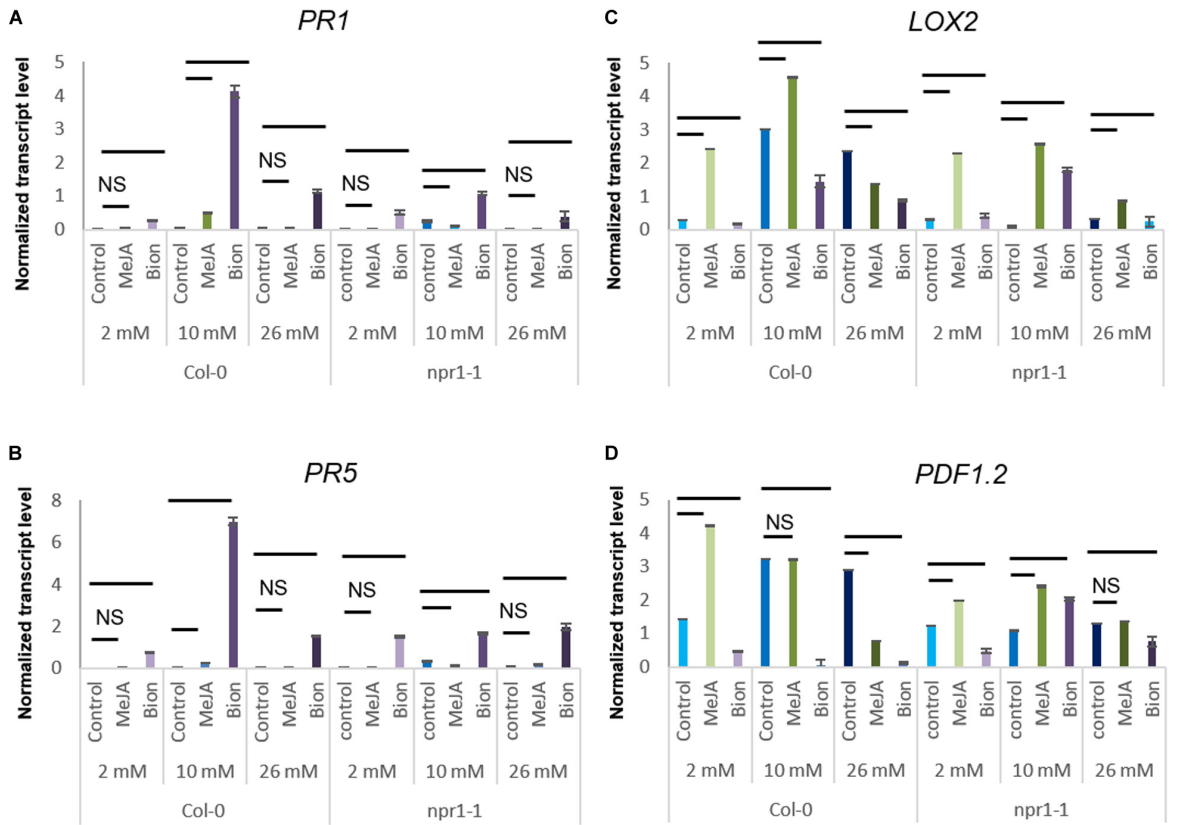


FIGURE 6 | Impact of nitrate nutrition on defense gene expression 48 h after elicitor treatment. Plants were cultivated until the developmental stage 2 under the indicated nutritional conditions (2, 10, 26 mM nitrate). They were treated with Bion®, MeJA or water as a control then harvested 48 h after treatment, 6 to 10 plants were harvested for each treatment in each experiment. Transcript levels of indictade genes, *PR1* **(A)**, *PR5* **(B)**, *LOX2* **(C)**, and *PDF1.2* **(D)** was monitored by qRT-PCR. Data represent normalized transcript levels using Clathrin as a reference gene. Bars indicate comparisons between control and plant defense stimulator treatments under the same nutritional condition. When only a bar is visible, the difference is significant ($p < 0.05$ as calculated by *t*-test). NS: statistically Non-Significant differences between control and plant defense stimulator treatments under the same nutritional condition.

induction by Bion®. In a transcriptomic approach to characterize the combined effect of pathogen and nitrogen deficiency, Farjad et al. (2018) showed that the upregulation of a set of defense related genes was higher under nitrogen limitation. Thus, depending on the biotic stress and the defense pathway considered, nitrogen deficiency can differentially affect immune responses. These studies did not investigate over-fertilization conditions.

Regulation of *LOX2* and *PDF1* transcript levels following MeJA treatment was strongly affected by nitrate supply and developmental stage (**Figures 2**, **3**). Up-regulation was not observed in all cases and it was surprising to observe repression of these markers following MeJA treatment in some cases (**Supplementary Figure 3**). Both ET and JA play important roles in plant development (Huang et al., 2017; Dubois et al., 2018). Thus, the differential expression observed between stage 1 and 4 may be in part due to their accumulation level during these key developmental phases. Interestingly, an up-regulation of *PR1* and *PR5* was observed following MeJA treatment although to a lower level than those observed following Bion® treatment. This dual activation of SA and JA pathways was also recently described in the context of plant resistance mediated by a specific resistance gene in Arabidopsis (Liu et al., 2016) and may be more common than usually assumed. The ET/JA pathway is recruited during induced systemic resistance (ISR) triggered by PGPR (Backer et al., 2018). It would be interesting to determine whether ISR is affected by plant developmental stage and nutrition.

The impact of nitrate supply on effective protection of MeJA and Bion® against two bacterial pathogens with different lifestyles was investigated. Bion® conferred protection against the hemibiotrohoic bacterium *P. syringae* pv. *tomato* under low and optimal nutrition but failed to protect under high nitrate. Conversely, MeJA treatment resulted in an increased plant susceptibility. Protection was conferred by MeJA against *D. dadantii* when plants were cultivated under optimal nitrate nutritional conditions with 10 mM nitrate, while no protection was observed under low or over-fertilization conditions. This optimal protection is not perfectly correlated with transcriptional activation of *PDF1.2* and *LOX2*, indicating that these two defense markers do not fully explain the protection at optimal nitrate nutritional conditions. Interestingly, *LOX2* expression, is up-regulated at stage 2 and under optimal nitrate nutrition

which correspond to the conditions where MeJA protects plants against *D. dadantii* (**Supplementary Figure 3**) indicating that the JA pathway could be acting here via ISR. These data are consistent with the fact that JA is involved in *A. thaliana* defense against *D. dadantii* (Fagard et al., 2007; Reverchon and Nasser, 2013). While MeJA conferred protection against *D. dadantii*, Bion® treatment resulted in an increased susceptibility. This increased susceptibility could be explained by the repression of ET/JA defenses we observed following Bion® treatment which activates the SA pathway (**Supplementary Figure 2**). An antagonistic effect of the SA pathway over the ET/JA pathway was previously described (Koornneef and Pieterse, 2008; Leon-Reyes et al., 2010; Van der Does et al., 2013; Caarls et al., 2017; Li et al., 2019; Yang et al., 2019). Care must be taken when fighting diverse bioagressors in the field since plant defense stimulators can have opposite effects.

In order to determine the defense signaling contribution in the plant protection mediated by MeJA against *D. dadantii*, the *npr1-1* mutant was used because this mutant was described as being affected in both the SA and the ET/JA defense responses (Pieterse and Van Loon, 2004; Withers and Dong, 2017; Barker, 2018; Backer et al., 2019). The enhanced susceptibility of the *npr1-1* mutant could be surprising since this gene is commonly known to activate SA response which is effective against biotrophs. In the *npr1-1* mutant, one could expect the increase in JA signaling leading to enhanced resistance to the necrotroph *D. dadantii*. However, several examples show that NPR1 overexpression leads to tolerance to necrotrophic pathogens (Wally et al., 2009; Ali et al., 2017). Our data show that MeJA mediated *A. thaliana* protection against *D. dadantii* requires *NPR1*. Similarly, Bion® mediated plant increased susceptibility to *D. dadantii* is abolished in the *npr1-1* mutant. It is intriguing that both increased and decreased protection involve *NPR1*. To tackle this issue, the role of the SA and ET/JA defense signaling pathways in the defense modulation by NPR1 was investigated by monitoring the expression of defense gene markers of these pathways in the *npr1*-1 mutant under the different nutritional conditions and following plant defense stimulator treatments. Interestingly, *LOX2* expression seems to both depend on *NPR1* and nitrate supply. Indeed, *LOX2* expression was strongly reduced in *npr1-1* mutant plants under 26 mM nitrate correlating with enhanced susceptibility to *D. dadantii*. Our data illustrate the complexity by which NPR1 is involved in the balance between the SA and the ET/JA signaling pathways that remains to be further investigated (Pieterse et al., 2011; Li et al., 2019).

The role of NPR1 in the nitrate dependent defense modulation by plant defense stimulators suggests a role of nitrate nutrition on defense signaling mechanisms. One possible mechanism by which nitrate nutrition can interact with defense signaling is via NO accumulation which can be a byproduct of nitrate reductase. *A. thaliana* plants fertilized with nitrate accumulated higher levels of NO than ammonium fed plants suggesting an involvement of NO in the higher tolerance of nitrate fertilized plants to the pathogenic bacterium *P. syringae* (Gupta et al., 2012). The role of NO could be related to the activity of the NPR1 protein which is known to be S-nitrosylated (Tada et al., 2008; Withers and Dong, 2017).

These data support the idea that the impact of nitrate nutrition in plant immunity is complex and probably involves interactions between defense signaling pathways and metabolic pathways.

Our data could be useful to the design of performant agronomic practices by choosing and adapting the best fitted conditions for the use of plant defense stimulators taking into account the stage of development and the nitrogen status.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AD and M-ES-M conceived conceptually the work. CV and AD wrote the manuscript. CV, ACRD, and MR performed the experiences. AD, M-ES-M, LR, and FG supervised the project.

ACKNOWLEDGMENTS

The authors want to thank the Staphyt Company and the National Agency of Research and Technology (ANRT) which funded the Ph.D. fellowship of CV supported by Paris Saclay University and the Jean-Pierre Bourgin Institut (IJPB) for the provision of scientific equipment and the reception within the institute that led to this work. The IJPB benefits from the support of Saclay Plant Sciences-SPS (ANR-17-EUR-0007). This work has benefited from the support of IJPB's Plant Observatory technological platforms.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00583/full#supplementary-material

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Conflict of Interest: ACRD, CV, MM, and FG were employed by the company STAPHYT.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 May 2020
doi: 10.3389/fpls.2020.00581

Quantitative Structure-Activity Relationship of Humic-Like Biostimulants Derived From Agro-Industrial Byproducts and Energy Crops

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *24 January 2020*
Accepted: *17 April 2020*
Published: *26 May 2020*

Citation:
Savy D, Brostaux Y, Cozzolino V, Delaplace P, du Jardin P and Piccolo A (2020) Quantitative Structure-Activity Relationship of Humic-Like Biostimulants Derived From Agro-Industrial Byproducts and Energy Crops. Front. Plant Sci. 11:581. doi: 10.3389/fpls.2020.00581

Humic-like substances (HLSs) isolated by alkaline oxidative hydrolysis from lignin-rich agro-industrial residues have been shown to exert biostimulant activity toward maize (*Zea mays* L.) germination and early growth. The definition of a quantitative structure-activity relationship (QSAR) between HLS and their bioactivity could be useful to predict their biological properties and tailor plant biostimulants for specific agronomic and industrial uses. Here, we created several projection on latent structure (PLS) regression by using published analytical data on the molecular composition of lignin-derived HLS obtained by both ^{13}C-CPMAS-NMR spectra directly on samples and ^{31}P-NMR spectra after derivatization of hydroxyl functions with a P-containing reagent (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane). These spectral data were used to model the effect of HLS on the elongation of primary root, lateral seminal roots, total root apparatus, and coleoptile of maize. The ^{13}C-CPMAS-NMR data suggested that methoxyl and aromatic moieties positively affected plant growth, while the carboxyl/esterified functions showed a negative impact on the overall seedling development. Alkyl C seems to promote Col elongation while concomitantly reducing that of the root system. Additionally, ^{31}P-NMR-derived spectra revealed that the elongation of roots and Col were enhanced by the occurrence of aliphatic hydroxyl groups, and guaiacyl and *p*-Hydroxyphenyl lignin monomers. The PLS models based on raw dataset from ^{13}C-CPMAS-NMR spectra explained more than 74% of the variance for the length of lateral seminal roots, total root system and coleoptile, while other parameters derived from ^{13}C-CPMAS-NMR spectra, namely the Hydrophobicity and Hydrophilicity of materials were necessary to explain 83% of the variance of the primary root length. The results from ^{31}P-NMR spectra explained the observed biological variance by 90, 96, 96, and 93% for the length of primary root, lateral seminal roots, total root system and coleoptile, respectively. This work shows that different NMR spectroscopy techniques can be used to build up PLS models which

can predict the bioactivity of lignin-derived HLS toward early growth of maize plants. The established QSAR may also be exploited to enhance by chemical techniques the bioactive properties of HLS and enhance their plant stimulation capacity.

Keywords: biostimulants, humic-like substance, biorefinery and agro-industrial byproducts, projection on latent structure regression, partial least square regression, solid-state ^{13}C-CPMAS NMR spectroscopy, liquid-state ^{31}P-NMR spectroscopy, 2-chloro-4,4,5,5-tetramethyl-1,3,2,-dioxaphospholane

INTRODUCTION

Plant biostimulants are a novel class of fertilizing products that improve the "plants' nutrient use efficiency, tolerance to abiotic stress, quality traits, and/or increasing the availability of confined nutrients in the soil or rhizosphere" (EU Fertilising Products Regulation, 1009/2019). Among several categories of biostimulants, Humic Substances (HS, including fulvic and humic acids) and HLSs are the most relevant and intensively studied (Grand View Research, 2019). They are extracted from soils, sediments, composted biomasses or agro-industrial residues and might be regarded as supramolecular associations of small, heterogeneous molecules held together in metastable structures by non-covalent interactions (van de Waals, π-π, H-bonds; metal bridges) (Piccolo et al., 2019; Wells and Stretz, 2019). Extracting HLS from energy crops or agro-industrial residues is also important from the environmental point of view, because it involves recycling precious photosynthate, which would be otherwise burnt or landfilled (Cherubini, 2010; Savy et al., 2020).

Both HS and HLS are reckoned to boost crop growth and yields, and protect plants from abiotic stresses, by triggering specific metabolic routes (Aguiar et al., 2016; Khaleda et al., 2017; Vinci et al., 2018). HS and HLS have also been applied to seeds or hydroponically-grown plants to study their bioactivity without the environmental complexities of field trials (Ertani et al., 2013; Savy et al., 2018; Spaccini et al., 2019).

For example, the positive bioactivity of HLS isolated by modifying the lignin contained in several agricultural residues or biorefinery wastes have been ascertained on maize (*Zea mays* L.) germination and early growth (Savy et al., 2015a, 2016a, 2017b). Although such HLS materials were not found to significantly promote seed germination percentage, a positive dose-dependent elongation of primary and lateral seminal roots, as well as of coleoptile, was recorded. As for other HS and HLS, the biostimulation caused by the above-mentioned lignin-derived HLS was related to their content of phenolic molecules and their role in affecting specific plant hormonal balances was suggested (Ray, 1986; Savy et al., 2017a). An important objective of the biostimulants industry is to understand the mode of action of a product allowing to predict its biological effect toward plant growth. In order to reach this aim, a QSAR should be derived. QSAR is a mathematical equation relating the biological properties of a material to some of its chemical and physical characteristics (Roy et al., 2015). Once a model for HLS bioactivity is defined, the expected bioactivity of other biostimulants from lignin-rich sources could be predicted. Furthermore, the production of humic-like biostimulants with the desired biological properties may be based on the selected QSAR model, thus reducing the risk of applying HLS with low bioactivity.

In order to optimize a QSAR model for a specific bioactive material, its molecular characterization should be as detailed as possible (Roy et al., 2015). Despite the efforts to define the molecular composition of the heterogeneous HS and HLS, unraveling their complex structure still remains a challenge (Nebbioso and Piccolo, 2011; Nebbioso et al., 2014; Drosos et al., 2017, 2018). Nevertheless, QSAR for HS had been obtained based only on NMR spectra of the bulk humic matter (Geyer et al., 1998; Steinberg et al., 2000; Aguiar et al., 2013). However, misleading interpretations may arise if linear regressions and multiple linear regressions are used to build the models, since they assume *non*-correlated predictors, whereas the HS and HLS chemical data used as predictors are expected to be correlated with each other. Conversely, Principal Component Regression and PLS (also known as Partial Least Square) regression provide accurate QSAR models even when dealing with highly correlated independent variables (Esbensen, 2001), and had been successfully applied to derive QSAR for HS from different sources (Canellas et al., 2012; García et al., 2016). Although both statistical tools are valid for developing prediction models, PLS regression is a more efficient technique than Principal Component Regression. In fact, the PLS regression sequential extraction of model components is a one-step process, and it is carried out by using both chemical and biological data simultaneously, whereas the Principal Component Regression extracts such components in a two-step process. Moreover, PLS regression usually requires fewer components than Principal Component Regression to achieve the same prediction level.

In this work, we derived a QSAR for HLS extracted from lignin-rich agricultural and biorefinery residues, by applying PLS regression to a dataset retrieved from previously published articles, in which both the chemical structure and the biological activity of such plant enhancers were reported (Savy and Piccolo, 2014; Savy et al., 2015a, 2016a, 2017b). In particular, HLS were chemically characterized by both solid-state ^{13}C-CPMAS and liquid-state ^{31}P NMR spectroscopy, whereas the HLS bioactivity was evaluated toward maize germination and early growth.

Abbreviations: ^{13}C-CPMAS, ^{13}C-cross polarization-magic angle spinning NMR spectroscopy; AD, humic-like substances isolated from giant reed; BYP 1 and BYP 2, humic-like substances isolated from two different biorefinery residues; CAR, humic-like substances isolated from cardoon; EUC, humic-like substances isolated from eucalypt; HLSs, humic-like substances; HS, humic substances; LIM, humic-like substances isolated from black poplar grown along the Limatola creek; MG, humic-like substances isolated from miscanthus; NMR, nuclear magnetic resonance; PLS, projection on latent structure; QSAR, quantitative structural-activity relationship; RIP, humic-like substances isolated from black poplar grown along the Ripiti creek; TMDP, 2-chloro-4,4,5,5-tetramethyl-1,3,2,-dioxaphospholane; VIP, variable importance plot.

Hence, our aims were: (i) to create two PLS models by relating spectral data from either ^{13}C-CPMAS-NMR or ^{31}P-NMR spectra of selected HLS to their bioactivity; (ii) to assess the accuracy of the achievable prediction by using the two models and (iii) to identify the most important HLS chemical features that exert the bioactivity on plants.

MATERIALS AND METHODS

Plant Biomasses

Cardoon (*Cynara cardunculus* L) was cropped on September 2012 at the University of Naples experimental farm in Bellizzi (Salerno, Italy), while eucalypt (*Eucalyptus camaldulensis* Dehnh.) was harvested on March 2012 at an experimental farm near Eboli (Italy). Black poplar (*Populus nigra* L.) was cropped on March 2012 from along either the Ripiti (Salerno, Italy) or the Limatola (Benevento, Italy) creeks. Miscanthus (*Miscanthus* x *giganteus* Greef et Deuter) was provided by Phytatec, Ltd. (United Kingdom), after being harvested on February 2007 in Aberystwyth (United Kingdom), whereas giant reed (*Arundo donax* L.) was cropped on January 2010 at the experimental farm of the University of Naples Federico II near Salerno (Italy). All the above-mentioned biomasses were selected because of their relevance in the production of either paper or energy (Benjelloun-Mlayah et al., 1997; Christersson, 2008; Cotana et al., 2015; Ge et al., 2016; Vilasboa et al., 2019). Finally, two biorefinery residues were obtained by hydrolyzing giant reed biomass to produce succinic acid (Savy et al., 2017b) and the hydrolyzed solid residues were then subjected to HLS extraction.

Extraction of Humic-Like Substances (HLS) by Alkaline Oxidative Hydrolysis

The HLS were isolated according to Sun et al. (2000). Briefly, an alkaline 2% H_2O_2 (v/v) aqueous solution (pH 11.6) was added to the lignocellulosic substrates and stirred overnight at 323 K. The mixture was then centrifuged (15,400 RCF x 20 min) and the supernatant dialyzed (1 kDa cut-off dialysis tubes) against deionized water, freeze-dried, and stored in dried conditions for further analyses. The amount of inorganic compounds in the HLS was previously showed to be negligible, i.e., the biostimulant effect observed is conceivably related only to the organic molecules they contained (Savy and Piccolo, 2014; Savy et al., 2015a, 2017b). HLS obtained from cardoon, eucalypt, poplars from Ripiti and Limatola, miscanthus, giant reed and the two biorefinery wastes will be referred to as CAR, EUC, RIP, LIM, MG, AD, BYP 1, and BYP 2, respectively.

Synthesis of 2-Chloro-4,4,5,5-Tetramethyldioxaphospholane and HLS Derivatization Prior to ^{31}P-NMR Spectroscopy

The derivatizing phosphorous reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (TDMP) was synthesized as described in Savy et al. (2017b). Briefly, TDMP was obtained by mixing the two following solutions: solution (A), prepared by dissolving 21.5 mL PCl_3 in 180 mL of dry n-hexane placed in a 250 mL three-necked round flask equipped with a condenser, and solution (B), prepared by dissolving 23.7 g pinacol in a mixture of 32 mL of dry pyridine and 150 mL of dry n-hexane placed in a conic flask. Solution (B) was added drop-wise to solution (A) using an addition funnel placed on the second neck of the round flask. The addition lasted 1 h under vigorous stirring in an ice bath, then and the mixture was left for 1 h at room temperature to complete the reaction. The solution was filtered on a filter paper, while the whitish residue on the filter was rinsed with 2 × 100 mL of n-hexane and the filtrates were evaporated under *vacuum* at 328 K. Finally, CTMP was separated from solution by *vacuum* distillation (b. p. 370 K at 4 mbar).

The hydroxyl (OH) groups in the HLS were derivatized with TDMP as it follows. A stock solution was prepared by adding 2.92 mg mL^{-1} cyclohexanol (used as internal standard), 10.0 mg mL^{-1} of triphenyl phosphate (as reference peak for the ^{31}P frequency axis calibration) and 0.6 mg mL^{-1} of chromium (III) acetylacetonate (as relaxation agent) to a pyridine and deuterated chloroform solution (1.6/1 v/v). The HLS (7.0 mg) were the dissolved in 750 μL of the stock solution and added with 50 μL of TDMP. All HLS were fully soluble in the solvent mixture used.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The solid-state CPMAS NMR spectra were acquired with a 300 MHz Bruker Avance magnet (Bruker Bio Spin GmbH, Rheinstetten, Germany), composed of a wide-bore system and equipped with a CPMAS (Cross-Polarization Magic-Angle-Spinning) probe, working at ^{13}C frequency of 75.47 MHz. Samples were loaded into 4-mm zirconia rotors, closed with Kel-F caps and spun at a rate of 10000 ± 1 Hz. Such spectra were acquired by applying a cross-polarization technique and consisted of 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 2 s, 5000 scans and 1 ms of contact time. The ^{13}C-CPMAS pulse sequence was conducted by using a ^{1}H Ramp pulse to account for the *non*-homogeneity of the Hartmann–Hahn condition. A TPPM15 scheme was applied to perform the ^{13}C-^{1}H decoupling. The free induction decay (FID) was transformed by applying a 4k zero filling and an exponential filter function with a line broadening of 100 Hz.

The ^{31}P-NMR spectra were obtained with a 400 MHz Bruker Avance spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm Bruker Inverse Broad Band (BBI) probe, working at ^{1}H and ^{13}C frequencies of 400.13 and 100.62. Such spectra were acquired on TDMP-derivatized HLS by applying an inverse gated pulse sequence including a 80 μs length (15.6 dB power level) Waltz16 scheme to decouple phosphorous from proton nuclei. In particular, spectra consisted in a 45° pulse length of 5.25 μs, a spectral width of 400 ppm (64,935.066 Hz), 10 s of recycle delay, 1600 transients, 8 dummy scans and 129,862 time domain points.

All NMR spectra were acquired at a temperature of 298 ± 1 K and processed by using either Bruker Topspin Software (v.2.1, Bruker Biospin, Rheinstetten, Germany) or MestReC

NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, MA, United States). Zero filling was applied during Fourier transform of FIDs.

Germination of Maize Seeds and Seedling Emergence

Maize (*Zea mays* L.) seeds were soaked in tap water overnight and fifteen (15) seeds were deposited for each replicate on round filter paper placed in a Petri dish. At least three replicates were used per each experiment. The filters were moistened with aqueous solution of HLS at various concentrations (ranging from 0 to 100 mg HLS L^{-1}) and the seeds were germinated in the dark at 298 K for 96 h. Thereafter, the plantlets were scanned with a modified flatbed scanner (Epson Perfection V700, Seiko Epson, Corp., Japan) and the length of primary root, lateral seminal roots, total root apparatus and coleoptile was measured by using the WinRhizo Pro software, version 2016 (Regent Instruments, Inc., Canada).

Projection on Latent Structure (PLS) Regression

Prior to run the PLS regression, the biological data were normalized with respect to the control mean for each specific experiment, which was set to 100. Furthermore, the models were built by selecting the treatment means that in most cases provided the longest value for the length of primary root, lateral seminal roots, total root system or coleoptile (Savy et al., 2015a, 2016a, 2017b). The dataset consisted of a matrix (8 × 20) with the HLS in columns and spectral data or biological parameters in rows. Specifically, 10 rows corresponded to the ^{13}C-CPMAS-derived data, 6 rows contained the ^{31}P-derived NMR data, and 4 rows showed the HLS-elicited biological response. The chemical information obtained by either ^{13}C-CPMAS- or ^{31}P-NMR spectra were employed as predictors of the biological results to build up two different PLS models per each biological parameter. Since the data from both the NMR techniques employed could not be always obtained due to technical and budget limitations, two different PLS models were created by using results from each NMR spectroscopic analysis, instead of building one only PLS model by combining results from both techniques. Hence, if the models sufficiently explain the variance of our biological data, their predictive power for future applications may be valid even though only one of the spectral techniques is available. The optimum number of latent factors was calculated by leave-one-out cross-validation. The latent variables (or factors) are variables that can capture an underlying phenomenon being investigated and, since cannot be directly measured, are calculated from the actual measurements; hence they are correlated with them. Also, the exploitation of latent variables represents a convenient mean to summarize the observed (X) variables by using much fewer factors (Bollen, 2002). All the statistical analyses were performed by using OriginPro, "2017" Version (OriginLab Corporation, Northampton, MA, United States).

RESULTS

Chemical Features and Bioactivity of the Different HLS

The molecular composition based on ^{13}C-CPMAS-NMR spectra of various HLS and their bioactive responses on maize germination are reported in **Table 1**, whereas the original spectra were shown in **Supporting Figure SF1**. Except for MG and AD, the HLS had generally large relative alkyl contents, with EUC showing the greatest value (38.0%). Conversely, both AD and MG contained larger methoxyl groups than the other HLS, with the exception of EUC, whose methoxyl amount was comparable to that of AD (**Table 1**). The *O*-alkyl groups, usually associated with the resonance of carbons in lignin lateral chains and carbohydrates, were larger in CAR- and LIM-derived materials (Robert, 1992; Spaccini et al., 2019). Furthermore, the occurrence of carbohydrates is confirmed by NMR signals in the 90–110 ppm range, attributed to the resonance of anomeric carbons (**Table 1**). The aromatic and phenolic content in HLS isolated from Liliopsida (MG, AD, BYP 1, and BYP 2) was more abundant than in those extracted from Magnoliopsida (CAR, RIP, LIM, and EUC) (**Table 1**). Finally, the relative amount of carboxyl/esterified C was larger in the two poplar-derived HLS, followed by EUC, BYP 2 and MG, which showed comparable amount of such units, and then by BYP 1, CAR and AD (**Table 1**).

Useful indicators of the hydrophobic or hydrophilic character of substrates are calculated from ^{13}C-CPMAS-NMR spectra as Hydrophobicity and Hydrophilicity (**Table 1**). In fact, Hydrophobicity corresponds to the sum of areas under the signals of alkyl, aryl and *O*-aryl C, while Hydrophilicity refers to the sum of signals areas for methoxyl, *O*-alkyl, anomeric and carboxyl C (Canellas et al., 2012). BYP 1, BYP 2, and EUC showed a larger Hydrophobicity than other HLS, mainly due to the significantly larger relative content of alkyl groups. The largest hydrophilic character of the remaining HLS can be mainly ascribed to the large relative amount of methoxyl, *O*-alkyl and anomeric C (**Table 1**).

The ^{31}P-NMR spectra of HLS previously derivatized with TMDP are used to quantitatively assess the amount of different hydroxyl (OH) functional groups present in HLS, namely in aliphatic, carboxyl and phenolic components (Granata and Argyropoulos, 1995). Besides allowing the qualitative and quantitative appraisal of the OH moieties, ^{31}P-derivatizaion of HLS significantly enhanced the spreading of NMR signals over a large width of ^{31}P-NMR spectra, with consequent reduction of signal overlapping (Savy et al., 2015b,c). All these characteristics highlight the advantages of this technique to thoroughly characterize HLS-contained OH groups.

The largest amount of aliphatic OH was found in CAR, followed by MG, AD and RIP, which showed comparable amount of these functions, followed in the order by LIM and EUC. The OH aliphatic content in both materials from biorefinery residues was instead markedly smaller than for the rest of HLS (**Table 1** and **Supporting Figure SF2**). EUC and LIM contained similar COOH amount, whereas CAR showed the smallest amount of carboxyl OH functions. The content of COOH groups for the

TABLE 1 | Carbon compounds and OH functional groups observed by ^{13}C-CPMAS- and ^{31}P-NMR spectra, respectively, for different lignin-derived HLS, and their bioactive responses toward maize germination.

	Peak attribution (chemical shift-ppm)	MG	AD	BYP 1	BYP 2	CAR	RIP	LIM	EUC
^{13}C-CPMAS-NMR	**Alkyl C (0-45)**	9.3	5.7	19.7	17.4	17.1	25.0	18.9	38.0
	Methoxyl C (45-60)	22.1	17.7	16.0	15.6	14.1	14.8	14.9	18.6
	***O*-alkyl C (60-90)**	24.0	24.6	17.9	20.5	33.9	25.2	29.1	19.4
	Anomeric C (90-110)	10.9	12.5	9.4	8.6	11.1	8.9	10.1	7.0
	Aryl C (110-145)	21.5	27.3	24.6	24.5	15.7	14.4	13.5	9.0
	***O*-aryl C (145-160)**	6.8	9.4	7.7	7.6	4.3	4.6	6.5	2.0
	Carboxyl/esterified C (160-190)	5.5	2.8	4.7	5.8	3.8	7.1	7.0	6.0
	Hydrophobicity	37.6	42.4	52.0	49.4	37.1	44.0	38.9	49.0
	Hydrophilicity	62.4	57.6	48.0	50.6	62.9	56.0	61.1	51.0
^{31}P-NMR	**Aliphatic OH groups (150.8-146.3)**	5.5	5.7	1.6	1.6	6.8	5.4	4.7	3.9
	Syringyl groups (143.3-142.2)	0.07	0.18	0.04	0.06	0.14	0.07	0.11	0.04
	Condensed phenolic groups (142.8-141.7)	ND	ND	0.07	0.10	0.14	0.04	0.04	0.11
	Guaiacyl groups (140.2-138.4)	0.46	0.63	0.28	0.30	0.11	0.07	0.14	0.04
	***p*-Hydroxyphenyl groups (138.6-136.9)**	0.28	0.18	0.13	0.15	ND	0.04	0.07	0.07
	Carboxyl OH groups (135.6-133.7)	0.9	1.0	0.9	0.7	0.4	1.0	1.2	1.4
Biological variables	**Primary root length**	141.2	149.7	115.0	109.7	107.7	116.4	98.4	88.0
	Lateral seminal root length	174.1	153.1	111.7	141.1	140.7	120.7	106.9	80.8
	Total root length	156.3	151.5	113.0	127.0	123.0	119.0	103.0	83.3
	Coleoptile length	146.5	131.9	105.1	113.6	175.0	109.8	103.8	95.3

The ^{13}C-CPMAS spectral data are expressed as percent of total spectral area, while ^{31}P-NMR data are reported as mmol OH g^{-1} of HLS. The biological results refer to raw data normalized with respect to the control mean, which was set to 100. Hydrophobicity = sum of signal areas corresponding to the resonance of alkyl, aryl and O-aryl C; Hydrophilicity = sum of signal areas corresponding to the resonance of methoxyl, O-alkyl, anomeric and carboxyl C. ND = Not Detected.

other HLS was intermediate between those shown by LIM and CAR (**Table 1**).

One of the main advantages of the ^{31}P-NMR technique is to enable both a qualitative and quantitative evaluation of different types of phenols contained in HLS (Granata and Argyropoulos, 1995; Savy et al., 2016b). In fact, it is possible to accurately estimate the content of lignin monomers, as well as that of condensed phenolic groups, thus providing a detailed molecular description of phenol-rich HLS. Lignin monomers commonly found in grasses are composed of guaiacyl, syringyl and *p*-hydroxyphenyl radicals (**Supporting Figure SF3**). Conversely, hardwood lignin is virtually composed only by guaiacyl and syringyl units (Monda et al., 2018). In fact, *p*-hydroxyphenyl-derived compounds found in EUC, LIM, RIP, and CAR were either present in very low concentration or even absent, whereas they were well-represented in HLS from grasses (**Table 1**). The amount of syringyl moieties was markedly larger in AD, followed by CAR and LIM, while the remaining HLS showed comparable syringyl content. Similar amount of condensed phenolic units were found in CAR, EUC and BYP 2, whereas their content was smaller in LIM and RIP and completely absent in AD and MG (**Table 1**). Conversely, the two latter HLS showed the largest amount of guaiacyl, followed by the two BYP materials and by the hardwood-extracted substances.

A HLS-dependent activity toward the elongation of both the root apparatus and the coleoptile was noted (**Table 1**). The largest primary root and total root length was promoted by addition of AD and MG, while BYP 1, BYP 2, CAR, and RIP elicited similar primary root elongation. Instead, the primary root growth induced by both LIM and EUC was similar to the control (**Table 1**). The development of lateral seminal roots was more pronounced for MG and AD, followed by BYP 2, CAR, BYP 1, RIP, LIM, and EUC (**Table 1**). Finally, CAR favored the largest coleoptile length, followed by MG and AD, whereas BYP 1, BYP 2, RIP, and LIM had the least effect. As for the other parameters, EUC addition did not significantly affected plant development compared to the control (**Table 1**) (Savy et al., 2015a).

Projection on Latent Structure (PLS) Regression

The optimal number of latent factors1 extracted using data from ^{13}C-CPMAS spectra was 3 for primary root elongation, 1 for both lateral seminal roots and total root length and 5 for coleoptile elongation, whereas the optimal number of latent components was 3 for primary root growth, 4 for the elongation of the lateral seminal roots and the total root system and 2 for coleoptile growth when the HLS bioactivity was predicted on the basis of ^{31}P spectra (**Table 2**).

The score and loading plots for the first two optimal latent factors extracted from the predictor variables related to ^{13}C-CPMAS-NMR spectra are reported in **Figure 1**. Since only one latent component was considered as optimal in the case of both LSR and TRL, only score plots and loading plots for primary root and Col are reported. In the case of primary root elongation, the HLS isolated from miscanthus, and raw or treated giant reeds were placed in the upper right-hand quadrant. Here, two clusters may be noticed: one is composed from the HLS

TABLE 2 | Number of latent factors, and percentage of the explained cumulative variance for both predictors (VarXcum) and dependent variables (VarYcum) related to ^{13}C-CPMAS-NMR and ^{31}P-NMR spectral data.

		Primary root length	Lateral seminal root length	Total root length	Coleoptile length
^{13}C-CPMAS-NMR	n° latent factors	3	1	1	5
	VarXcum	98.83	72.81	72.93	99.97
	VarYcum	83.24	74.68	81.51	98.89
^{31}P-NMR	n° latent factors	3	4	4	2
	VarXcum	99.9	99.98	99.98	98.6
	VarYcum	89.63	95.8	96.43	92.74

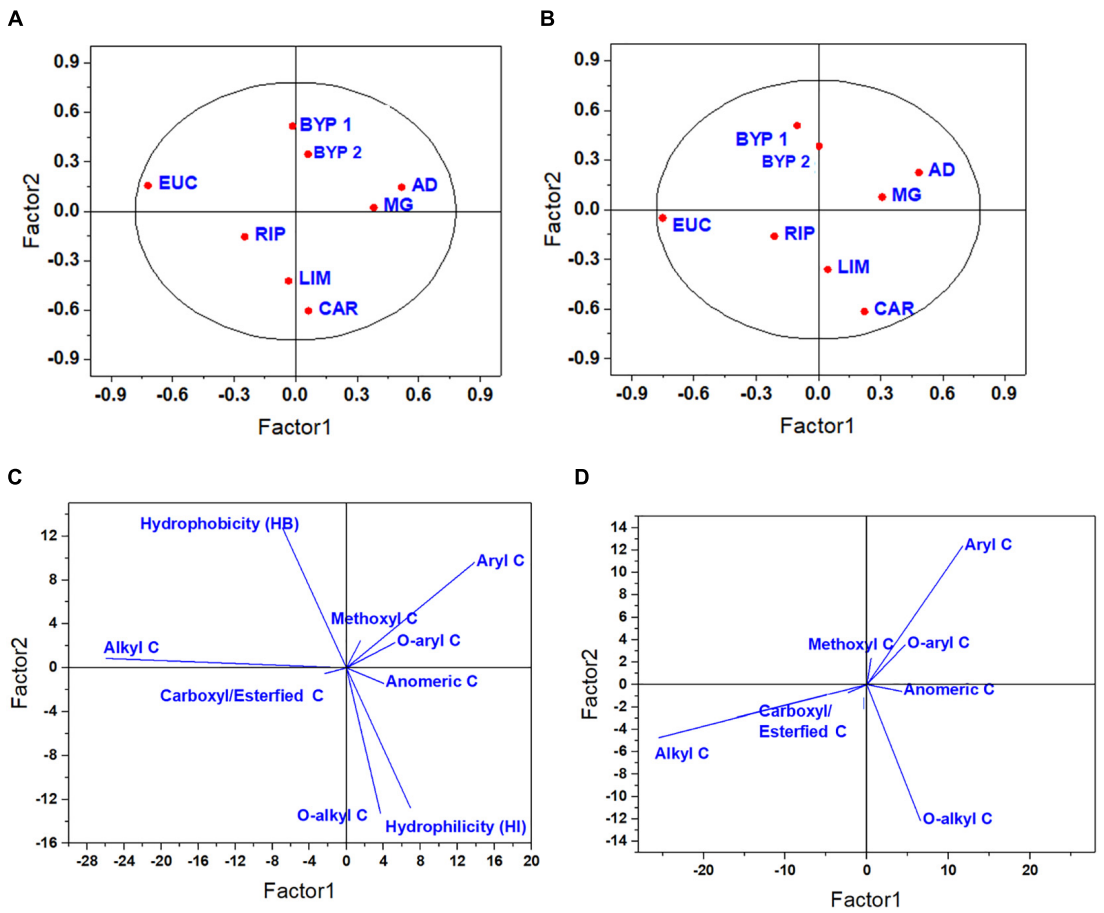


FIGURE 1 | Score plot **(A,B)** and loading plot **(C,D)** for the first two latent components for primary root **(A,C)** and coleoptile **(B,D)** elongation, as related to ^{13}C-CPMAS-NMR spectral data.

derived from biorefinery residues, whereas the other is formed by the HLS from miscanthus and giant reed. These four HLS were placed in the same quadrant due, to their large amount of lignin-related moieties Instead, the HLS from other sources spread across the other quadrants (**Figure 1A**). LIM and CAR were grouped together due to their HI and large content of *O*-alkyl C, while RIP was associated with carboxyl/esterified C, and EUC did not cluster with any HLS, showing the largest alkyl C content (**Figure 1C**). An even clearer separation was achieved in the case of coleoptile growth. For this biological variable, the second latent factor discriminated between HLS isolated from Magnoliopsida and Liliopsida, due to their similar relative amount of lignin-related moieties, such as methoxyl, aryl and *O*-aryl C (**Figures 1B,D**).

The PLS model calculated by using the ^{31}P-NMR spectra also resulted in the formation of several clusters (**Figure 2**). The primary root -related score plot showed that the first latent factor clustered BYP 1 and 2 together, while the second component enabled the discrimination between HLS obtained from herbaceous Angiosperms (MG, AD, BYP 1, and BYP 2)

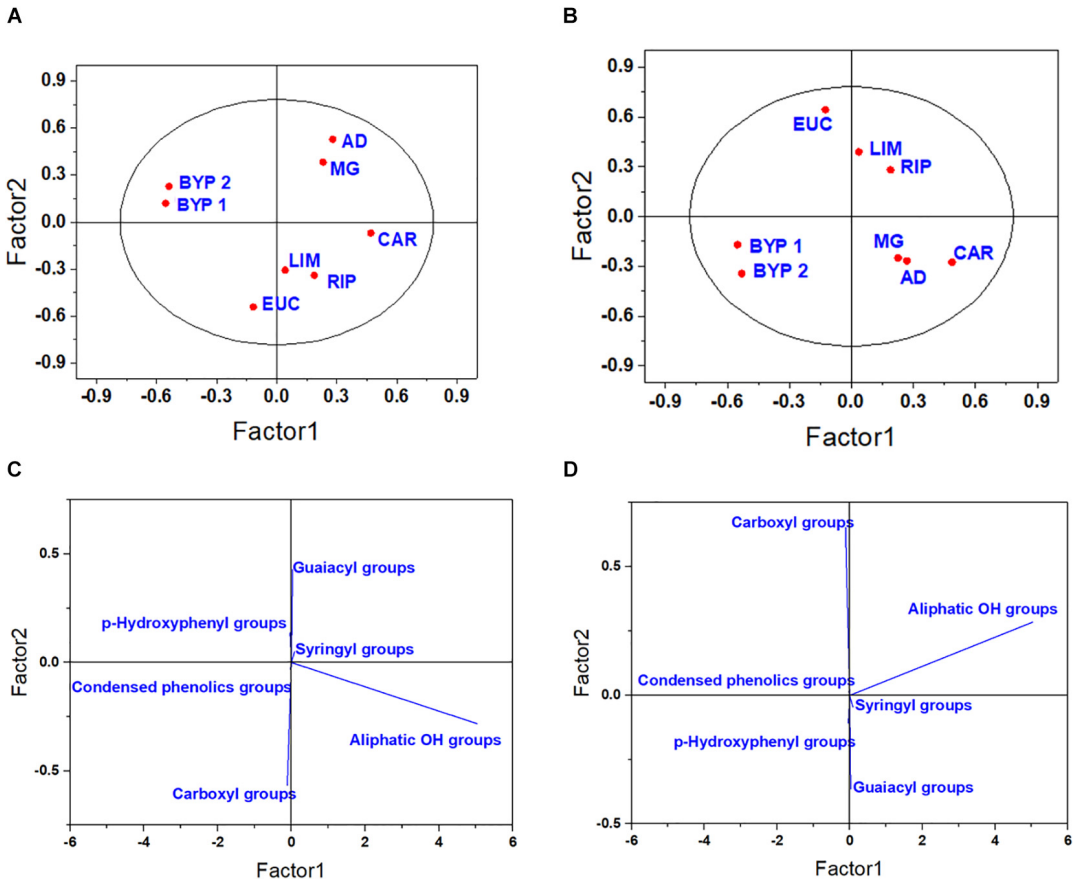


FIGURE 2 | Score plot **(A,B)** and loading plot **(C,D)** for the first two latent components for the elongation of primary root **(A,C)**, and lateral seminal root **(B,D)**, as related to ^{31}P-NMR spectral data.

from those isolated from hardwood (CAR, EUC, RIP, and LIM) (**Figures 2A,C**). Since the score and loading plots calculated for the elongation of lateral seminal roots, total root system and coleoptile were all similar, only those obtained for development of lateral seminal roots are reported (**Figures 2B,D**), while plots for both total root system and coleoptile length are shown in **Supporting Figure SF4**. The score plots for LSR showed that BYP 1 and 2 were separated from the other products, and CAR tended to cluster with MG and AD, instead ofbeing grouped with other Angiosperm-derived HLS (**Figures 2B,D**).

Despite our models relied on a relatively limited number of HLS, the residuals for all the biological variables were found to be randomly distributed around zero, no matter of the predictor employed (not shown). This indicates that there is no drift in the process and that the model is reliable. Moreover, the PLS models calculated by using either ^{13}C-CPMAS- or ^{31}P-NMR spectra provided satisfactory predictions for all evaluated biological traits (**Table 2**). The explained cumulative variability for both predictors and biological parameters based on ^{13}C-CPMAS-NMR spectra was larger than 70%, and reached almost 100% when modeling Col length. Conversely, the explained cumulative variability for the predictors and the biological parameters were always larger than 89% and even reached 100% when HLS bioactivity model was built by employing data from ^{31}P spectra (**Table 2**). Since the varYcum indicates the extent of biological variability explained by the proposed model, it also corresponds to the coefficient of determination obtained by plotting the measured biological variables versus the predicted values (**Supporting Figures SF5, SF6**).

In order to ascertain the statistical significance of each independent variable with respect to its effect on the generated model, the VIP was reported in **Figure 3**. The VIP describes the extent by which a model relies on each predictor, and indicates the contribution of each X variable in predicting the independent variables. The variables with larger values contribute to the model more than those with smaller values, thus entailing a greater predictive power. The most useful variables for all biological parameters when modeled on ^{13}C-CPMAS-NMR spectral data are alkyl C and aryl C (**Figure 3A**). Furthermore, Hydrophobicity and Hydrophilicity, as well as methoxyl C had a large impact on length of primary root. Methoxyl C, together with *O*-alkyl, *O*-aryl and carboxyl/esterified C showed an important effect on coleoptile length (**Figure 3A**). The guaiacyl and *p*-hydroxyphenyl compounds, together with the aliphatic OH and carboxyl groups were among the most important predictors for the development of the root

apparatus (**Figure 3B**). Instead, coleoptile development was mainly affected by OH groups in aliphatic and carboxyl moieties. Smaller effects were recorded for syringyl and condensed phenols (**Figure 3B**).

Although VIP is essential to unravel how well each descriptor predicts biological parameters, it does not inform on whether such predictors may positively or negatively affect the dependent variables. In order to understand the effect of the X variables toward the Y variables, the calculated regression coefficients should be considered. When basing ^{13}C-CPMAS spectral data to run the PLS model, positive regression coefficients were obtained for the methoxyl and aryl C for both the root system (primary root, lateral root, total root) and coleoptile (**Table 3**). Also, length of lateral seminal roots, total root system and coleoptile showed positive coefficients for the *O*-alkyl groups, while a negative coefficient was calculated for length of primary root. As for the *O*-aryl C, a positive coefficient was derived for length of lateral seminal roots and total root system, while negative ones were found for coleoptile and primary root elongation, the latter being anyhow almost negligible (**Table 3**). In the case of anomeric C, positive coefficients were found for the root-related parameters, whereas a negative one was calculated for coleoptile. Moreover, the coefficients for alkyl groups were negative for length of primary root lateral seminal roots and total root system, whereas it was positive for coleoptile. Carboxyl/esterified C showed negative coefficients for all the evaluated biological traits evaluated (**Table 3**). Finally, the same but opposite coefficients were obtained for both Hydrophobicity and Hydrophilicity, being Hydrophobicity negative and Hydrophilicity positive (**Table 3**). It is noteworthy that Hydrophobicity and Hydrophilicity were used as predictors for length of primary root, while they were not applied for other models, since their exclusion provided larger values of the explained cumulative variability for both predictors and biological parameters (data not shown).

The ^{31}P-NMR results for TMDP-derivatized HLS suggested a positive role of aliphatic OH, and G and P groups on the elongation of all the studied plant organs, while negative coefficients were derived for carboxyl moieties (**Table 3**). Furthermore, the PLS model calculated positive coefficients for syringyl functionalities in the case of primary root and coleoptile elongation, while negative coefficients were observed for the length of both lateral seminal root and total root system. Finally, negative coefficients were derived for condensed phenols with respect to root-related measurements, whereas positive coefficients were calculated for coleoptile (**Table 3**).

DISCUSSION

Pros and Cons of ^{13}C-CPMAS- or ^{31}P-NMR Spectroscopy for QSAR Derivation

Several models were created here to obtain a QSAR between HLS extracted by lignin-rich agro-industrial residues and their biological effect on maize early development. In the PLS model, ^{13}C-CPMAS-NMR or ^{31}P-NMR spectra were used as predictor variables, which enabled explanation of a large percentage of variance for all modeled biological parameters, thus indicating that these physical-chemical techniques provide an accurate prediction of HLS bioactivity (**Table 2**).

The explained cumulative variance based on ^{31}P-NMR spectra appeared to better relate predictors and dependent variables than the ^{13}C-CPMAS technique did, and allowed to acquire detailed and quantitative information on the most reactive groups of HLS. However, both methods have advantages and disadvantages. The derivatization technique applied to obtain ^{31}P-NMR spectra encompasses several drawbacks. In fact, routine ^{31}P analyses by this method may be difficult and costly, since the TMDP derivatizing reagent is not widely commercially available and it is rather expensive (Meng et al., 2019). Moreover, although TMDP can be synthetized in the laboratory, this is achieved through a complex procedure, and hazardous and/or deuterated solvents (pyridine and chloroform) are still required for ^{31}P spectral acquisitions (Hatzakis and Dais, 2008). On the other hand, the ^{13}C-CPMAS technique can be applied on samples without any preliminary treatment, but it requires a specific NMR probe for solid-state experiments, and that is not always available in routine NMR laboratories. Moreover, the solid-state technique provides only semi-quantitative data, thereby limiting its reliability in the complete characterization of a material. In spite of these drawbacks, such analytical tool was already successfully exploited to derive QSARs for HS (Canellas et al., 2012; Aguiar et al., 2013; García et al., 2016).

Despite the drawbacks showed by ^{13}C-CPMAS- and ^{31}P-NMR, the spectral results obtained by each technique have helped in shedding light on the molecular composition of HLS responsible for different biological activities (**Table 1**). The structural dissimilarities among HLS should be attributed to the different molecular nature of the original lignocellulosic biomasses and their different reactivity during the HLS extraction. For example, *p*-hydroxyphenyl moieties were not found in CAR, since cardoon lignin is composed only by syringyl and guaiacyl units (Lourençao et al., 2015), whereas lignin in giant reed, miscanthus, eucalypt and poplar contain all three monolignols, although their content vary significantly among these materials (Faix et al., 1989; Fukushima and Terashima, 1990; Evtuguin et al., 2001). Furthermore, though the AD and the BYP substrates were ultimately isolated from giant reed biomass, several discrepancies in composition were observed between the latter and the two former lignocellulosic extracts. For example, the relative content of alkyl and carboxyl C was larger in BYP1 and 2, whereas aliphatic OH functions and syringyl monolignol were larger in AD (**Table 1**). These differences should be attributed to the biorefinery treatments applied on giant reed, which first underwent a steam-explosion pre-treatment and then two different and enzymatic hydrolyzes, providing the two BYP products (Garbero et al., 2010; De Bari et al., 2013; Cimini et al., 2016). Hence, the hydrolytic procedures should have modified the bioactivity of the giant reed substrate, by making both aliphatic- and phenolic-containing molecules more prone

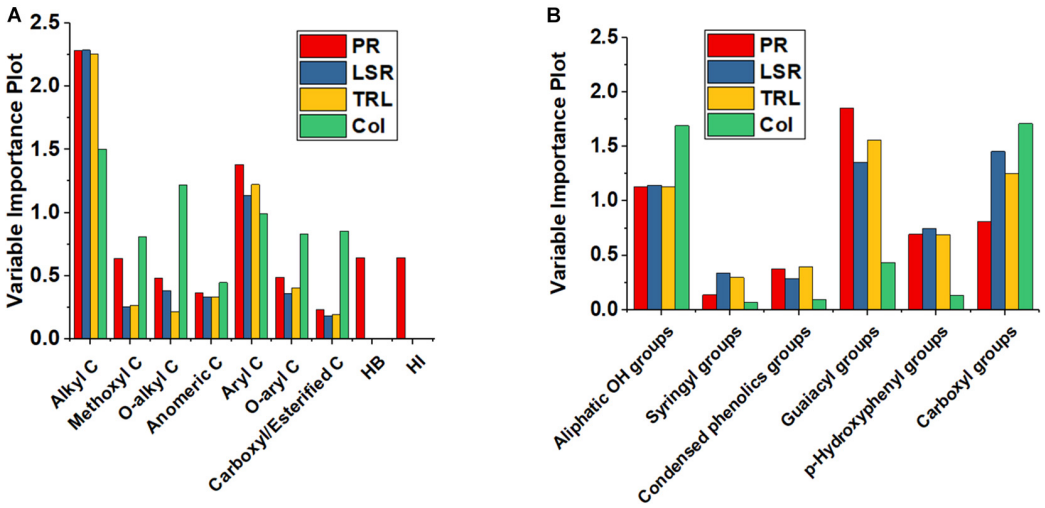


FIGURE 3 | Variable Importance Plot based on ^{13}C-CPMAS-NMR **(A)** and ^{31}P-NMR **(B)** results.

TABLE 3 | Regression coefficients from PLS regression for different biological variables.

		Primary root length	Lateral seminal root length	Total root length	Coleoptile length
^{13}C-CPMAS-derived predictors	**Alkyl C**	−1.17	−1.91	−1.59	0.32
	Methoxyl C	1.50	0.21	0.19	4.09
	***O*-alkyl C**	−1.07	0.32	0.15	6.78
	Anomeric C	0.32	0.28	0.23	−4.09
	Aryl C	0.71	0.95	0.86	6.67
	***O*-aryl C**	−0.03	0.30	0.29	−11.92
	Carboxyl/Esterified C	−0.26	−0.15	−0.14	−1.84
	Hydrophobicity*	−0.49			
	Hydrophilicity*	0.49			
	Intercept	117.85	131.77	125.58	−119.40
31P-derived predictors	**Aliphatic OH groups**	2.92	6.31	4.45	8.10
	Syringyl groups	4.27	−71.15	−49.26	2.33
	Condensed phenolic groups	−17.21	−62.79	−71.18	3.26
	Guaiacyl groups	74.27	34.76	49.25	15.56
	***p*-Hydroxyphenyl groups**	28.57	154.27	94.04	4.59
	Carboxyl OH groups	−8.41	−59.53	−37.35	−61.32
	Intercept	89.53	141.33	123.55	140.52

**The regression coefficient for both Hydrophobicity and Hydrophilicity were used as predictors only to model the primary root length.*

to degradation, thus explaining their significantly lower amount in BYP 1 and 2 (Savy et al., 2017b).

Different Chemical Functionalities Display Diverse Effects Toward Plant Growth

In line with previous findings, our models highlighted the dependence of HLS bioactivity upon specific chemical groups and functions (**Figure 3** and **Table 3**). The positive role of lignin-derived aryl moieties was already reported by Canellas et al. (2012) and Aguiar et al. (2013). These authors additionally indicated the negative impact on lateral root formation of both alkyl C and carbohydrates groups present in the humic acids applied to maize. We observed positive regression coefficients for the *O*-alkyl functions that were used to model the elongation of lateral seminal roots, and the total root and coleoptile length. Our findings agree with results found by García et al. (2016), who studied the structure-activity relationship of both HS and humic acids by Principal Component Regression, and ascribed the elongation of smaller rice (*Oryza sativa* L.) roots to HS-contained labile and more functionalized structures, such as *O*- and *N*-alkyl chains.

A negative regression coefficient was found here for the Hydrophobicity, in agreement with Canellas et al. (2012). The value of Hydrophobicity coefficient was related to the large negative impact of the alkyl groups (**Figure 3** and **Table 3**), which may have either a direct negative effect on plant growth or a role in reducing the positive bioactivity of polar molecules. In fact, hydrophilic bioactive molecules may become trapped within the hydrophobic domains of HLS, thus limiting their interactions with plant tissues and ultimately and significantly affecting HLS biological properties (Spaccini et al., 2002; Piccolo et al., 2019). In contrast to our results, García et al. (2016) reported a positive effect of alkyl C on plant development. This discrepancy may be attributed to specific differences in the types of alkyl functions between our materials and those of García et al. (2016). Moreover, it should be noted that the values of the regression coefficients based on the semi-quantitative ^{13}C-CPMAS-NMR technique are also dependent on other X variables, since the values of predictors are inter-dependent and sum to 100%. For this reason, the regression coefficient for each X variable is strongly affected by the raw value of other predictors, since when an X value increases other(s) necessarily decreases. Hence, the other regression coefficients could actually account for the negative effect of alkyl moieties, the effect of which is then not reflected in the calculated regression coefficient for such chemical group. Besides the structural diversity of the various HS, it should be noted that differences in their biological activity could be related to the heterogeneous ways the bioactivity was assessed. Indeed, the biological effect of HS had been evaluated on different plants at different phenological states, grown in different conditions. Therefore, the lack of homogeneity in the employed bioassays may also explain the discrepancies between our results and those of the cited studies (Zandonadi et al., 2013).

In line with the PLS regression based on ^{13}C-CPMAS-NMR spectra, the QSAR derived from ^{31}P-NMR spectra indicated the positive effect of aliphatic OH groups, and suggested an important role of both guaiacyl and *p*-hydroxyphenyl units in modulating the HLS bioactivity (**Figure 3** and **Table 3**). The positive biological effect of these compounds toward root development had already been described earlier and confirmed thereafter (DeKock and Vaughan, 1975; Kuiters, 1989; Reigosa et al., 1999). *p*-Coumaric, *p*-hydroxybenzoic, and vanillic acids, and *p*-vanillin were found to promote the root growth of six plant species in a dose-dependent manner. In particular, small concentrations stimulated root growth or were ineffective, whereas larger concentrations inhibited plant development (Kuiters, 1989; Reigosa et al., 1999). The positive effect of phenols on plant development may be due to their hormone-like activity in germination, as well as in root and coleoptile elongation. Savy et al. (2017a) showed the gibberellic-like effect of AD, as assessed by a specific bioassay. Pizzeghello et al. (2006) and Nardi et al. (2000) also reported the gibberellic-like effect of vanillin, and *p*-hydroxybenzoic and vanillic acids. Moreover, it was also found that a phenolic mixture from grape (*Vitis vinifera* L.) had a stimulating effect of on the activity of α-amylase, β-amylase, catalase and protease, which are enzymes known to be involved in the germination process (Tudose, 2002). Some of the listed phenolic compounds had been identified in our HLS, and might be responsible for their biological activity (Savy and Piccolo, 2014; Savy et al., 2015a, 2016a, 2017b).

Moreover, it should be kept in mind that the validity of our models may vary according to the HLS mode of application. If HLS are used as seed coaters, our models would likely accurately predict the HLS bioactivity. Instead, if HLS are applied to soil, some molecules may be selectively adsorbed onto clay surfaces or absorbed into pre-existing clay-humic complexes, hence significantly affecting HLS bioactivity (Xiao et al., 2019). Finally, the HLS may show a different bioactivity if sprayed on plant leaves rather than applied to the seeds, due the different interactions of such materials with the plant organs (Zandonadi et al., 2013; Sleighter et al., 2015).

QSAR Derivation May Support in Designing the Next-Generation Biostimulants

Altogether, our findings point out that HLS bioactivity was positively affected by the presence of lignin-derived monomers, while hydrophobic, alkyl components and free carboxyl groups had a negative influence (**Figure 3** and **Table 3**). Hence, the application of lignocellulosic biomasses containing large amount of guaiacyl and *p*-hydroxyphenyl units, such as herbaceous Angiosperms, can be recommended. Additionally, longer alkaline hydrolysis times may be adopted to reach a more extensive lignin depolymerization in order to increase the concentration of free phenolic units in HLS (Guizani and Lachenal, 2017), although a selective oxidation of lignin is difficult to achieve. In order to overcome undesired reactions and control the HLS oxidation, the implementation of specific oxidative strategies are required (Cha et al., 2017; Guo et al., 2018; Jeong et al., 2018). Finally, a removal of alkyl moieties from HLS may be envisaged to reduce the amount of such inhibitory compounds, for example by extracting them from the lignocellulosic matrix. However, because HLS bioactivity may be due to compounds bearing different chemical groups, further research should be carried out in order to implement analytical techniques for the isolation of the biologically active compounds present in the HLS (Nebbioso et al., 2014; Sleighter et al., 2015). Humeomics is an emerging protocol useful to progressively reduce the large chemical heterogeneity of HS and HLS (Nebbioso et al., 2014). Alternatively, preparative size exclusion chromatography can also be exploited to obtain fractions with similar hydrodynamic *radius* (Conte et al., 2007). Once the biological activity of HLS is understood, new biological trials should be carried out with the separated HLS fractions, and new, more robust PLS models could be obtained. These results may be then useful to select or modify HLS structure in order to fine-tune their bioactivity toward plant development.

CONCLUSION

Several PLS models were created in order to derive a QSAR between maize early growth and HLS isolated from lignin-rich agro-industrial residues, based on their molecular

characteristics evaluated by ^{13}C-CPMAS-NMR spectra and by ^{31}P-NMR spectroscopy after ^{31}P-derivatization. The developed models explained more than 72% of the cumulative variance regardless of the employed predictors, and suggested the relevant positive role of aryl-containing molecules and *O*-alkyl groups of lignin origin on root and coleoptile elongation. Moreover, positive regression coefficients were calculated for both guaiacyl- and *p*-hydroxyphenyl –derived molecules. Conversely, the PLS regression indicated the negative role of alkyl groups and free carboxyl/esterified functions on plant development. Since our models were built to predict the bioactivity of HLS at the early growth stage of maize, they provided information on the chemical characteristics of lignin-derived HLS relevant for seedling establishment and the plant organs impacted by HLS application. Further studies are needed to relate HLS molecular structure to specific, biostimulant-related plant traits, such those relevant to nutrient use efficiency, tolerance to abiotic stress and/or crop quality, especially for plants at more advanced growth stages. Moreover, further research should be devoted to the implementation of separation techniques in order to attempt the isolation of the biologically active compounds present in the HLS. Once the biologically active molecules have been identified, new, more robust PLS regressions can be calculated. Then, the researchers may adopt chemical technologies to control the molecular composition of HLS with the aim to enhance the functions responsible for their bioactivity. Tailored-made HLS with the desired bioactivity toward plant physiology and development may be therefore developed, with the aim to exert efficient and sustainable biostimulant capacities to improve crop yields and their resilience to adverse environment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DS conceived the manuscript, carried out the statistical analyses, and drafted the article. YB supervised the statistical tests and revised the manuscript. PD and VC contributed to the statistical analyses and revised the manuscript. PJ and AP revised the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00581/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 May 2020
doi: 10.3389/fpls.2020.00633

Rhizospheric Organic Acids as Biostimulants: Monitoring Feedbacks on Soil Microorganisms and Biochemical Properties

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The biostimulant potential of three different organic acids (OAs) present in the rhizosphere, specifically lactic, oxalic, and citric acids, have been studied. The results showed a rapid and complete metabolism of these three acids with soil microorganisms using them as a source of carbon and energy. Biostimulation was confirmed by soil biochemical studies which showed an increase in enzymatic activities, such as dehydrogenase and phosphatase, lactic and citric acids being those that produced the greatest biostimulation. With regard to microbiota composition, amplicon sequencing of the 16S rRNA gene showed changes in the structure of soil microbial communities. Applying OAs produced a decrease in richness and diversity indices, inducing specific changes in the structure of the microbiological communities. Applying lactic acid induced rapid changes in microbiota composition at both phylum and family taxonomic levels, favoring the proliferation of microorganisms involved in its degradation and soil fertility, such as the genus *Bacillus* and the family Micrococcaceae. Once the lactic acid was degraded, the biodiversity tended to return to similar phyla, but specific distinctive families and genera remained, leaving a pattern of induction of taxa described as plant growth-promoting bacteria (PGPB), such as the *Sinorhizobium* and *Lysobacter* genera, and the Pseudomonaceae family. Similar behavior was found with citric acid, which favored the proliferation and dominance of microorganisms of the Clostridiaceae family, involved in its degradation, as well as microorganisms of both the Micrococcaceae and Pseudomonadaceae families which were found on day 7, leaving a similar pattern of induction as that found after the mineralization of lactic acid. On the other hand, oxalic acid induced long-lasting changes in the bacterial community composition. This was characterized by an increase in the proportion of the Burkholderiales order, which includes microorganisms involved in the degradation of this acid and microorganisms described as PGPB. This study presents evidence supporting the use of OAs as potential soil fertility inducers, due both to their effects in enhancing the dominance of taxa described as PGPB and to their stimulating soil microbial activity.

Keywords: organic acids, biodiversity, DNA metabarcoding, PGPB, edaphic biostimulation

OPEN ACCESS

Edited by:
Andrew Merchant,
The University of Sydney, Australia

Reviewed by:
Peter Ryan,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia
Robert Koller,
Helmholtz-Verband Deutscher Forschungszentren (HZ), Germany

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *20 November 2019*
Accepted: *24 April 2020*
Published: *28 May 2020*

Citation:
Macias-Benitez S, Garcia-Martinez AM, Caballero Jimenez P, Gonzalez JM, Tejada Moral T and Parrado Rubio J (2020) Rhizospheric Organic Acids as Biostimulants: Monitoring Feedbacks on Soil Microorganisms and Biochemical Properties. Front. Plant Sci. 11:633. doi: 10.3389/fpls.2020.00633

INTRODUCTION

Soil microorganisms, because they determine soil biochemical properties and soil physicochemical properties such as organic matter, are considered important indicators of soil fertility and productivity (Van Der Heijden et al., 2008; Liang et al., 2018). Plants are able to modify their surroundings by root exudation into the rhizosphere of a large range of compounds that alter soil physical and chemical properties and mediate the interactions between plants and rhizospheric microorganisms (Nihorimbere et al., 2011; Huang et al., 2014). Root exudates are often divided into two classes of compounds: low molecular weight compounds, such as amino acids, organic acids (OAs), sugars, and phenolic compounds, and other secondary metabolites, as well as high molecular weight compounds, such as polysaccharides and proteins (Narasimhan et al., 2003; Bais et al., 2006; Badri and Vivanco, 2009; Huang et al., 2014). Many low molecular weight compounds have been hypothesized to present a functional significance in regulating ecosystem productivity, particularly in the rhizosphere. More specifically, low molecular weight carboxylic acids (LMWOAs) play a significant part in the rhizosphere as essential factors for nutrient acquisition, mineral weathering, and alleviation of anaerobic stress in roots (Blaylock and James, 1994; Zhou et al., 2007; Wang et al., 2015; Adeleke et al., 2017). These LMWOAs (ranging from 46 to 100 Da) are characterized as weak acids that contain a chain of carbon atoms associated with at least one functional acid group (Perminova et al., 2003; Dinh et al., 2017). A number of different OAs have been found in soils (e.g., oxalic, citric, formic, lactic, acetic, etc.; Mimmo et al., 2008; Jiang et al., 2012), the average total content of these acids is estimated as being as much as 10% of the total dissolved organic carbon (Van Hees and Lundstrom, 2000). In a wide range of soils, the concentration of these products in soil solution ranges from 1 to 50 μM (Strobel, 2001), an upper value that is not infrequent (Fox and Comerford, 1990).

The variety of organic compounds released by plants has been postulated as being a key factor in influencing microorganism diversity in the rhizospheres of different plant species (Rovira, 1969; Bowen and Rovira, 1991; Bolton et al., 1992), primarily by selecting organisms able to utilize the carbon source profile produced by the roots (Grayston et al., 1998, 2001). LMWOAs are intimately linked to the carbon cycle and P solubilization and acquisition and are a significant part of the water-soluble fractions of organic molecules released in the rhizosphere not only by root exudates, but also by soil microbial metabolites and organic matter decomposition (Adeleke et al., 2017). OAs are also considered key drivers in bacterial chemotaxis from bulk soil to the rhizosphere (Jones et al., 2003). In this way, a bidirectional relationship is established in which plant roots are able, at least partially, to induce a specific microbial community, their exudates facilitating the formation of a plant-friendly rhizosphere and promoting plant growth-promoting bacteria (PGPB) as well as microbial metabolites that affect soil physical-chemical and biological properties (Swinnen, 1994; Lemanceau et al., 1995; Latour et al., 1996). Therefore, the soil bacterial community structure and diversity could be affected by the application or different LMWOAs.

Soil contains highly diverse microbial communities and it is well-known that the information given by culture-dependent techniques is limited (Nisiotou et al., 2014). At present, the method of preference to survey complex microbial communities is based on 16S rRNA gene amplicon sequencing (Huse et al., 2008; Parlapani et al., 2018). Sample multiplexing is achieved by using sample-specific barcoding tagging which adds great versatility and makes the simultaneous analysis of multiple samples feasible.

The aim of this work is to study whether the treatment of soil with rhizospheric OAs, such as lactic, citric and oxalic acids, may have an effect on both soil physicochemical performance and on the composition of the associated microbial community, and their potential use as soil prebiotics.

Prebiotics are substrates that are selectively metabolized by microorganisms, inducing specific changes in the composition and/or activity of highly diverse microbial communities (Adam et al., 2016). These substrates could be used to stimulate a selected group of beneficial bacteria of interest, enhancing their growth and establishment in certain environments such as soils (Teitelbaum and Walker, 2002; Saulnier et al., 2008). As described above, plants can establish highly specific interactions with soil microorganisms by exudation of rhizospheric compounds, among which OAs seem to play a key role (Teplitski et al., 2000; Jones et al., 2003; Badri et al., 2009; Adeleke et al., 2017).With this in mind, we proposed the addition of OAs in soil, used as a selective source of food capable of stimulating specific bacteria and inducing changes in soil microbiota that could have a beneficial effect on plants.

MATERIALS AND METHODS

Experimental Design

In order to investigate the effects of OAs on biochemical and biological properties of soil, an experimental design was established according to Rodríguez-Morgado et al. (2017). Two hundred and fifty grams of soil were pre-incubated in semi-closed microcosms in the incubation chamber at 30–40% of their water holding capacity, under darkness and at 25°C for 7 days. After this phase, soil samples were mixed with OAs such as: L-lactic (BP, Ph. Eur.) pure, pharma grade (PanReac AppliChem, Barcelona, Spain) (L), oxalic acid 2-hydrate pure (PanReac AppliChem, Barcelona, Spain) (O), and citric acid (Sigma–Aldrich, St. Louis, MO, United States) (Ci).

Each OA was dissolved in distilled water, filtered using 0.2 μm cellulose acetate filters (Sartorius Stedim Biotech), to ensure sterility, and applied to the pre-treated soils at a final concentration of 0.05 mmol/g of dry soil. Soil without OA was used as control (C). Each treatment was replicated three times. Distilled water was added to each soil to reach 60% water-holding capacity.

All treated soil samples were placed in semi-closed microcosms and incubated in the incubation chamber under darkness at 25°C for 0, 1, 5, 7, 12, 21, and 28 days. The samples

were removed from the incubation microcosm at each incubation time. Soil samples from 0 time point were collected a few minutes after adding and thoroughly mixing each OA.

For each sample, 10 g of soil were taken, and stored in small glass jars (50 ml) at 4°C for chemical and biochemical analysis, whereas 2 g of soil subsamples were pooled and stored at −80°C in sterile polypropylene centrifuge tubes until DNA extraction.

Soil Chemical and Biochemical Analysis

The soil used in this study was a Plagic Anthrosol (IUSS Working Group WRB, 2015). The main soil characteristics are shown in **Table 1**. Soil pH was determined in distilled water with a glass electrode (soil/H_2O ratio 1:2.5) (MAPA. Métodos Oficiales de Análisis, 1986).

Organic acids were extracted according to the method described by Bolan et al. (1994). One gram of soil was incubated with 10 ml of extraction buffer (1 N H_2SO_4), stirring for 30 min at room temperature. The samples were then centrifuged at 12,000 × *g* for 15 min at 4°C, to remove particles in suspension, and supernatants were filtered through a 0.2 μm regenerated cellulose filter. Concentration of OAs present in each extract were analyzed by RP-HPLC using a LC-4000 JASCO system equipped with a Nova-Pak C18 4 μm (4.6 × 300 mm) reverse phase column (Waters) coupled to a JASCO HPLC UV-475 UV/VIS detector. 20 μl of each sample was injected into the chromatograph and eluted isocratically at a constant flow of 0.7 ml/min using 0.2 M KH_2PO_4 + 1.5% methanol adjusted with phosphoric acid at pH 2 as the mobile phase. OAs were detected at a wavelength of 215 nm. Analytes concentrations were determined based on the standard corresponding OAs' calibration curves. Standards of the three OAs were supplied by Sigma–Aldrich.

Soil dehydrogenase activity was measured by reducing 2-p-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride to iodonitrophenyl formazan (INTF) according to Tabatabai (1994). Product resulting from the reduction was measured at 485 nm using a GeneQuant 1300 spectrophotometer (GE Healthcare Bio-Sciences AB, United States).

Soil acid phosphomonoesterase activity was assayed (Tabatabai, 1994) using p-nitrophenyl phosphate as substrate and modified universal buffer (MUB) substrate buffer (pH 6.5). Then 0.5 M $CaCl_2$ and 0.5 M NaOH were added to stop the reaction and to extract the product, p-nitrophenol (PNF), whose concentration was determined photo-metrically at 410 nm.

TABLE 1 | Soil properties (mean ± standard error).

Soil properties	
pH (H_2O)	7.9 ± 0.2
CO_3^{-2}(g kg^{-1})	204 ± 10
Fine sand (g kg^{-1})	139 ± 33
Coarse sand (g kg^{-1})	385 ± 24
Silt (g kg^{-1})	244 ± 17
Clay (g kg^{-1})	232 ± 9
Clay types	Smectite 64%
	Kaolinite 24%
	Illite 12%
Organic matter (g kg^{-1})	± 0.4
Humic acid-C (mg kg^{-1})	18.2 ± 2.6
Fulvic acid-C (mg kg^{-1})	9.6 ± 1.3
Total N (g kg^{-1})	0.5 ± 0.2
Olsen P (g kg^{-1})	8.6 ± 0.9
Fe (mg kg^{-1})	35.6 ± 3.8
Cu (mg kg^{-1})	9.5 ± 1.2
Mn (mg kg^{-1})	11.1 ± 2.4
Zn (mg kg^{-1})	8.3 ± 1.3
Cd (mg kg^{-1})	6.2 ± 1.5
Pb (mg kg^{-1})	0.39 ± 0.15
Ni (mg kg^{-1})	3.1 ± 0.5
Cr (mg kg^{-1})	5.5 ± 0.7

Soil DNA Extraction and Illumina MiSeq Sequencing

Total genomic DNA was extracted from soil samples using the DNeasy Power-Soil DNA isolation kit (Qiagen) according to manufacturer's instructions. DNA was resuspended in a final volume of 100 μl, and a DNA extraction blank was included in each extraction round to check for cross contamination during the DNA extraction process.

For library preparation, the V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primer pair Bakt 341F (5' CCTACG GGN GGC WGC AG 3')/Bakt 805R (5' GAC TAC HVG GGTATC TAA TCC 3') (Herlemann et al., 2011) as the forward and reverse primers with the Illumina-specific sequencing sequences attached to their 5' ends.

The PCR was performed in 25 μl final volume containing 12.5 μl of Supreme NZYTaq 2x Green Master Mix (NZYTech), 0.5 μM each primer, 2.5 μl of template DNA, and ultrapure water. The PCR program consisted of an initial heating step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min.

The barcoding sequences required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions but with only five cycles and with 60°C as the annealing temperature. A negative control containing no DNA was included in order to check for contamination during library preparation.

The correct library size was checked by gel electrophoresis and then the libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), and pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific). Finally, the pool was paired-end sequenced in an Illumina MiSeq PE300 platform.

Analysis of Microbial Community Composition

Sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0) as described by Caporaso et al. (2010). Raw FASTQ files were demultiplexed, trimmed by CUTADAPT 1.3 (Martin, 2011), merged by FLASH (Magoč and Salzberg, 2011), and quality–filtered and labeled by

QIIME 1.9.0 with the following criteria: (i) sequences whose overlap exceeded 30 bp were merged according to their overlap sequence; (ii) primers were matched allowing two nucleotide mismatches, (iii) reads shorter than 300 nucleotides were removed; and (iv) merged reads were quality-filtered at minimum Phred quality score of 20.

All chimeric sequences were identified and removed by the UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH, using the Greengenes reference database (DeSantis et al., 2006). The sequences were then clustered into operational taxonomic units (OTUs) using the *de novo* approach at the $\geq$ 100% identity threshold. Singleton OTUs were filtered out, and the representative sequence for each OTU was assigned to a microbial taxon using the RDP classifier (Wang et al., 2007) with a confidence threshold of 97%.

Alpha diversity indices Chao, Good's coverage, Simpson, Shannon, and phylogenetic diversity were calculated to analyze the complexity of species diversity in each sample.

Operational taxonomic unit data files generated by QIIME were imported in R version 3.5.1 (R Core Team, 2018), so as to further process and visualize results. OTUs counts and taxonomic assignments were merged to a phyloseq object with phyloseq R package (McMurdie and Holmes, 2013).

Rarefaction, relative abundance, and heatmap plots were generated using a combination of Vegan (Oksanen et al., 2018) and ggplot2 (Wickham, 2016) R packages. The principal coordinate analysis (PCoA) using the Weighted-Unifrac distance metric was applied to visualize the microbial community structure in relation to each treatment for each time, and Venn diagrams were generated with VennDiagram (Chen, 2018) R package to depict all possible comparisons of shared, common, and/or unique OTUs, among samples.

The original sequence data have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB35168.

Statistical Analysis

The differences in soil chemical and biochemical properties among different treatments were compared using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test using agricolae R package (Mendiburu, 2019). All significance levels were set at $p < 0.05$.

RESULTS

Influence on pH and Mineralization of Organic Acids on Soil

The addition of OAs induced a decrease in soil pH (**Figure 1**), whose magnitude was dependent on the OA added. Lactic acid produced the highest decrease in pH, followed by citric acid and oxalic acid, respectively. The initial pH decrease returned to control sample values 12 days after applying the OAs.

This pH value decrease was independent of acid strength. Lactic acid a weak monoprotic acid (pKa: 3.86) was the acid with the greatest capacity to reduce soil pH, from 7.91 to 7.18. Oxalic acid, with two diprotic carboxylic acid groups and citric acid, a triprotic carboxylic acid had a smaller capacity to acidify the soil, reaching to pH 7.61 and 7.50, respectively.

The three OAs used were completely mineralized within days of being applied (**Figure 2**). About 70% of the lactic acid and more than 50% of the citric and oxalic acids had been degraded after 5 days. The lactic and oxalic acids became undetectable after 7 days, whereas the citric acid had a slower mineralization rate. Mineralization of the OAs resulted in a return to control samples pH values.

Influence of Organic Acids on Soil Enzyme Activities

Soil enzymes are the mediators and catalysts of a wide range of soil biological processes and provide an integrative biological assessment of soil functions (Nannipieri et al., 2002). In our present work, two enzymatic activities were measured; dehydrogenase activity, commonly used as an indicator of biological activity in soils, and phosphomonoesterase activity, that has a crucial role in P cycle and correlates with soil phosphate bioavailability (Karaca et al., 2011; Navnage et al., 2018) (**Figures 3**, **4**). Samples treated with lactic and citric acids showed a significant stimulation of dehydrogenase activity with respect to control samples, whereas samples treated with oxalic acid presented an insignificant effect on this activity.

Although both lactic and citric acids produced maximum peaks of dehydrogenase activity around the twelfth experimental day [lactic acid (L), 283.7 μmoles $INTFg^{-1}min^{-1}$; citric acid (Ci), 425.7 μmoles $INTFg^{-1}min^{-1}$], the samples treated with citric acid resulted in a highest peak of activity which was slightly delayed with respect to the lactic acid-supplemented samples.

From day 12 of the experiment dehydrogenase activity decreased progressively and by the end of the incubation period, the values of treated and control soil samples both presented similar levels (**Figure 3**).

After 7 days of incubation phosphomonoesterase activity (**Figure 4**) was decreased due to the addition of the three OAs [L, -18.38%; Ci, -15.65%; oxalic acid (O), -28.55%] 12 (L, -28.57%; Ci, -30.07%; O, -18.69%) with respect to control samples. The results showed that the decrease in phosphomonoesterase activity coincides with the pH drop. At the end of the assay when pH has reverted to the control values and OAs have been completely metabolized phosphomonoesterase activity remained at higher values than controls in all OAs-treated samples (L, + 41.14%; Ci, + 66.15; O, + 17.44%).

Effects on Soil Bacterial Community Diversity

A total of 85,549 quality bacterial sequences were obtained with a range of 8191–12,534 sequences per sample. To perform the downstream analyses, each sample was normalized to the minimum depth of sequences, which was 8191.

The good coverage indices for all samples were greater than 0.93 (**Table 2**), and their rarefaction curves (**Supplementary Figure S1**) approached a level-off indicating that our analysis captured most of the bacterial diversity representative of the samples.

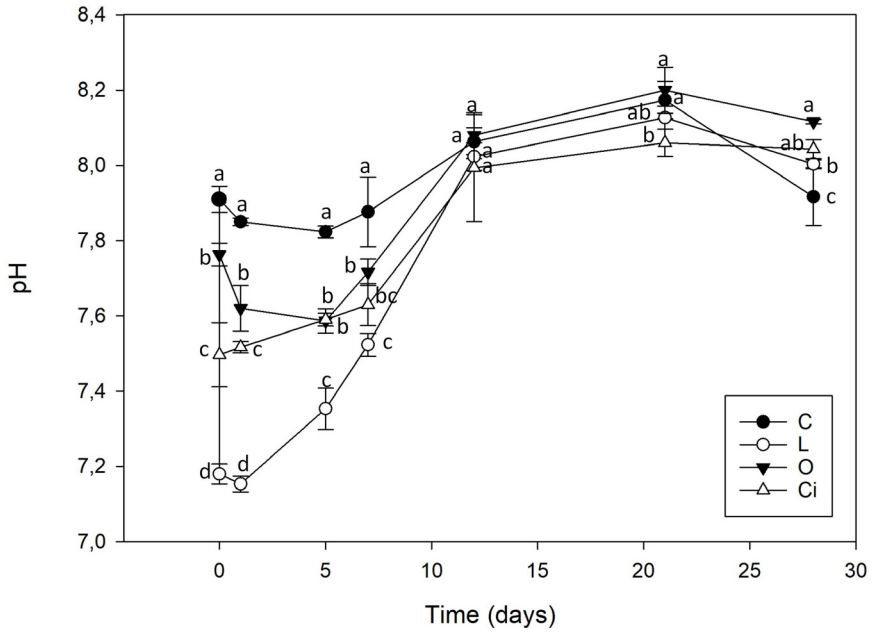


FIGURE 1 | Soil pH after organic acids supplementation. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. Different letters indicate significant difference among treatments ($p < 0.05$).

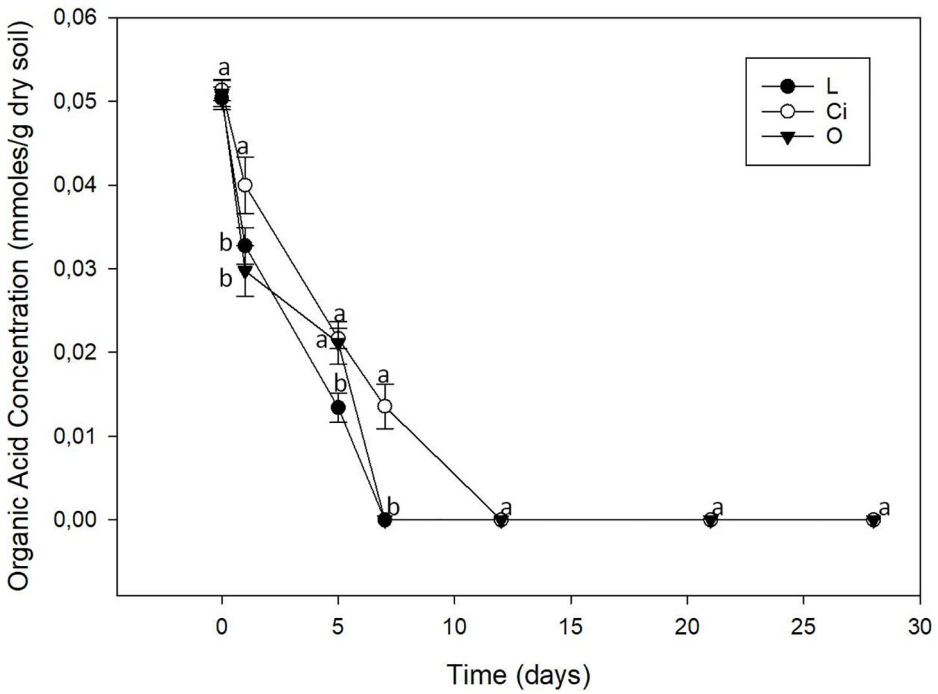


FIGURE 2 | Organic acids content of soil. L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. Different letters indicate significant difference among treatments ($p < 0.05$).

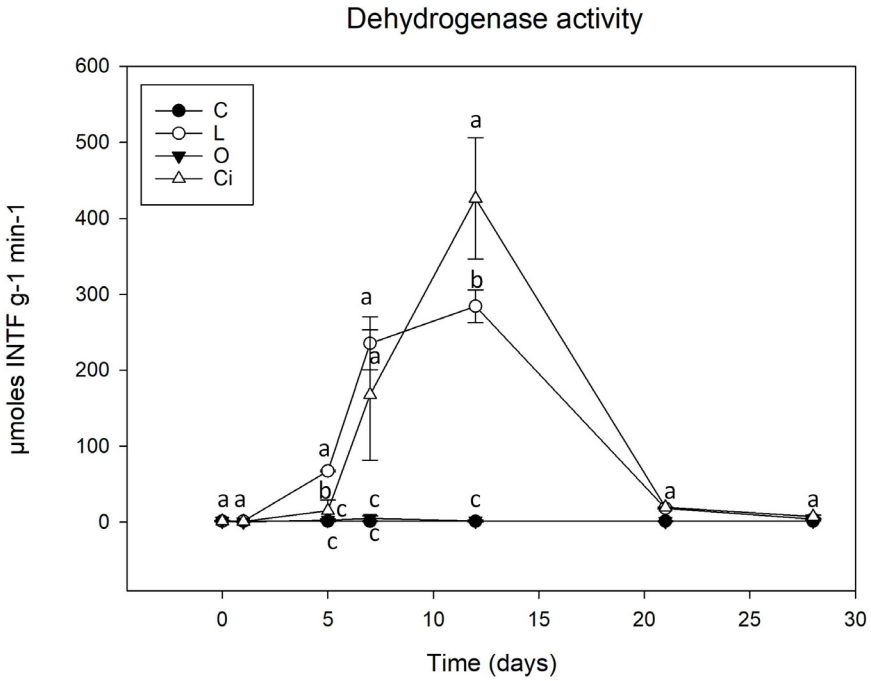


FIGURE 3 | Dehydrogenase activity in soil samples. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. Different letters indicate significant difference among treatments ($p < 0.05$).

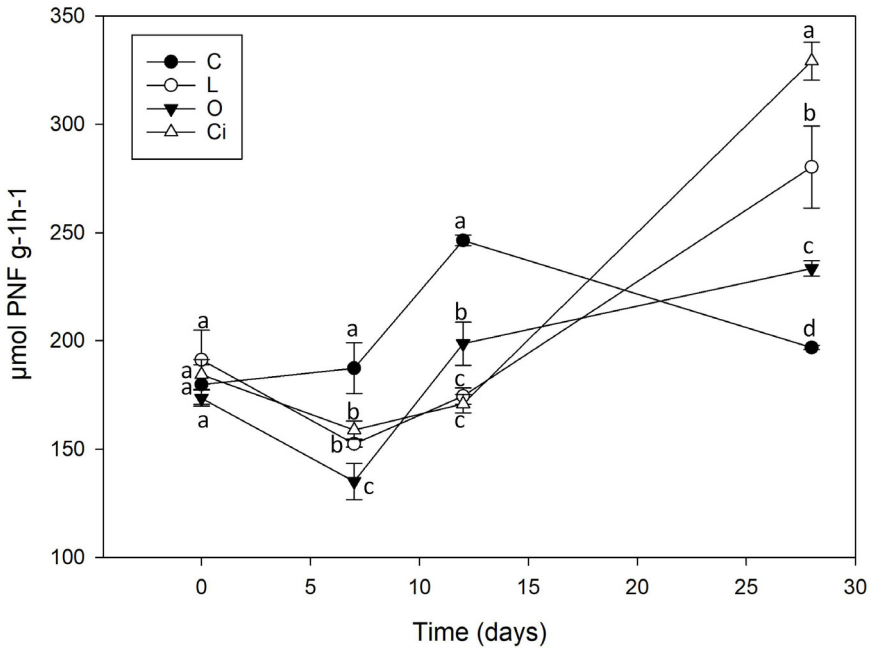


FIGURE 4 | Acid phosphomonoesterase activity in soil samples. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. Different letters indicate significant difference among treatments ($p < 0.05$).

TABLE 2 | α-Diversity indices of soil samples.

Treatment	Time	Coverage	Observed OTUs	Shannon	Simpson	PD_whole_tree	Chao1
Control	7	0.944	1497	9.2	0.995	67.9	1837
	28	0.946	1480	9.3	0.996	67.5	1793
Lactic	7	0.938	1135	7.4	0.973	56.2	1672
	28	0.945	1212	8.1	0.986	61.8	1620
Oxalic	7	0.940	1317	8.3	0.983	62.9	1723
	28	0.945	1297	8.5	0.983	64.9	1700
Citric	7	0.947	890	5.9	0.884	50.5	1434
	28	0.944	1150	7.8	0.981	59.3	1611

Coverage, non-parametric coverage estimator; Observed, OTUS observed operational taxonomic units; Shannon, non-parametric Shannon diversity index; Simpson, non-parametric Simpson diversity index; PD_whole_tree, Faith's Phylogenetic Diversity index; Chao1, richness of the Chao1 estimator.

Control samples had the highest number of observed OTUs and the highest richness and diversity indices.

Organic acid supplementation produced a decrease in the richness and diversity of the bacterial communities, being more pronounced in the samples treated with citric acid and lactic acid at 7 days. Specifically, on day 7, the sample treated with citric acid showed both the lowest number of OTUs (890) and the lowest values for the diversity indices of Shannon (5.9), Simpson (0.884), and phylogenetic diversity (PD_whole_tree) (50.5) (**Table 2**).

Venn diagrams (**Figure 5**) were used to represent the shared and unique OTUs among all the samples after treatment with OAs on both days 7 and 28. Thus, on day 7, all samples shared 459 (23.1%) OTUs, the control sample had 164 (8.56%) unique OTUs and 419 (21.87%) OTUs were specific to the application of the three OAs, of which 28 OTUs were shared among the three OA treatments and 75 (3.85%), 105 (5.48%), and 51 (2.67%) OTUs were unique to the treatments of lactic, oxalic, and citric acid, respectively (**Figure 5A**). On day 28, all of the samples had 609 (31.8%) OTUs in common, 144 (7.5%) OTUs were found exclusively in the control sample and 435 (22.7%) OTU were detected in samples treated with OAs only, of which 40 OTUs were shared by the three OA treatments, and 60 (3.13%), 84 (4.38%), and 66 (3.44%) OTUs were unique to the samples treated with lactic, oxalic, and citric acids, respectively (**Figure 5B**).

The Venn diagrams show the existence of OTUs specific to each OA treatment. Microbial communities differed between each other and differed from the control on both days 7 and 28.

The effect of the application of OAs on the composition of bacterial communities was analyzed by two-dimensional PCoA of weighted UniFrac distance (**Figure 6**), which explained 63.8% (PCoA1) and 13.7% (PCoA2) of the total variance and confirmed a differentiation between the samples treated with the different OAs and the control samples. On days 7 and 28, samples treated with oxalic acid were grouped in the same cluster, whereas samples treated with lactic and citric acids showed notable differences in the composition between days 7 and 28.

Applying the three OAs studied induced changes in bacterial communities, finding that these communities are different not only from control but also between each other. With regard to OAs, oxalic acid produced a long-lasting change, showing almost identical populations both on days 7 and 28, although samples treated with lactic and citric acids showed different communities on different days during the study.

Effects on Soil Bacterial Community Composition

Analysis of the taxonomic composition of samples treated with OAs showed that all samples shared a total of 10 phyla. The main phyla for all of them were Proteobacteria, Actinobacteria, Acidobacteria, and Firmicutes, but their abundances differed according to OA supplementation and incubation time (**Figure 7**).

The soil microbiota in the samples treated with lactic acid on day 7 were dominated by the Firmicutes, Actinobacteria, and Proteobacteria phyla, in this order, which represented 90.7% of the total abundance (**Figure 7**).

At phylum level, lactic acid produced an increase of 28.7 and 11.1% in the relative abundances of Firmicutes and Actinobacteria, respectively, as well as a decrease of 17.8 and 8.4% in the abundances of Proteobacteria and Acidobacteria.

The increase of Firmicutes was due mainly to the increase of 16.1 and 12.6% in the Bacillales and Clostridiales, whose main identified genera were *Bacillus* and *Pelosinus*, respectively (**Figure 8** and **Supplementary Table S1**).

The increase in the relative abundance of the Actinobacteria phylum corresponded to an increase in the relative abundance of the Actinomycetales order, with an increase of 16.9% in the relative abundance of the Micrococcaceae family in particular (**Figures 8**, **9**).

Within the Proteobacteria phylum, the 17.3% decrease in the relative abundance of the Alphaproteobacteria class should be noted and was paralleled by a decrease in the relative abundance of the Rhizobiales [Lt7 (lactic acid time 7 days), 4.7%; Ct7 (control time 7 days), 9.2%], Rhodospirillales (Lt7, 3.7%; Ct7, 6.3%), and Sphingomonadales (Lt7, 5.8%; Ct7, 17.9%) orders and a 3%increase in the relative abundance of the Gammaproteobacteria, class corresponding to an increase in the relative abundance of the Pseudomonadaceae family (Lt7: 3.8%, Ct7: 0%). On day 28 of incubation, when the OAs had been metabolized, the microbial community of samples treated with lactic acid seemed to restore its bacterial composition with respect to the control at the phylum level. Thus, Proteobacteria became the dominant phylum followed by the Actinobacteria

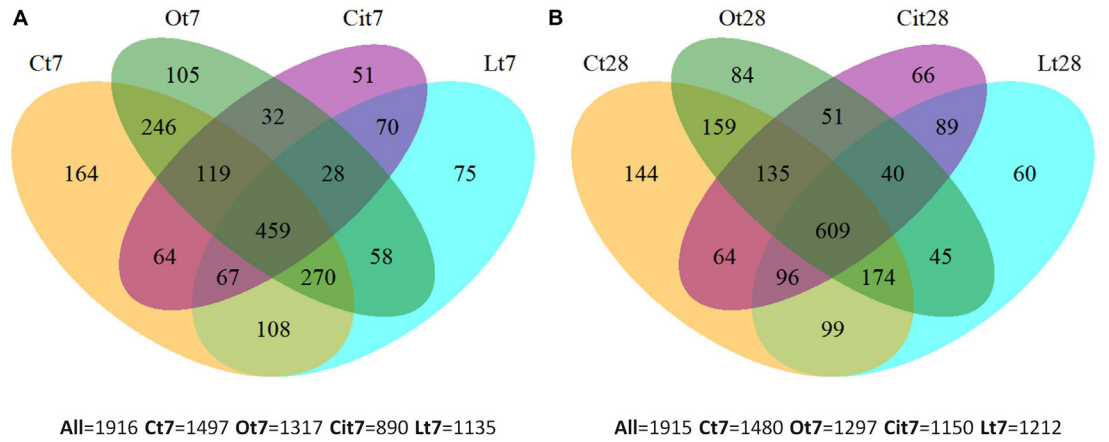


FIGURE 5 | Venn diagrams of unique and shared OTUs among samples at **(A)** 7 and **(B)** 28 days. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample.

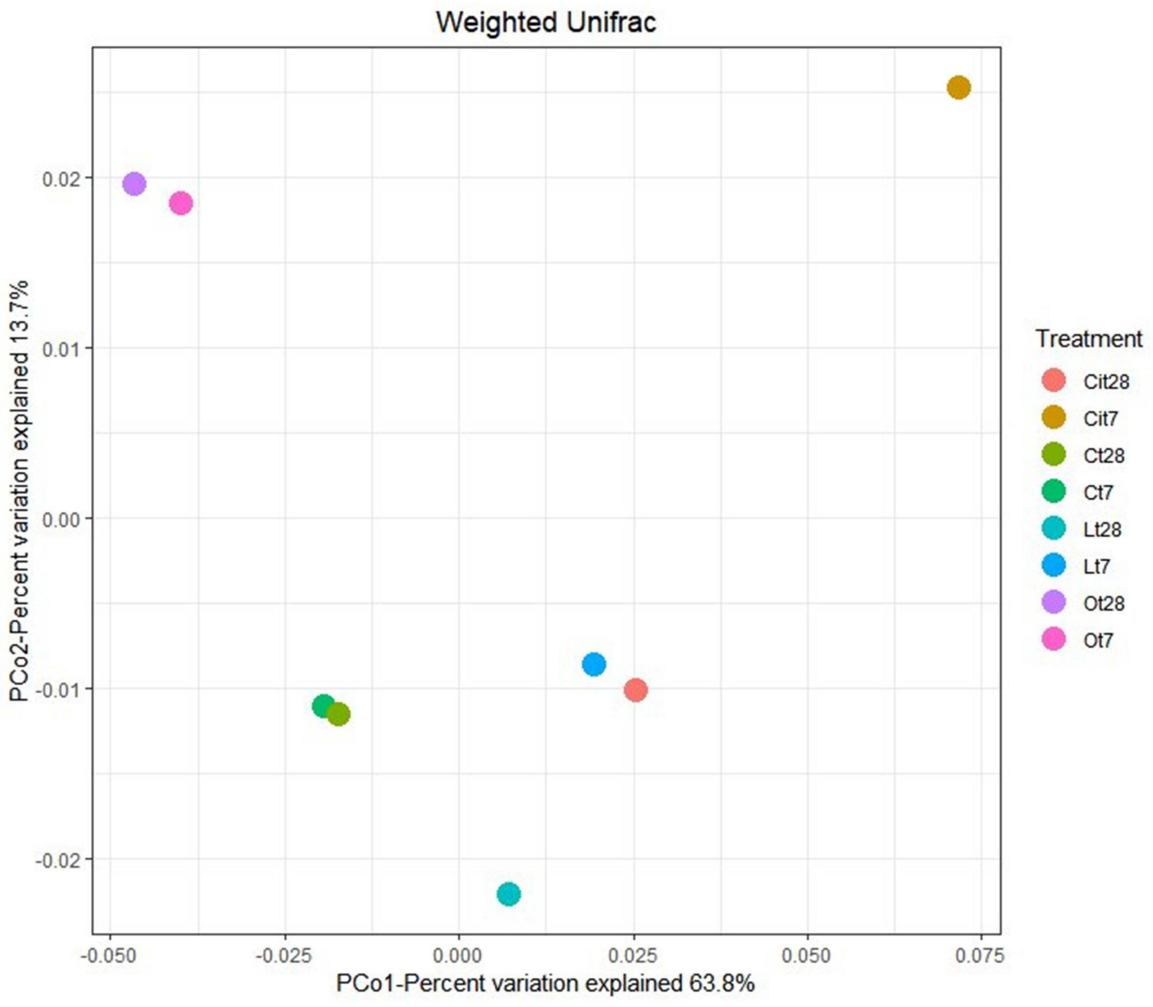


FIGURE 6 | Principal coordinate analysis (PCoA) based on the Weighted Unifrac distance. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. t7: day 7; t8: day 28.

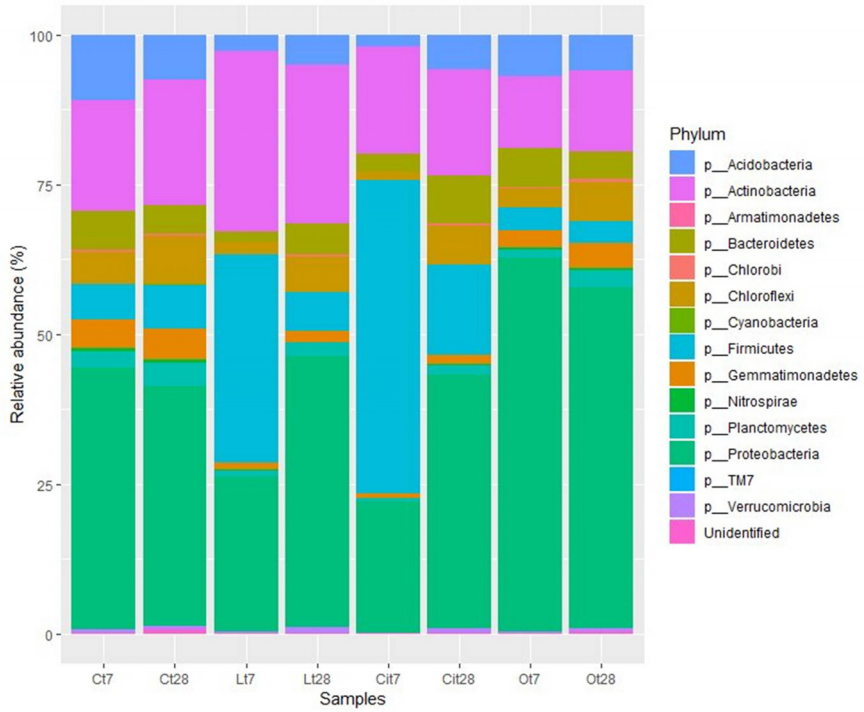


FIGURE 7 | Bacterial community composition at phylum level. Sequences that could not be classified into any known group were designated "Unidentified." C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. t7: day 7; t8: day 28.

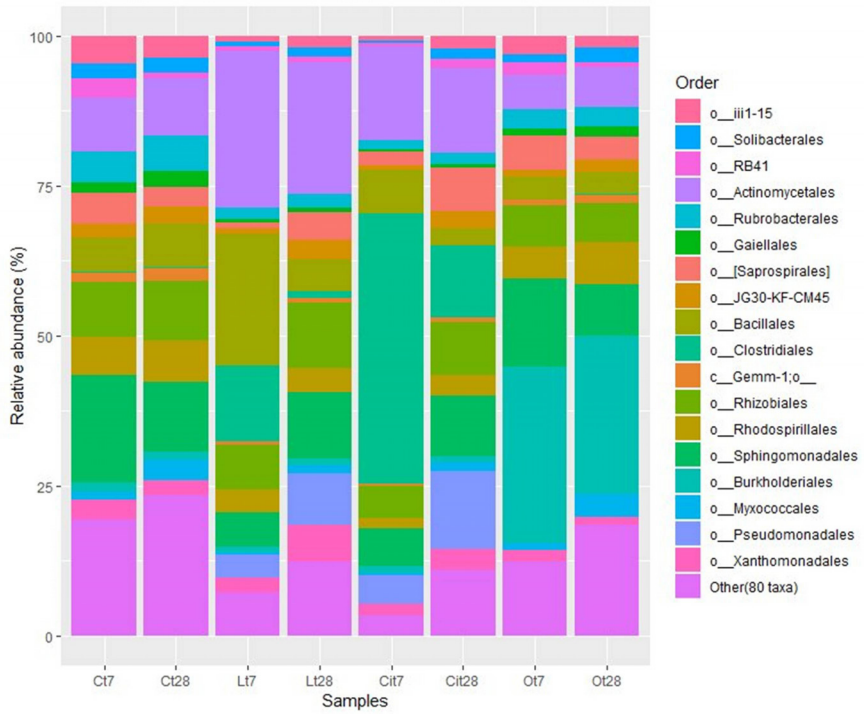


FIGURE 8 | Major bacterial orders. Orders with a total abundance of less 1.0% were collected into "Other (80 taxa)." C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. t7: day 7; t8: day 28.

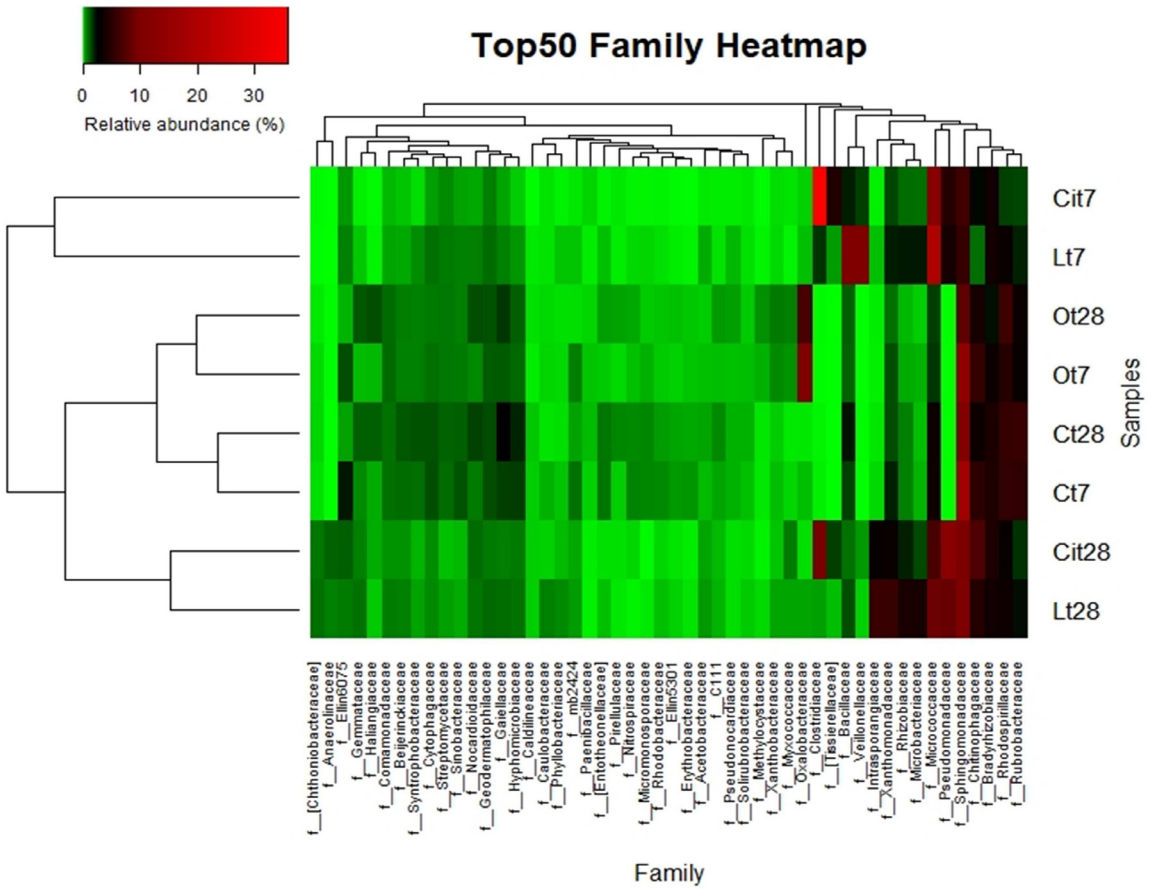


FIGURE 9 | Top 50 most-abundant identified bacterial families. Double hierarchical dendogram of bacterial distribution. Average linkage hierarchical clustering based on Bray–Curtis dissimilarity. C, control sample; L, lactic acid treated sample; O, oxalic acid treated sample; Ci, citric acid treated sample. t7: day 7; t8: day 28.

and Firmicutes phyla, with similar relative abundances to those detected in control samples (45.1, 6.5, and 26.1%, respectively).

Looking into changes at lower taxonomical categories during the lactic acid supplemented experiment, we can observe a few differences, mainly in the Proteobacteria and Actinobacteria phyla. Within the Proteobacteria phylum, there was an increase in the relative abundances of the families Rhizobiaceae, Pseudomonadaceae, and Xhantomonadaceae, which presented percentages of relative abundances: 3.6, 12.2, and 4.5%, respectively, higher than control samples.

Within the Actinobacteria phylum the Actinomycetales order maintained a relative abundance similar to that detected on day 7 12.1% higher than that of the control sample, with special stimulation of the Micrococcaceae (Lt28, 8.9%; Lt7, 20.4%; Ct28, 3.4%), Microbacteriaceae (Lt28, 4%; Lt7, 2.2%; Ct28, 0.3%), and Intrasporangiaceae (Lt28, 6.8%, Lt7, 0.2%; Ct28, 0.1%) families (**Figures 8**, **9** and **Supplementary Table S1**).

Similar to lactic acid-supplemented samples, the soil microbiota in the samples treated with citric acid on day 7 were dominated by the Firmicutes, Proteobacteria, and Actinobacteria phyla, representing 92.4% of the total abundance (**Figure 7**).

By day 7, citric acid produced an increase of 46.4% in the relative abundances of phylum Firmicutes (Cit7, 52.3%; Ct7, 5.9%), as well as a decrease of 21.8 and 9.1% in the abundances of Proteobacteria (Cit7, 22%; Ct7, 43.8%) and Acidobacteria (Cit7, 1.8%; Ct7, 10.9%) phyla, respectively.

Considering lower taxonomical categories, the increase in Firmicutes corresponded almost entirely to a 38.4% increase in the relative abundance of the family Clostridiaceae. Within the Proteobacteria phylum, the samples treated with citric acid resulted in a decrease of 10.9% (Cit7, 6.3%; Ct7, 17.2%) and an increase of 4.8% (Cit7, 4.8%; Ct7, 0%), of the families Sphingomonadaceae and Pseudomonadaceae, respectively (**Figure 9**).

Even though on day 7 the Actinobacteria phylum did not present differences respect to the control at phylum level, a marked increase of 9.5% in the relative abundance of the Micrococcaceae family was observed. As in the samples treated with lactic acid, the Acidobacteria phylum decreased its relative abundance.

In parallel to the increase in microbial activity, the presence of citric acid induced a rapid stimulation of specific

bacterial populations, as in the case of the Clostridiaceae, Pseudomonadaceae, and Micrococcaceae families (**Figure 9**).

Like lactic acid, at the end of the incubation period (28 days) when the citric acid had been completely degraded, the composition of the microbial community at the phylum level tended to return to the communities present in the control samples.

On day 28 in the samples treated with citric acid, the Proteobacteria (Cit28, 42.4%; Cit7, 22%; Ct28, 40.2%) phylum had increased its relative abundance by 20.4%, becoming the dominant bacterial phylum, and the Firmicutes (Cit28, 14.9%; Cit7, 52.3%; Ct28, 7.4%) phylum had decreased its relative abundance by 37.4% with respect to day 7. The relative abundance of the Actinobacteria phylum (Cit28, 17.8%; Cit7, 18.1%; Ct28, 21%) remained constant during this period (**Figure 7**).

By day 28, the Proteobacteria phylum had increased its relative abundance with respect to day 7 and to control samples through the Sphingomonadaceae (Cit28: 9.8%, Cit7: 6.3%, Ct28: 11.1%), Rhizobiaceae (Cit28, 2%; Cit7, 0.4%; Ct28, 0.5%), and Pseudomonadaceae (Cit28, 13%; Cit7, 4.8%; Ct28, 0%) families (**Figure 9**).

Within the Actinobacteria phylum, by day 28, there was an increase in the relative abundances of the Intransporangiaceae (Cit28, 3%; Cit7, 0%; Ct28, 0.1%) and Microbacteriaceae (Cit28, 1.5%; Cit7, 0.8%; Ct28, 0.3%) families, both with respect to day 7 and to control. Moreover, a decrease of 6.3% in the relative abundance of the family Micrococcaceae (Cit28, 6.7%; Cit7, 13%; Ct28, 3.4%) was observed with respect to day 7.

Within the Firmicutes phylum, there was a 33% decrease in the relative abundance of the order Clostridiales respect to day 7, although it remained 11.9% higher than in the untreated control, samples. This change corresponded to the variations observed in the Clostridiaceae (Cit28, 9.7%; Cit7, 38.4%; Ct28, 0%), Tissierellaceae (Cit28, 1.6%; Cit7, 4.5%; Ct28, 0%), and Veillonellaceae (Cit28, 0.4%; Cit7, 1.5%; Ct28, 0%) families. Within the Firmicutes phylum, the Bacillales (Cit28, 3%; Cit7, 7.4%; Ct28, 7.3%) order also decreased.

The soil microbiota in the samples treated with oxalic acid showed a taxonomic composition differs very much from those of the untreated control and the samples supplemented with lactic and citric acid. After 7 days of treatment, the effect of the oxalic acid resulted in a marked increase of 18.5% in the relative abundance of the Proteobacteria phylum. This phylum was dominant with a representation of 62.3%. This samples presented a decrease in the relative abundances of the Firmicutes (Ot7:3.8%, Ct7: 5.9%), Actinobacteria (Ot7, 11.9%; Ct7, 18.6%), and Acidobacteria (Ot7, 6.9%; Ct7, 10.9%) phyla (**Figures 7–9**).

That increase within the Proteobacteria phylum was mainly due to the increase in the Burkholderiales (Ct7, 1.6%; Ot7, 29.4%/increase of 27.8%) order (**Figures 7**, **8**).

Unlike in the cases of lactic and citric acids, the addition of oxalic acid produced a long-lasting modification of soil microbiota. After 28 days of incubation, when the oxalic acid had been completely mineralized, the soil bacterial community was highly similar to that at day 7.

On days 7 and 28 of incubation, the samples treated with oxalic acid were dominated by the Burkholderiales order, representing, in both cases, more than 25% of the communities in these samples, but this order only represented less than 1% in the other treatments. Within this order, the Oxalobacteraceae family (Ot7, 10.2%; Ot28, 6.9%, in the rest of treatments around 0.2%) was dominant (**Figures 7–9** and **Supplementary Table S1**).

DISCUSSION

Organic acids are one of the main components of rhizospheric exudates. They are strongly involved in the solubilization and acquisition of essential nutrients such as P are used as carbon and energy sources by soil microorganisms, and act as key drivers in rhizospheric bacterial chemotaxis (Jones et al., 2003). In short, OAs are associated with different processes that seem to play a key role both in soil fertility and in the development and growth of plants.

The present study focuses on monitoring the effects produced by the addition to the soil of three specific OAs present in rhizospheric exudates and on their characterization as edaphological biostimulants and soil prebiotics. In order to achieve the proposed objectives, different chemical and biochemical analyses of the soil were carried out and the composition of the soil microbiota was evaluated using a metabarcoding approach. Results have important implications for both soil microbiological process and plant growth.

OAs Fate, Soil Chemical, and Biochemical Properties

The concentration of OAs used in this study falls within the range described in soil extracts (Strobel, 2001). Nevertheless, the concentration of these products varies greatly and by different abiotic and biotic stresses, such as physiological stress, nutrient stress, and some physical changes in soil also have an influence (Chen and Liao, 2016; Dinh et al., 2017; Jiang et al., 2017). Moreover, it is suggested that plant roots' production of OAs vary among plant species and is influenced considerably by the plant's developmental stage (Dinh et al., 2017).

The ability to lower soil pH is specific to each type of OA and this behavior may be explained due to a differential chemical interaction between the acids and other soil constituents. The participation of citric and oxalic acids dissolving soil carbonates has been described to buffer pH (Duquène et al., 2008). Furthermore, previous reports mentioned that the application of citric and oxalic acids at low concentrations increased soil pH (Duquène et al., 2008). Therefore, citric and oxalic acids would be participating in the dissolution of carbonates, precipitating in the form of oxalate and calcium citrate, releasing CO_2 and H_2O, and increasing bicarbonate levels, which would greatly buffer the potential decrease of soil pH.

The return of pH values to those of control samples was in parallel with the mineralization of the OAs. The three OAs were completely mineralized, probably due to their consumption by soil microflora, soil microorganisms using them as a source of carbon and energy (Rodríguez-Morgado et al., 2017).

With regard to enzyme activities, changes in dehydrogenase activity reflect soil status (Doi and Ranamukhaarachchi, 2009) and have been proposed as a good indicator of soil microbial activity. It is, therefore, widely used as an integrated measure of soil quality (Maliszewska and Smreczak, 2003). As described, dehydrogenase activity was stimulated after treatment with lactic and citric acid (**Figure 3**). The stimulation would justify the degradation of the citric and lactic acids by soil microorganisms, the citric acid being the richest carbon source, and producing a greater induction of microbial metabolism.

On the other hand, oxalic acid, despite being completely metabolized, did not produce a significant increase in dehydrogenase activity. This behavior could be due to the fact that oxalic acid would be a carbon source used by a small fraction of the microbial community of oxalotrophic bacteria, including Burkholderiales order (Sahin et al., 2009; Palmieri et al., 2019). As a result, oxalic acid could be favoring a small group of specific bacteria that could be metabolizing it without increasing its biomass during the process, and consequently without producing an increase in dehydrogenase activity (Landi et al., 2006).

The changes in phosphomonoesterase activity found in our study may be associated to P bioavailability. Increase of P bioavailability in soils is linked to an inhibition of the enzymatic activity due to the fact that production and excretion of hydrolytic enzymes is stimulated by several signals that convey information about the utility of these enzymes during nutrient-limited growth conditions (Cezairliyan and Ausubel, 2017). Accordingly, previous works described that LMWOAs were able to increase the bioavailability of P in soils during the initial stage of incubation, increasing P bioavailability for microbial growth (Palomo et al., 2006; Clarholm et al., 2015). So the higher P bioavailability due to OAs treatment would explain the inhibition of phosphatase enzymes, as P requirements for growth are being satisfied by its availability in soils. The ability of OAs to mobilize P is already known, so it is reported that low molecular weight OAs such as citric and oxalic acids are among the most commonly produced root and microbial exudates which affect rhizosphere P availability (Clarholm et al., 2015; Menezes-Blackburn et al., 2016). These two OAs induce a higher increase in P mobilization than other OAs (Gerke et al., 2000; Giles et al., 2012) through a different mechanism such as rhizosphere acidification which increases P availability and also forms stable complexes with metal cations and competes with P for adsorption sites on soil colloids (Menezes-Blackburn et al., 2016).

During the experimental incubation, the OAs' mineralization led to a progressive decrease in the availability of P and the microorganisms reactivated their own production of phosphatases to satisfy their current demand of P (Heitkötter et al., 2017) as shown by our results (**Figure 4**). Thus, these OAs may contribute to the release of bioavailable P while simultaneously stimulating microbial growth and P sequestration for microbial biomass.

Although oxalic acid did not induce an increase in microbial metabolic activity, the increase in phosphomonoesterase activity detected at the end of the assay could be explained by the induction of specific taxa, in particular the Burkholderiales order and the Oxalobacteraceae family described as effective P solubilizers (Silva et al., 2017).

OAs and Soil Microbiota

One of the main objectives of this study was to verify how three specific OAs affect the soil microbiota. To this end, bacterial abundance and diversity in soil were assessed using the culture-independent analysis by 16S amplicon sequencing using the Illumina MiSeq platform, the analysis enabling observation of how applying the three OAs tended to produce a decrease in both the diversity and the richness of the microbiota (**Table 2**). This could be explained by inducing that these supplementary carbon sources are stimulating the proliferation of specific, possibly specialized microorganisms that could be involved in their mineralization—these microorganisms belonging to certain taxonomic categories that would dominate the population.

In agreement with this hypothesis, previous studies have already shown that the application of oxalic and citric acids produced changes in the soil microbial community which were more marked than those produced by the addition of other carbon sources such as glucose and glycine, justified by the fact that these OAs could only be mineralized by certain specialized microorganisms while glucose and glycine could be used by a large part of the microorganisms present in the soil (Falchini et al., 2003; Landi et al., 2006; Eilers et al., 2010). Shi et al. (2011) using DGGE and PhyloChip analyses also showed that OAs stimulated the soil microbial community and produced changes in its composition that were considerably greater than those produced by sugars only. But in contrast to our results, they observed that the OAs produced an increase in diversity and richness indices, something that could be explained by the fact that they used mixtures of OAs with sugars instead of pure OAs.

Supporting our previous explanation, we were also able to verify how applying OAs promoted the proliferation of unique OTUs that were not present in untreated samples. Some of these were found in all of the samples treated with the three LMWOAs, while others were specific to each one, something which evident both on days 7 and 28 of incubation (**Figure 5**). In Venn diagrams, we could also observe the existence of specific OTUs in the control samples, such taxa would be responding negatively to the application of OAs. This negative impact may result from the direct inhibition of microorganisms due to the presence of OAs. Thus these taxa were outcompeted by the rapid growth of other microorganisms (Paterson et al., 2007; Shi et al., 2011). Moreover, PCoA ordination analysis showed large differences between bacterial community structure with the samples treated with the three OAs and the untreated samples throughout the assay (**Figure 6**).

At high taxonomic rank composition, Proteobacteria, Actinobacteria, Acidobacteria, and Firmicutes were the predominant phyla in all samples (**Figure 6**). This is to be expected, since they have been described as some of the phyla that recapitulate most of the diversity of contrasting soil biomes (Fierer and Jackson, 2006; Bulgarelli et al., 2013). But the three OAs induced changes in the representation of these phyla compared with control samples. Lactic and citric acids produced a reversible increase in the relative abundances of

the Actinobacteria and Firmicutes phyla, and of the Firmicutes phylum, respectively, returning to initial values at the end of the assay, while oxalic acid produced an increase in the relative abundance of the Proteobacteria phylum that remained unchanged throughout the entire assay.

By delving into the taxonomic categories, we were able to verify how at 7 days of incubation, the stimulation of the *Bacillus*, *Pelosinus*, and *Sinorhizobium* genera in the samples treated with lactic acid stood out, and in the samples treated with citric acid that of the Clostridiaceae family. Interestingly, this effect was simultaneous, with high values of stimulation of the dehydrogenase activity of the samples treated with lactic and citric acids. A common induction of the Micrococcaceae and Pseudomonadaceae families after lactic and citric application was observed (**Figure 9** and **Supplementary Table S1**).

While the *Bacillus* and *Pelosinus* genera would be involved in the degradation of lactic acid. These genera have been characterized by proliferating in acidic environments (Hansel et al., 2008) and by the use of lactate as a source of C (Mosher et al., 2012; Beller et al., 2013); the Clostridiaceae family—which would account for almost 50% of the relative abundance of the bacterial population of samples treated with citric acid—would probably be directly involved in the degradation of this acid (Antranikian and Gottschalk, 1982). On the other hand, the application of both citric and lactic acids would also be promoting the proliferation of beneficial bacteria of agricultural importance by stimulating microorganisms belonging to the *Bacillus* and *Sinorhizobium* genera in the case of lactic acid, as well as the Micrococcaceae and Pseudomonadaceae families in the case of both acids, since PGPB microorganisms have been described within these taxa due to their involvement both in biocontrol, as in the production of ammonium, indolacetic acid, atmospheric N_2 fixation, P solubilization, etc. (Kumar et al., 2012; Souza et al., 2015; Karličić et al., 2016; Turan et al., 2016; Radhakrishnan et al., 2017).

In a previous study with lactic acid applied to soil, DGGE analyses showed microbial community changes similar to those observed in this study by barcoded, amplicon sequencing data. Specifically, DGGE bands related to the *Pseudomonas*, *Bacillus*, and *Rhizobium* genera were observed (Rodríguez-Morgado et al., 2017).

On day 28, once the lactic and citric acids had already been completely mineralized (**Figure 2**), biodiversity tended to return to the initial levels when assessing data on phyla (**Figure 7**), but an induction pattern of PGPB microorganisms, such as the genera *Sinorhizobium* and *Lysobacter*, and the Pseudomonaceae family was observed and maintained at the end of the experiment—suggesting an important involvement of this LMWOAs in soil fertility (**Figure 9** and **Supplementary Table S1**) (Islam, 2011; Gupta et al., 2015; Prasad et al., 2015; Souza et al., 2015; Sharma et al., 2017).

Applying oxalic acid to soil produced long-lasting effects in the taxonomic composition of soil bacterial communities, inducing the proliferation of specific microorganisms involved in its mineralization, despite its not producing a remarkable induction of its metabolic activity. These observations are in agreement with the findings of Landi et al. (2006), who observed that oxalic acid influenced the bacterial communities of the rhizosphere although net soil ATP remained relatively stable, suggesting insignificant changes in bacterial biomass. These small changes in biomass could be the result of the activation of only small fractions of the community, specifically the oxalotrophs (Messini and Favilli, 1990; Palmieri et al., 2019) which represent those bacteria able to mineralize oxalic acid. Other authors have also shown changes in soil bacterial communities induced by oxalic acid (Falchini et al., 2003; Yuan et al., 2018) in spite of a limited modification of the overall soil bacterial biomass.

The Burkholderiales order dominated the samples treated with oxalic acid from day 7 onward. Within this order, the detection of microorganisms belonging to the family Oxalobacteraceae should be highlighted (**Figures 8**, **9**). These taxa could be involved in the mineralization of oxalic acid and it could also play a potentially important role in soil fertility because this order includes numerous representatives such as effective P solubilizers and PGPB (Baldani et al., 2014; Souza et al., 2015; Silva et al., 2017).

CONCLUSION

In summary, we conducted an exhaustive study of the influence of OAs common in the rhizospheres, which provided fundamental knowledge for studying their potential use as soil prebiotics. The stimulating effect of OAs on microbial activity in soils is illustrated by the induction of specific bacterial groups with known PGPB roles in soils. In this sense, our study highlights the potential use of rhizospheric OAs as biostimulants to enhance crop yield in a sustainable way.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in The European Nucleotide Archive (ENA) under accession number PRJEB35168.

AUTHOR CONTRIBUTIONS

SM-B and JP designed the study. SM-B, JP, and PC performed research. SM-B, AG-M, PC, JG, MT, and JP analyzed data. SM-B, AG-M, and JP wrote the article.

FUNDING

This study was supported by the Ministry of Science, Innovation and Universities (Spain), Proyectos I + D + i <<Retos investigación>> Convocatoria 2018 RTI2018-097425-B100.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00633/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 June 2020
doi: 10.3389/fpls.2020.00836

A Biostimulant Seed Treatment Improved Heat Stress Tolerance During Cucumber Seed Germination by Acting on the Antioxidant System and Glyoxylate Cycle

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 30 January 2020
Accepted: 25 May 2020
Published: 17 June 2020

Citation:
Campobenedetto C, Grange E, Mannino G, van Arkel J, Beekwilder J, Karlova R, Garabello C, Contartese V and Bertea CM (2020) A Biostimulant Seed Treatment Improved Heat Stress Tolerance During Cucumber Seed Germination by Acting on the Antioxidant System and Glyoxylate Cycle. Front. Plant Sci. 11:836. doi: 10.3389/fpls.2020.00836

Seed enhancement technologies have the potential to improve germination and seedling growth under environmental stress. The effects of KIEM®, an innovative biostimulant based on lignin derivatives and containing plant-derived amino acids and molybdenum, were investigated on cucumber (*Cucumis sativus* L.) seed germination. To determine the metabolic targets of this product, biometric, transcriptional and biochemical analyses were carried out on both non-treated and KIEM®-treated seeds incubated for 24 and 48 h under standard (28°C) and heat stress (35°C) conditions. The application of the biostimulant as a seed treatment increased the percent germination (+6.54%) and fresh biomass (+13%) at 48 h, and decreased the content of H_2O_2 in treated seeds at 28°C (−70%) and at 35°C (−80%). These changes in biometric and biochemical properties were accompanied by changes in expression levels of the genes coding for ROS-producing (RBOH) and scavenging (SOD, CAT, GST) enzymes and their specific activity. In general, the treatment with KIEM® in heat-stress condition appeared to stimulate a higher accumulation of three scavenger gene transcripts: *CuZnSOD* (+1.78), *MnSOD* (+1.75), and *CAT* (+3.39), while the *FeSOD* isoform was dramatically downregulated (0.24). Moreover, the amount of non-protein thiols, important antioxidant molecules, was increased by the biostimulant after 48 h (+20%). Taken together these results suggest that KIEM® acts through mitigation of the effects of the oxidative stress. Moreover, after 48 h, the pre-sowing treatment with KIEM® increased the transcription levels (+1.5) and the activity of isocitrate lyase (+37%), a key enzyme of the glyoxylate cycle, suggesting a potential effect of this product in speeding up the germination process. Finally, the chemical characterization of KIEM® identified five essential and three non-essential amino acids, and others bioactive compounds, including five organic and inorganic acids that might be potentially involved in its activity. Based on these data, insights on the potential mechanism of action of the biostimulant, suggested that there are broader applications as a product able to increase seed tolerance to different abiotic stress typical of adverse environmental conditions.

Keywords: biostimulant, seed treatment, *Cucumis sativus*, antioxidant molecules and enzymes, isocitrate lyase, gene expression

INTRODUCTION

Plants may encounter a variety of abiotic stresses during their life cycle and these factors may have a significant impact on plant growth and final productivity. Different approaches have been employed to enhance plant stress tolerance. Some treatments can be particularly time-consuming (e.g., conventional breeding), while others are not accepted by all countries in the world (e.g., plant genetic modification). Seed priming could represent an alternative tool to prepare plants to counteract more successfully abiotic stress conditions (Filippou et al., 2013).

Recent studies suggest that different molecules have the potential to act as a biostimulant against different abiotic stresses. The application of amino acids, hormones, reactive oxygen–nitrogen–sulfur species or just water can be effective in enhancing plant tolerance to different abiotic stresses (Savvides et al., 2016). Plants can be pretreated at different developmental stages (e.g., vegetative or reproductive stage). However, in the past decades attention has been focused on seed enhancements to alleviate environmental stress on germination and early seedling growth (Taylor et al., 1998). Seed treatment technologies (Taylor, 2003) provide methods to apply synthetic or natural compounds, aimed to increase the uniformity and vigor of seedlings and to enhance the tolerance of plants to different abiotic stresses. The treatment at the seed stage has relatively low application costs, as it requires only a single treatment and often leads to a prolonged protection (Savvides et al., 2016). Biostimulant pretreatments generally cause a faster germination and a faster field emergence, which have practical agronomic implications, notably under adverse conditions (Yildirim et al., 2000).

The search for new substances able to act as biostimulant has become an important target for both the academic and seed industry. Among these new products, biostimulants could play a key role as a seed-treatment agents (Masondo et al., 2018). Modern biostimulants may be complex mixtures derived from raw materials of highly diverse origin, including waste from food and paper industries. They are considered safe for the environment and possess a broad spectrum of biological activities (Bulgari et al., 2019). In the last 25 years, plant biostimulants have received considerable attention since these innovative products offer a potentially novel approach for the modulation of physiological processes in plants to stimulate growth, to enhance stress tolerance, and to increase yield (du Jardin, 2015). For these reasons, they have found an important application in modern crop production (Yakhin et al., 2017). In general, changes in temperature significantly affect seed germination through the inhibition of radicle emergence and post-germination growth in seedlings (Probert, 2000). For this reason, the use of biostimulants to overcome heat stress became an important method to preserve the final crop production and yield (Bulgari et al., 2019).

Cucumber (*Cucumis sativus* L.) is an important vegetable crop, mainly produced in Asia and Europe, also used as model organism. Along with tomato, onion, and melon, cucumber is the most widely cultivated vegetable species in the world (Bisognin, 2002). Cucumber germination and development is negatively affected by adverse conditions, including high temperature (Kurtar, 2010; Baninasab and Ghobadi, 2011).

In this study, we evaluated the potential effects of KIEM®, an innovative biostimulant based on lignin derivatives (lignosulphonates) and containing plant-derived amino acids and the nutrient molybdenum (Mo) on cucumber seed germination under heat stress conditions. More than 90% of aminoacid-based biostimulants employed in agriculture is related to animal-derivative hydrolysates (Colla et al., 2015), while those of plant-origin are less common, due to their recent introduction into the biostimulant market (Colla et al., 2014). However, there are several reports that plant amino acid-based biostimulants have positive effects on seed in the early stages of germination (Yildirim et al., 2000; Ugolini et al., 2015; Amirkhani et al., 2017). Moreover, Mo is also used for seed treatments and known to be helpful during the germination process. In legumes, Mo is able to help the formation of root nodules, involved in nitrogen fixation, while in non-legume plants this micronutrient enhances the use of nitrates absorbed from the soil (Farooq et al., 2012). Application of Mo directly on seeds seems to be more effective than soil application and it could be also involved in seed protection against abiotic stress conditions, by increasing the activity of Mo-containing enzymes (Babenko et al., 2015).

In order to determine the effects and the metabolic targets of this innovative product, biometric, gene expression (qPCR) and biochemical (ROS-scavenging system) analyses were carried out on both cucumber untreated and KIEM®-treated seeds incubated for 24 or 48 h at 28 or 35°C (heat stress condition). Finally, in order to correlate the composition of KIEM® with its possible mechanism of action, a partial chemical characterization of the amino acid fraction of this product was obtained by GC-MS analysis. All results taken together provide insights on the mechanism of action of KIEM® and on its application as a seed biostimulant able to increase tolerance to heat stress and potentially to other abiotic stress typical of adverse environmental conditions. The use of KIEM® as a pre-sowing agent, could be of paramount importance for reducing the number of treatments and thus the final management costs.

MATERIALS AND METHODS

Plant Material and Biostimulant

Cucumis sativus L. (cucumber) seeds var. Vert Petit de Paris were purchased and certified OGM free by OLTER® (Piacenza, Italy) and treated with the biostimulant KIEM®, developed by Green Has Italia S.p.A (Canale, Italy). This product contains 2% w/w of organic nitrogen, 2% w/w of molybdenum and 21% w/w of organic carbon. The pH (1% acq. sol. w/w) and Electrical Conductivity (acq. sol. 1 g L^{-1}) were 4.00 ± 0.50 u. pH and 200 $\mu S\ cm^{-1}$, respectively.

Seed Treatment and Germination Parameters

Cucumber seeds were treated by following the protocol provided by Embrapa in 2005 (Henning, 2005) and currently used in Brazil for the seed treatment with different products, including phytochemicals and biostimulants (dos Santos et al., 2018). This application method allowed the use of the minimum amount of product still ensuring its homogeneous distribution on cucumber

seed surface. Moreover, due to the low dosage of the product, this application method is estimated to be one of the most efficient and safe for both seeds and environment (Braccini et al., 2015; de Andrade et al., 2018; dos Santos et al., 2018). Briefly, 2 mL of KIEM® solution was diluted in distilled water in order to reach the final volume of 8 mL. The KIEM®-diluted solution was then added drop by drop to 2.5 g of dried seeds kept in continuous shaking until the complete and visible distribution of the product on the seed surface was obtained. Following the treatment, seeds were dried at room temperature and then placed in glass Petri dishes (20 cm Ø) containing two filter papers saturated with 15 mL of distilled water. Seeds treated with the same protocol, but with distilled water instead of the biostimulant, were employed as controls. For both treatment and control, three replicates were used. Each replicate was composed by 100 seeds, placed in five different Petri dishes (20 seeds × 5 Petri Dishes). Finally, the Petri dishes were incubated in the dark at standard (28°C) or heat stress (35°C) conditions for 24 or 48 h. At 48 h, germination percentage and fresh biomass were measured in order to evaluate differences between KIEM®-treated and untreated seeds. Before performing the following experiments, teguments were removed from seeds and cotyledons were dry-blotted on filter paper.

Hydrogen Peroxide Content

The hydrogen peroxide levels were detected according to Velikova et al. (2000). Powdered seeds (0.5 g) were homogenized with 5 mL of 0.1% (w/v) Trichloroacetic Acid (TCA). The samples were centrifuged at 12000 × *g* for 15 min and 0.5 mL of supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance was read at 390 nm and the H_2O_2 content was determined based on a standard curve.

Total Soluble Protein Content

The soluble protein concentration was evaluated by the method of Bradford (1976) using bovine serum albumin as a standard (Bradford, 1976).

Non-protein Thiol Content

The assay was carried out by mixing 500 μL of crude extract prepared for enzymatic analysis (antioxidant enzyme extraction), to 100 μL of 25% (w/v) TCA. The samples were centrifuged at 12000 × *g* for 20 min at 4°C. Then, 300 μL of supernatant were added to 2.7 mL of 0.6 mM 5,5′- dithiobis (2-nitrobenzoic acid) (DTNB) prepared in 0.1 M sodium phosphate buffer (pH 8.0). The absorbance was detected at 412 nm (Jain and Bhalla-Sarin, 2001).

Extraction and Activity of Antioxidant Enzymes

Antioxidant (ROS-producing and scavenging) enzymes were extracted and analyzed according to Contartese et al., 2016 using 0.5 g of powdered seeds (Contartese et al., 2016). All steps were carried out at 4°C. The extraction buffer used contained: 50 mM sodium phosphate, pH 7.5, 250 mM Sucrose, 1.0 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT), and 1% (w/v) polyvinylpolypyrrolidone (PVPP) in a 1:10 proportion (w/v). The homogenate was mixed by pipetting and then centrifuged 20 min at 25000 × *g* (4°C). The supernatants were directly used for enzymatic assays.

NADPH Oxidase (RBOH; EC 1.6.3.1)

The activity of RBOH was measured spectrophotometrically by reading the changes in absorbance at 530 nm (Ozawa et al., 2009). A standard assay mixture contained 40 mM NADPH, 0.02% (w/v) Triton X-100, 100 mM nitroblue tetrazolium (NBT) and buffer (20 mM Tris–chloride, pH 7.5, 3 mM $MgCl_2$) to make a total volume of 1 mL in a quartz cuvette. An additional 30 μM DPI (diphenyl iodonium) was added to the reaction mixture. The specific activity was calculated using an absorption coefficient of 12.8 mM^{-1} cm^{-1}.

Superoxide Dismutase (SOD; EC 1.15.1.1)

Superoxide dismutase activity evaluation was based on the ability of this enzyme to inhibit the reduction of nitro blue tetrazolium, thanks to the superoxide anion, generated photochemically (Krishnan et al., 2002). The reaction consisted in 1 mL containing 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), 2 μM riboflavin, 0.1 mM EDTA, and enzyme extract. To avoid degradation, riboflavin was added last. The samples were placed 30 cm under a light source (4000 lux) and the reaction was run for 15 min. Two blanks were prepared: one without enzyme extract, placed under the light to totally develop the reaction and, the other one, containing the enzyme extract placed in the dark to avoid the reaction. The last one was used as control. The absorbance was detected at 560 nm.

Catalase (CAT; EC 1.11.1.6)

Catalase activity was detected spectrophotometrically. The absorbance at 240 nm was measured for 120 s for evaluating the change due to the decreased absorption of H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}). The reaction was prepared in 1 mL final volume, containing 50 mM sodium phosphate, pH 7.0, 15 mM H_2O_2, and enzyme extract. The reaction was started by addition of H_2O_2.

Glutathione-S-Transferase (GST; EC 2.5.1.18)

The 1-Chloro-2,4-dinitrobenzene (CDNB) was used as reaction substrate. The enzyme activity was evaluated by monitoring the absorbance variation at 340 nm for 15 min. One mL of reaction solution contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (10 mM CDNB dissolved in 50% acetone stock solution), and enzyme extract. The reaction was started by adding CDNB (Jain and Bhalla-Sarin, 2001).

Extraction and Activity of Isocitrate Lyase

All steps were carried out at 4°C. The plant material was homogenized in two volumes of extraction buffer containing 40 mM Hepes [N-(2-hydroxyethyl) piperazine-N%-(2-ethanesulfonic acid)] buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM

EDTA, 1 mM DTT and Tween 20 (1% v/v) (Maffei et al., 1999). The homogenate was centrifuged 30 min at 15000 × *g* (4°C) and the resulting supernatants were brought to 30% saturation with solid $(NH_4)_2SO_4$. After stirring for 2 h the solution was centrifuged at 10000 × *g* for 20 min and solid $(NH_4)_2SO_4$ was added slowly to the supernatant to 50% saturation. After stirring for 2 h, the enzyme-enriched pellets were collected by centrifugation (10000 × *g* for 20 min), resuspended in a small volume of 40 mM Hepes (pH 7.0) and used for enzymatic assays.

Isocitrate lyase (ICL; EC 4.1.3.1) activity was recorded following NADH oxidation at 340 nm in the presence of an excess of lactate dehydrogenase (LDH) according to the protocol of Giachetti et al. (1987). The reaction mixture, in a final volume of 1 ml, contained: 40 mM Hepes buffer (pH 7.0), 6 mM $MgCl_2$, 45 IU LDH, 0.28 mM NADH, 2 mM isocitric acid and enzyme extract. The reaction was started with isocitric acid.

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted by using the NucleoSpin® RNA Plant Isolation Kit (Macherey-Nagel, Germany) according to manufacturer's instructions. RNA concentration was measured using an UV/visible spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Sweden). Total RNA quality was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, United States) according to manufacturer's instructions.

First strand cDNA synthesis was accomplished with 1 μg of total RNA and random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States), according to the manufacturer's instructions.

Gene Expression Analysis by qPCR

All qPCR analyses were run on a Stratagene Mx3000P Real-Time System (Agilent Technologies, United States) using SYBR Green I with ROX as reference dye. The reactions were performed with 10 μL of mixture consisting of 5 μL of 2XMaxima^{TM} SYBR Green qPCR Master Mix (Thermo Fisher, United States), 0.5 μL of cDNA and 100 nM primers (Integrated DNA Technologies, United States). Thermal conditions were as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 57°C, and 30 s at 72°C. Fluorescence was read after each annealing and extension phase. All runs were followed by a melting curve analysis from 55 to 95°C. *Ubiquitin* (*UBI*) was used as a reference gene to normalize the results. Primers for *RBOH, CuZnSOD*, *MnSOD*, *FeSOD, CAT, GST*, *ICL,* and *UBI* used in this work are reported in **Supplementary Table S1**. All amplification plots were analyzed with the MX3000P^{TM} software (Agilent Technologies, United States) to obtain Ct values. The relative expression levels of each gene were estimated using the method previously described by Pfaffl (2001).

Biostimulant Chemical Characterization

Targeted and untargeted metabolomics was performed on KIEM® in order to identify polar metabolites as previously described by Lisec et al. (2006). Some adaptations were made to the protocol according to Villafort Carvalho (Villafort Carvalho et al., 2015). Briefly, polar metabolites were extracted from 50 mg of the biostimulant using methanol, followed by a 2-phase separation using chloroform. Aliquots of the polar phase were dried by vacuum centrifugation and the dried samples were derivatized online according to the protocol of Lisec et al. (2006) using a Triplus RSH autosampler system (Thermo Fischer scientific) that was coupled to the GC/MS system (Lisec et al., 2006). The derivatized samples were analyzed by gaschromatography (GC) (Thermo Trace 1300) coupled to mass spectrometry (MS) (Thermo TSQ Duo) system.

The chromatographic separation was performed using a VF−5MS capillary column [Agilent, 30 m × 0.25 mm (internal diameter) × 0.25 μm (film thickness)] including a 10-m guardian column with helium as carrier gas at a constant column flow rate of 1 ml min^{-1}. The GC oven temperature was isothermal for 2 min at 70°C, followed by a 10°C min^{-1} ramp to 310°C, and then held at this temperature for 10 min. The transfer line temperature was set at 280°C. The column effluent was ionized by electron impact at 70 eV. Mass spectra were acquired using selected reaction monitoring (SRM) as scan type, with preselected SRM transitions and collision voltage. The ion source was set at a temperature of 290°C. A solvent delay of 420 s was set up. The detector voltage was set at 1500 V.

Each sample was injected in two different concentrations in order to better detect and quantify the different compounds. External calibration curves of each amino acid were used for the identification and quantification. On the other hand, other polar compounds were tentatively identified using the in-house metabolite database.

Statistical Analysis

Data are expressed as mean values ± standard deviation (SD) of three biological replicates. Hydrogen peroxide and non-protein thiol content were expressed as nmol or μmol g^{-1} of fresh weight (FW). Enzymatic activities were expressed as nKat mg^{-1} protein, as previously described (Maffei et al., 1999). Concerning molecular data, gene expression was calculated using the method previously described by Pfaffl (2001). For all determinations, significant differences ($p \leq 0.05$) among the samples were evaluated by performing one-way ANOVA followed by Tukey-Kramer's s HSD test using SPSS ver. 24 software.

RESULTS

Seed Germination and Fresh Biomass

The biostimulant treatment was investigated on cucumber seeds at the early germination phase under heat stress conditions, the percent germination and fresh weight were measured on control and KIEM®-treated seeds at 48 h after incubation at 28°C and 35°C. At 48 h, both untreated and KIEM®-treated seeds incubated at 28°C showed similar germination percentage (**Table 1**). At 24 h, seeds were imbibed and the cotyledons appeared healthy and uniform among the different replicates. Moreover, at 48 h both germination and radicle length appeared uniform. With regard to fresh weight, a significant lower weight was recorded for treated seeds compared to untreated ones ($p \leq 0.05$). However, KIEM® treatment prompted a

TABLE 1 | Germination percentage and fresh weight at 48 h after seed incubation.

Seed treatment	Germination (%)	Fresh weight (g)
28°C Untreated	100 ± 0.58^{a}	3.11 ± 0.05^{a}
28°C Treated	99 ± 0.6^{a}	2.85 ± 0.01^{b}
35°C Untreated	92 ± 1.5^{b}	1.74 ± 0.02^{d}
35°C Treated	98 ± 0.6^{a}	2.00 ± 0.02^{c}

Values are expressed as a mean (±SD). Different letters indicate significant differences among samples (ANOVA, Tukey's post hoc test, $p \leq 0.05$).

TABLE 2 | H_2O_2 (nmol of H_2O_2 g^{-1} FW) and non-protein thiol (μmol of thiols g^{-1} FW) levels at 24 and 48 h after seed incubation.

		H_2O_2		Non-protein thiols	
Treated	**Stressed**	**24 h**	**48 h**	**24 h**	**48 h**
x	x	1.95 ± 0.12^{a}	1.94 ± 0.25^{a}	41.0 ± 3.22^{a}	38.6 ± 2.92^{a}
✓	x	0.61 ± 0.06^{b}	0.79 ± 0.04^{b}	56.4 ± 0.58^{b}	36.0 ± 2.64^{ab}
x	✓	15.0 ± 0.57^{c}	19.1 ± 0.15^{c}	245 ± 11.2^{c}	34.2 ± 2.54^{b}
✓	✓	3.04 ± 0.90^{d}	1.86 ± 0.70^{a}	174 ± 4.84^{d}	45.3 ± 3.18^{c}
		nmol of H_2O_2 g^{-1} FW		*μmol of thiols g^{-1} FW*	

Each value is the mean ± SD of three biological replicates. For each column, different lowercase letters indicate significant differences ($p \leq 0.05$), as measured by one-way ANOVA followed by Tukey's post hoc test.

significant increase in the germination percentage and in the fresh biomass of the germinating seeds with respect to non-treated controls (**Table 1**).

Endogenous H_2O_2 Content

Heat stress potentially generates a condition of oxidative stress in seeds, leading to the overproduction of ROS. For this reason, the levels of endogenous H_2O_2 were evaluated in untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and heat stress condition (35°C) for 24 and 48 h. The results are reported in **Table 2**. Heat stress on untreated cucumber seeds caused an increase in endogenous H_2O_2, both after 24 and 48 h. On the other hand, KIEM®-treated seeds showed strong reduction of endogenous H_2O_2 levels, at both incubation temperatures. Indeed, after 24 h a decrease from 1.94 ± 0.12 to 0.61 ± 0.05 nmol g^{-1} FW, and from 15.09 ± 0.57 to 3.04 ± 0.89 nmol g^{-1} FW was recorded, at 28 and 35°C, respectively. A similar trend was also observed after 48 h, in which the H_2O_2 content decreased from 1.94 ± 0.25 to 0.79 ± 0.04 at 28°C, and from 19.09 ± 0.15 to 1.86 ± 0.69 at 35°C. In particular, after biostimulant treatments, endogenous H_2O_2 decrease ranged between −70% (at 28°C) and −80% (at 35°C) after 24 h, and between −60% (at 28°C) and −90% (at 35°C) after 48 h.

Non-protein Thiol Content

The levels of non-protein thiols were evaluated in untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and heat stress condition (35°C) for 24 and 48 h. The results are reported in **Table 2**. After 24 h, heat stress on untreated cucumber seeds caused a strong increase of non-protein thiol content (from 40.96 ± 2.56 to 245.26 ± 11.23 μmol g^{-1} FW). When the non-protein thiol content was measured on treated-seeds, a slight increase (+40%) was recorded with respect to untreated seeds (**Table 2**). On the other hand, treated seeds at 35°C displayed an opposite effect, and clearly led to a reduction in thiols (−30%) compared to the non-biostimulant control. In particular, the content was reduced from 245.23 ± 11.23 to 174.11 ± 4.84 μmol g^{-1} FW. Interestingly, an opposite trend was observed after 48 h, where an increase of the level of non-protein thiols (+35%) was observed at 35°C in KIEM®-treated cucumber seeds (**Table 2**). The delayed effect under high temperature conditions suggests a possible activation in the biostimulant-depending production of thiol molecules.

Antioxidant Enzyme Activities and Gene Expression

To gain more insight into the seed response to heat stress and H_2O_2 production during the early phases of germination, the transcript levels and the activities of several ROS producing and scavenging enzymes namely, NADPH-Oxidase (RBOH), superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST) were evaluated.

The gene expression analysis was carried out on *RBOHD,* three *SOD* isoforms (*CuZnSOD, MnSOD, FeSOD*), *CAT* and *GST* on untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and in heat stress conditions (35°C) for 24 and 48 h. **Figure 1** reports the data as fold-change values, as described by Pfaffl (2001).

In general, the treatment with KIEM® at 28°C did not exert a strong effect on antioxidant gene expression level, after 24 and 48 h. In particular, after 24 h only *FeSOD* was slightly activated (+1.15), while the other antioxidant genes were downregulated with respect to the control (**Figure 1A**). A different expression profile was obtained at 48 h, in which the biostimulant treatment stimulated a higher accumulation of three scavenger gene transcripts: *CuZnSOD* (+1.74), *MnSOD* (+1.22) and *CAT* (+1.30). Interestingly, the *FeSOD* isoform, upregulated at 24 h, was dramatically downregulated at 48 h (0.11) (**Figure 1B**).

Also, when cucumber seeds were treated with KIEM® and incubated at 35°C for 24 h, downregulation of antioxidant genes, similar to that recorded at 28°C, was observed (*MnSOD*, 0.82; *FeSOD*, 0.26, and *CAT*, 0.65) (**Figure 1A**). The downregulation of the genes coding for antioxidant enzymes might be correlated to the capacity of this biostimulant to slow down the consequence of heat stress. Indeed, at 24 h all the genes coding for the antioxidant enzymes in analysis, except *GST*, were downregulated. On the other hand, at 48 h, KIEM® exerted a stronger effect at expression level (**Figure 1B**). Indeed, the treatment led to upregulation of the expression of all the antioxidant genes (*CuZnSOD*, +1.78; *MnSOD*, +1.75; *CAT*, +3.39, and *GST*, +1.7). This effect is probably due to the potential of KIEM® in preparing the seedlings to be more active to counteract the effects of heat stress.

With regard to the enzymatic assays, in general, a lower activity of the ROS producing and scavenging enzymes compared to the controls was recorded at both temperatures and incubation

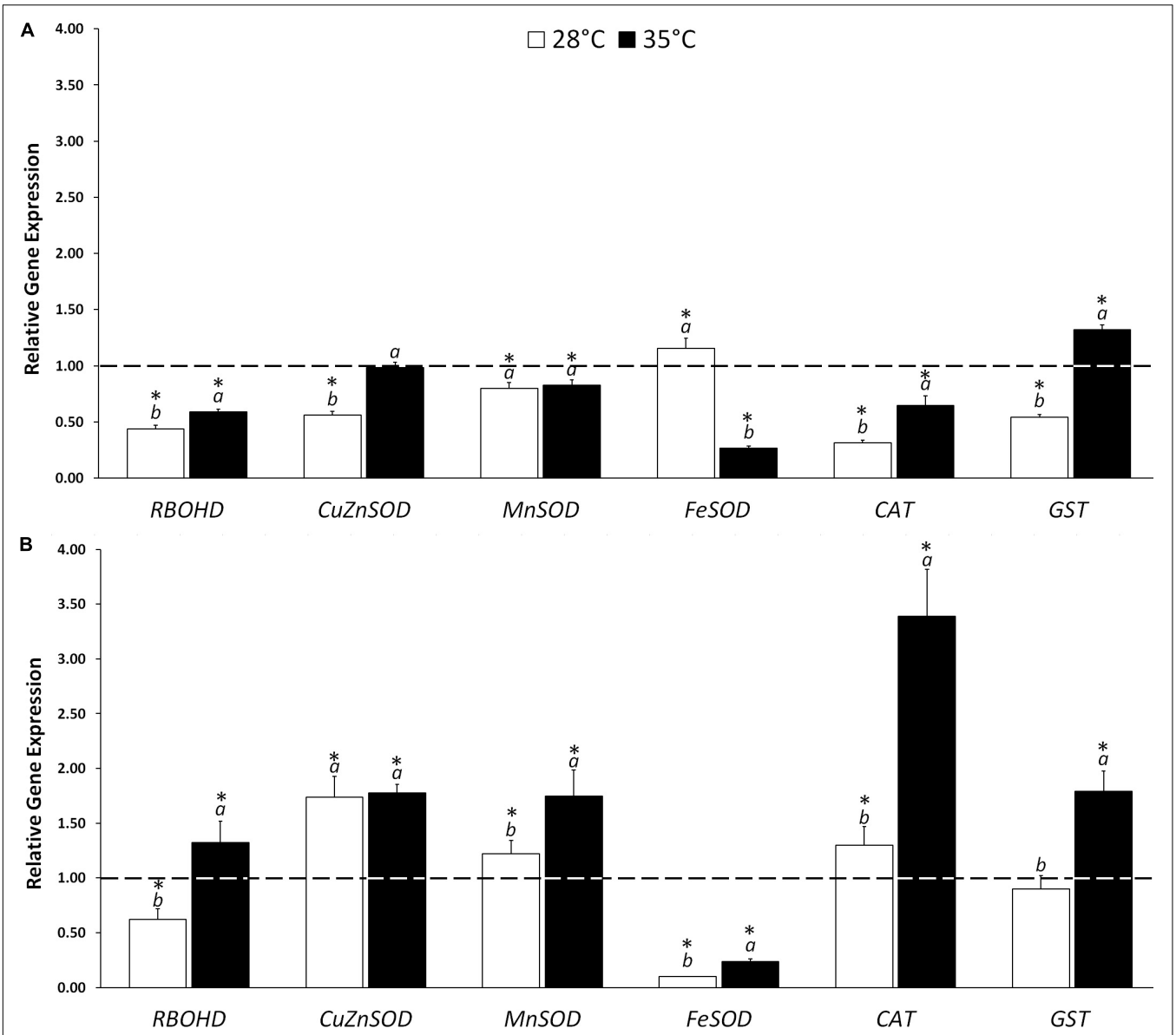


FIGURE 1 | Effect of KIEM® on expression levels of genes coding for ROS producing (*RBOHD*) and scavenging (*CuZnSOD*, *MnSOD*, *FeSOD*, *CAT,* and *GST*) enzymes after 24 **(A)** and 48 h **(B)** from seed incubation. Values are expressed as a relative gene expression obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the mean ± SD of three biological replicates. For each bar, different lowercase letters indicate significant differences ($p \leq 0.05$) between treatments with the biostimulant at the two different temperatures (28 and 35°C), as measured by ANOVA followed by Tukey's *post hoc* test. Asterisks (*) indicate significant differences ($p \leq 0.05$) between KIEM®-treated samples and the correspective untreated control at the same temperature condition, as measured by *t*-test.

times, indicated a positive action of KIEM® in mitigating the effects of the oxidative stress. The data are reported in **Figure 2**. In particular, at 24 h (**Figure 2A**), the enzymatic activity profile followed the same trend as the gene expression pattern, since all enzymes showed a very low activity compared to the controls. This effect was particularly evident at 35°C, in which significant differences compared to the activity registered at 28°C were observed. After 48 h (**Figure 2B**) from the application of the biostimulant in heat stress conditions, a higher activity was observed for all antioxidant enzymes, compared to 24 h. The enzymatic profiles follow the observations for gene expression and data were also in agreement with the lower amount of H_2O_2 measured in KIEM®-treated seeds (**Table 2**).

Isocitrate Lyase Enzymatic Activity and Gene Expression

In order to evaluate the effect of KIEM® in modulating the germination process, the level of expression and the enzymatic activity of isocitrate lyase were analyzed on cucumber cotyledons

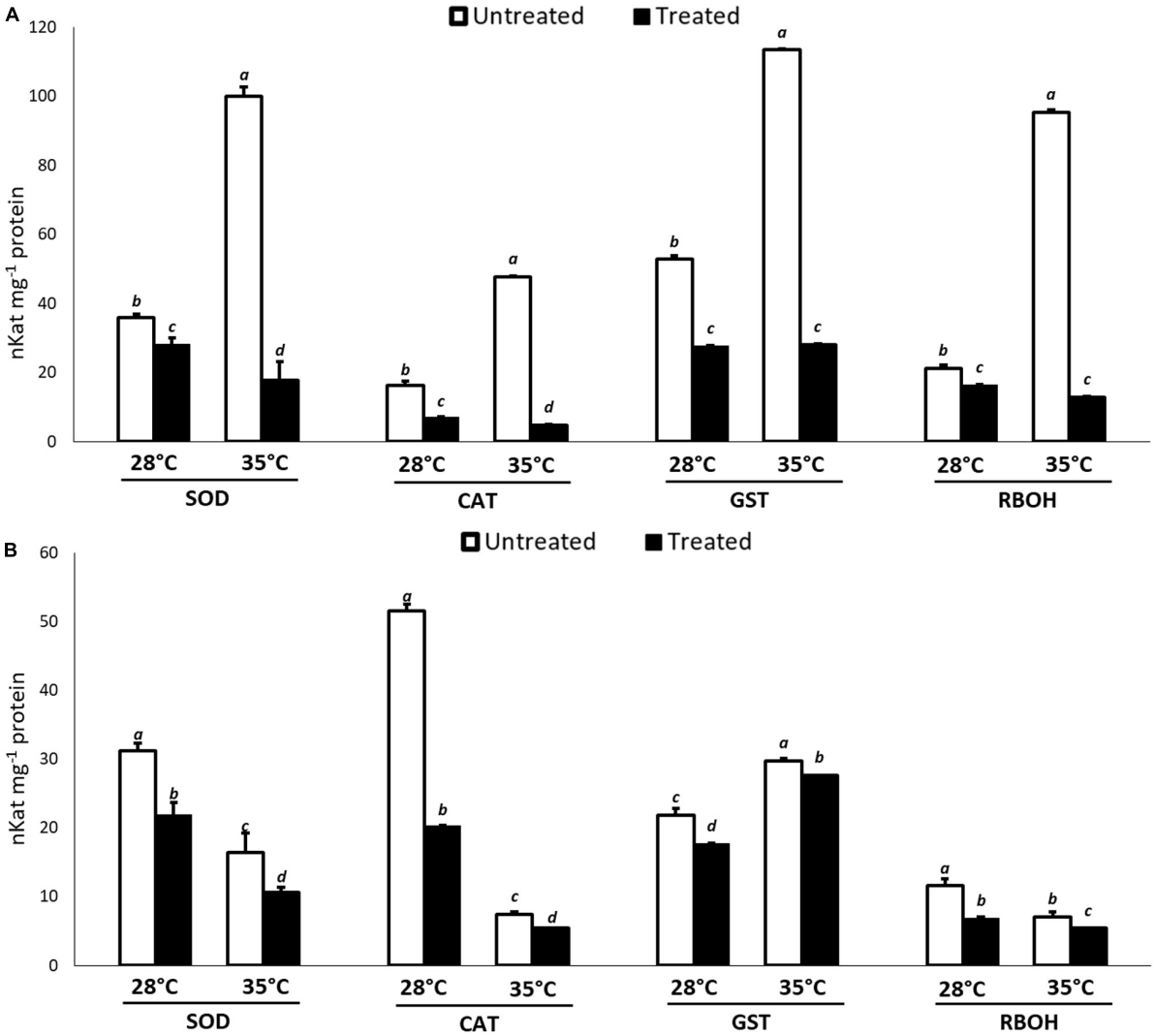


FIGURE 2 | Effect of KIEM® on enzymatic activities of RBOH, SOD, CAT, and GST at 24 **(A)** and 48 h **(B)** after seed incubation. Values are expressed as nKat mg^{-1} protein. Bars represent the mean ± SD of three different biological replicates. For each bar, different lowercase letters indicate significant differences ($p \leq 0.05$), as measured by one-way ANOVA followed by Tukey's *post hoc* test.

treated and untreated with KIEM® and incubated for 24 and 48 h at 28°C or 35°C. Isocitrate lyase, which is part of the glyoxylate pathway, is characteristic for metabolic activity of the germinating seed, catalyzing the cleavage of isocitrate to succinate and glyoxylate (Yuenyong et al., 2019). In standard conditions (28°C), KIEM® did not affected the level of *ICL* expression at 24 h, but a significant ($p \leq 0.05$) up-regulation was observed at 48 h (+1.35) when compared to control (**Figure 3**). The most pronounced effect was obtained when cucumber seeds were treated with KIEM® at 35°C. At this temperature condition, the application of the biostimulant was able to promote *ICL* upregulation (+1.13 and +1.59, at 24 and 48 h, respectively). These data were statistically ($p \leq 0.05$) different not only compared to control, but to the values observed at 28°C. This suggests a positive effect of this biostimulant in enhancing the germination process, at least when judged from *ICL* expression level, at high temperature conditions.

With regard to biochemical results, the changes in ICL-activity were evaluated in untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and heat stress condition (35°C) for 24 and 48 h. The results are reported in **Figure 4**. In general, a lower ICL enzymatic activity was observed in cucumber seeds treated with KIEM® compared to controls at both incubation times and temperatures. However, the observed values showed a similar trend of the gene expression profile (**Figure 4**).

Characterization of the Biostimulant Amino Acid Fraction

The main polar metabolites present in KIEM® were analyzed through targeted and untargeted analysis using GC-MS. GC-MS

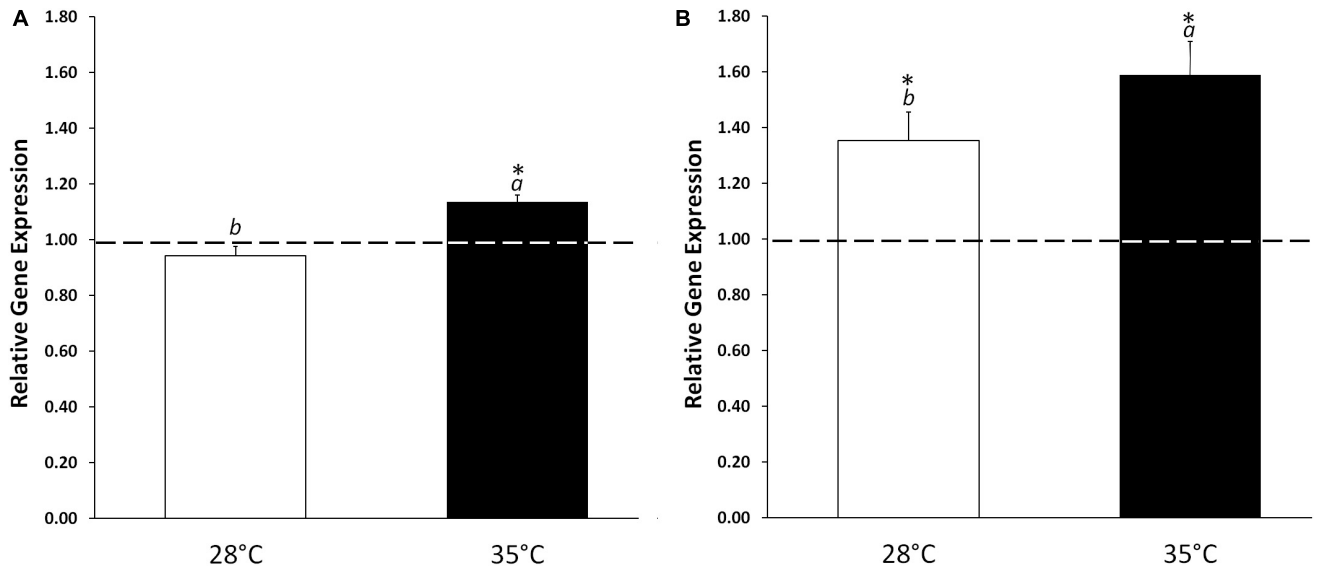


FIGURE 3 | Effect of KIEM® on *ICL* expression levels at 24 **(A)** and 48 h **(B)** after seed incubation. Values are expressed as a relative gene expression obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the mean ± SD of three biological replicates. For each bar, different lowercase letters indicate significant differences ($p \leq 0.05$) between treatments with the biostimulant at the two different temperatures (28 and 35°C), as measured by one-way ANOVA followed by Tukey's *post hoc* test. Asterisks (*) indicate significant differences ($p \leq 0.05$) between KIEM®-treated samples and the correspective untreated control at the same temperature condition, as measured by *t*-test.

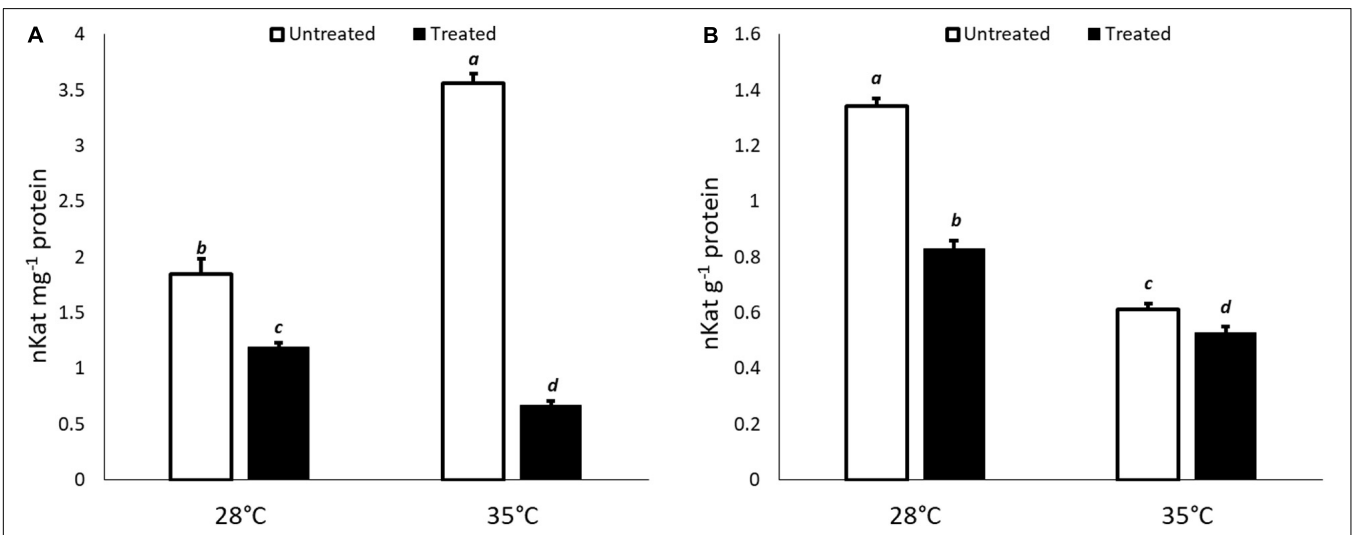


FIGURE 4 | Effect of KIEM® on ICL enzymatic activity at 24 **(A)** and 48 h **(B)** after seed incubation. Values are expressed as nKat mg^{-1} protein. Bars represent the mean ± SD of three different biological replicates. For each bar, different lowercase letters indicate significant differences ($p \leq 0.05$), as measured by one-way ANOVA followed by Tukey's *post hoc* test.

analysis revealed the presence of five essential (#5, leucine; #7, isoleucine; #9, threonine; #10, methionine; and #12, phenylalanine) and three non-essential amino acids (#2, alanine; #8, serine; #14, glutamic acid) in detectable amount (**Table 3**). Moreover, other polar metabolites were tentatively identified comparing their retention time (RT), molecular weight (MW) and mass fragmentation (m/z) to literature data. Among them five organic and inorganic acids (#1, lactic acid; #3, sulfuric acid; #6, phosphoric acid and #15, citric acid), two sugars (#16, fructose and #18, galactose), myoinositol (#19), oxoproline (#11) and glycerol (#4) were identified. However, due to the lower and not significant amount with respect to amino acid compounds, the quantification was performed only for the amino acid fraction. GC-MS data are reported in **Table 3**. The most abundant amino acid found in KIEM® is glutamic acid (#14) followed by methionine (#10). The sums of these two compounds counted for more of 55% of the total amino acid content.

TABLE 3 | GC-MS analysis of the polar metabolites content in the biostimulant.

#	RT	Compound	μg per mL of KIEM®
1	8.21	Lactic acid	*
2	8.83	Alanine	269.2 ± 19.67^{e}
3	9.79	Sulfuric acid	*
4	11.14	Glycerol	*
5	11.19	Leucine	902.3 ± 15.49^{c}
6	11.19	Phosphoric acid	*
7	11.51	Isoleucine	948.4 ± 62.75^{c}
8	12.32	Serine	131.2 ± 12.61^{e}
9	12.65	Threonine	431.1 ± 32.44^{d}
10	13.81	Methionine	1371 ± 60.28^{b}
11	14.59	Oxoproline	*
12	15.77	Phenylalanine	743.1 ± 18.83^{d}
13	15.84	Arabinose	*
14	17.27	Glutamic acid	2930 ± 197.9^{a}
15	17.67	Citric acid	*
16	18.28	Fructose	*
17	18.42	Glucose	*
18	18.61	Galactose	*
19	20.29	Myo-inositol	*

** indicates the tentatively identified and not quantified compounds. Quantification of amino acids was performed using an external calibration curve of pure standards. Values are expressed as μg ± SD of three different replicates. Data with different lowercase letters indicate significant different values at $p \leq 0.05$ as measured by ANOVA test followed by Tukey's post hoc test. Letter "a" denotes the highest value. The symbol "*" categorizes the compounds that were tentatively identified simply on the base of their mass fragmentation and comparison with literature data.*

DISCUSSION

The use of biostimulants to counteract the effect of abiotic stress has been documented and their capability to promote plant defenses against adverse environmental conditions were reported (Alzahrani and Rady, 2019; Rady et al., 2019). Seed treatment with biostimulants is a technology to counteract environmental stress at the time of sowing, and improving yield, all starting from seed germination (Rady et al., 2019). This is a faster method in comparison to conventional breeding or plant genetic modification and could be useful for seed treatment in countries, where high temperature at sowing could be a limiting factor (Savvides et al., 2016).

In this work, the potential effects of the biostimulant KIEM® was tested on cucumber seeds germinated under standard (28°C) and heat stress (35°C) conditions by using different methodologies, such as morphological, biochemical and transcriptional (qPCR) analyses.

With regard to biometric data (**Table 1**), our results suggest a potential effect of KIEM® in promoting germination and seedling growth under heat stress. The final germination percentage was higher in KIEM®-treated seeds at 48 h after incubation at 35°C, while 99% of germination percentage was observed at 28°C (**Table 1**). Therefore, KIEM® is not harmful to seeds and has not shown phytotoxicity as biostimulants used as other studies (Yildirim et al., 2000; Masondo et al., 2018).

In seed physiology, reactive oxygen species (ROS) are usually considered as toxic molecules, whose accumulation leads to cell injury with consequent problems in seed germination and development (Jeevan Kumar et al., 2015). However, there is the increasing evidence that ROS, at low concentrations, can act as signaling molecules involved in a wide range of responses to various stimuli (Bailly, 2004; El-Maarouf-Bouteau and Bailly, 2008; Barba-Espín et al., 2012). The dual function of ROS in plants mainly relies to the cellular antioxidant machinery, which involves detoxifying enzymes (Alscher and Hess, 2017) and antioxidant compounds (Gershenzon, 1984). Such mechanisms can scavenge potentially toxic ROS, generally produced under stressful conditions, or rather tightly control ROS concentrations in order to regulate various signaling pathways. Among ROS, hydrogen peroxide plays a key role during germination process, however, high levels of H_2O_2 can be toxic for the seeds (Wojtyla et al., 2016). The ability of seeds to survive to this oxidative condition during germination phases is related, at least partly, to their ability to activate different detoxification systems, including both the neo-synthesis of soluble antioxidants and the activation of gene expression of enzymatic defense (Lehner et al., 2006). Our study indicates that the level of H_2O_2 is reduced in KIEM®-treated seeds (**Table 2**), suggesting a possible role of the biostimulant in preventing the accumulation of this reactive oxygen species. The lower amount of H_2O_2 observed in KIEM®-treated seeds can be linked to the expression level of genes coding for ROS-scavenging enzymes. The effect of KIEM® seemed to be stronger at 48 h and at 35°C (**Figure 1**). In heat stress conditions, KIEM® led to an increase in the transcription levels of all antioxidant genes, except *FeSOD*.

In addition to the scavenging enzyme machinery, plants possess a number of antioxidant molecules that are able to counteract the effects of different stress, such as non-protein thiols, the most important source of sulfur in different seeds, a fundamental element involved in metabolic pathways, nutritional quality and plant productivity.

Plants can respond to an increase in ROS production through the synthesis of soluble antioxidants (Gershenzon, 1984). Among them, non-protein thiols are considered important molecules in counteracting the effect of oxidative stress (Zagorchev et al., 2013). Thiols, such as glutathione (GSH) together with its regulation in redox signaling and defense processes, are important components for the heat stress tolerance (Szalai et al., 2009). The glutathione pool was shown to be associated with the response to heat stress of maize (Kocsy et al., 2002), *Coleus blumei* and *Fagus sylvatica* L. (Peltzer et al., 2002), *Triticum aestivum* (Nieto-Sotelo and Ho, 1986) and *Vigna radiata* (Nahar et al., 2015). Directly linked to thiols, are glutathione-S-transferases (GSTs), proteins playing important roles in enzymatic thiol-dependent ROS scavenging mechanisms (Zagorchev et al., 2013) since they catalyze the conversion of H_2O_2 by using glutathione or homoglutathione as substrates. In our study, the levels of non-protein thiols were higher in KIEM®-treated seeds compared to untreated seeds in standard conditions at 24 h and in heat stress at 48 h (**Table 2**), and in correlation with the expression levels of *GST* (**Figure 1**). The trend observed for non-protein thiols, which an initially decrease followed by an increase upon KIEM®

treatment, might be explained with the double role played by these molecules. These compounds are soluble antioxidants able to mitigate ROS production in several stress conditions, but they also play an important role as substrates for the synthesis of proteins and enzymes (Zagorchev et al., 2013). Moreover, GSH, the main non-protein thiol, is used as a reducing substrate in the synthesis of ascorbate (Dixit et al., 2001).

During cucumber seed germination, the glyoxylate cycle plays a key role in the mobilization of triacylglycerides located in storage tissue during post-germinative growth to effect net gluconeogenesis from the acetyl-CoA derived by β-oxidation (Lamb et al., 1978; Reynolds and Smith, 1995; Dunn et al., 2009). During early germination phases, enzymes of the glyoxylate cycle such as isocitrate lyase increase their activity during maximum fat metabolism in specialized microbodies (glyoxysomes) located in the storage tissue of germinating seeds (McLaughlin and Smith, 1994).

For these reasons, the upregulation of the gene coding for ICL is essential for the seed health status, and its down-regulation might be linked to particular stress conditions.

In general, the application of KIEM® promoted a strong accumulation of *ICL* transcripts, especially at 48 h, suggesting a positive action of this biostimulant in enhancing cucumber seed germination.

In the recent years, several scientific studies reported the beneficial effects of the application of plant-derived protein hydrolysates as biostimulant in order to increase the growth, yield and fruit quality of agricultural crops (Shafeek et al., 2015). Since the beneficial properties of biostimulants were largely linked to their content of amino acids and other polar metabolites (Nardi et al., 2016), investigation about the chemical profile is actually essential to elucidate the possible mechanism of action of these products. The chemical profile of these formulations depend clearly on the raw material used for their manufacture processes, and the use of different raw materials determines changes both in metabolite profile and in plant physiological activity. With regard to seed germination, Amirkhani and co-workers showed that broccoli seeds coated by plant protein lysates enhanced seedling shoot and root growth compared to uncoated seeds (Amirkhani et al., 2016).

Despite the high content of methionine and glutamic acid in the amino acid fraction of this biostimulant, we cannot exclude that the displayed effects both on the balance of oxidative status, and on the expression of genes coding for antioxidant enzymes and isocitrate lyase might also depend on other compounds (i.e., lignin derivatives) present in the formulation of KIEM®, which were, however, more difficult to analyze. Probably, the effects discussed in this paper were the consequences of a synergic action of the different and several metabolites.

CONCLUSION

Plant-based biostimulants are an excellent choice for a more sustainable agriculture. In this work, we showed that KIEM®, an innovative biostimulant, was able to increase the percent germination and restore the oxidative balance in cucumber seeds under heat stress conditions. The balancing effect is displayed not only through the reduction of endogenous H_2O_2 but also through the activation of antioxidant defenses. Indeed, the pre-sowing treatment with KIEM® is able to restore the capacity of synthesizing the soluble antioxidants and modulate the expression of genes coding for antioxidant enzymes. Moreover, our study provided also the experimental evidence that this biostimulant is able to regulate positively the *ICL* expression, a gene coding for a key enzyme involved in the germination process. Finally, comparing the effects displayed after 24 and 48 h, it is interesting to note that the most significant protective effects occurred after 48 h from the application of the biostimulant in heat stress condition. Probably, the effects of the biostimulant were the consequence of a synergic action of the different and several metabolites in the formula. This new product may improve tolerance to heat and crop productivity by triggering different responses, along with the advantage of reducing the number of treatments and thus the final management costs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CB, VC, and CG conceived and designed the experiments. VC and CG provided free of charge the biostimulant object of this study. CC, EG, JB, RK, and JA performed the experiments. GM, CC, and EG analyzed the data. GM, CC, EG, and CB wrote many parts of the manuscript. CB, CG, and VC revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

FUNDING

This work was supported both by funding from Regione Piemonte (Fondo Sociale Europeo – Apprendistato di Alta Formazione e di Ricerca, 2016–2020) for the research activity of CC and assigned to CB, and by the Local Research Grants (ex-60%, 2018–2019) from the University of Turin, Italy and assigned to CB.

ACKNOWLEDGMENTS

The authors are grateful to Alexander Ciattoni for his technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00836/full#supplementary-material

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Conflict of Interest: VC and CG were employed by Green Has Italia S.p.A. CC was a Ph.D. student of the Ph.D. Program in Pharmaceutical and Biomolecular Sciences of the University of Turin, and in apprenticeship in Green Has Italia S.p.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 June 2020
doi: 10.3389/fpls.2020.00807

Ascophyllum nodosum Extract Biostimulant Processing and Its Impact on Enhancing Heat Stress Tolerance During Tomato Fruit Set

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *28 February 2020*
Accepted: *19 May 2020*
Published: *25 June 2020*

Citation:
Carmody N, Goñi O, Łangowski Ł and O'Connell S (2020) Ascophyllum nodosum Extract Biostimulant Processing and Its Impact on Enhancing Heat Stress Tolerance During Tomato Fruit Set. Front. Plant Sci. 11:807. doi: 10.3389/fpls.2020.00807

The application of biostimulants derived from extracts of the brown seaweed *Ascophyllum nodosum* has long been accepted by growers to have productivity benefits in stressed crops. The impact of the processing method of the *A. nodosum* biomass is also known to affect compositional and physicochemical properties. However, the identification of the mechanisms by which processing parameters of *Ascophyllum nodosum* extracts (ANEs) affect biostimulant performance in abiotically stressed crops is still poorly understood. In this study, we performed a comparative analysis of two carbohydrate-rich formulations derived from *A. nodosum*: C129, an ANE obtained at low temperatures through a gentle extraction and the novel proprietary PSI-494 extracted under high temperatures and alkaline conditions. We tested the efficiency of both ANEs in unstressed conditions as well as in mitigating long-term moderate heat stress in tomato (*Lycopersicon esculentum*, cv. Micro Tom) during the reproductive stage. Both ANEs showed significant effects on flower development, pollen viability, and fruit production in both conditions. However, PSI-494 significantly surpassed the heat stress tolerance effect of C129, increasing fruit number by 86% compared to untreated plants growing under heat stress conditions. The variation in efficacy was associated with different molecular mass distribution profiles of the ANEs. Specific biochemical and transcriptional changes were observed with enhanced thermotolerance. PSI-494 was characterized as an ANE formulation with lower molecular weight constituents, which was associated with an accumulation of soluble sugars, and gene transcription of protective heat shock proteins (HSPs) in heat stressed tomato flowers before fertilization. These findings suggest that specialized ANE biostimulants targeting the negative effects of periods of heat stress during the important reproductive stage can lead to significant productivity gains.

Keywords: tomato (*Lycopersicon esculentum*), plant biostimulants, *Ascophyllum nodosum* extracts, abiotic stress tolerance, heat stress, flowering, carbohydrates, heat shock protein

INTRODUCTION

In the last decade, reports show that climate change has significantly impacted on overall food security due to a reduction in viable land areas, global yields of many staple crops, and an increase in both biotic and abiotic stresses (Ainsworth and Ort, 2010; FAO, 2016). In the field, abiotic stresses can occur in isolation or together to impact phenotypical, physiological, biochemical, and/or molecular aspects of crop development. Heat stress, can have significant implications on important plant activities, such as seed germination, plant development, photosynthesis, and reproduction, which leads to reductions in plant growth and crop yield (Rieu et al., 2017; Nadeem et al., 2018). The most recent IPCC report predicts a global increase of more than 4°C until the end of the century and a more frequent occurrence of severe heat waves (Change, 2014). Under these conditions, it has been predicted that a 1°C increase in global temperature could decrease the production of important commodity crops between 4.1 and 6.4% (Zhao et al., 2017). An assessment of heat stress risk at a global level for four key crops (wheat, maize, rice, and soybean) suggests that the global warming impact on agriculture production would not only occur in sub-tropical and tropical regions, but also in important agricultural regions such as Eastern China, the Northern United States, South-Western Russian Federation, and Southern Canada (Teixeira et al., 2013).

A key factor in the successful implementation of agronomic strategies to enhance crop thermotolerance is a better understanding of the mechanisms by which heat stress alters plant metabolism and leads to crop yield losses. While heat stress typically occurs when temperatures rise 5–15°C above the optimum for plant growth, the impact of high temperatures on crop yield is defined by the intensity, duration, and rate of the temperature change. Generally, two types of high temperature stresses can be distinguished. A short period of very high temperatures (e.g., >15°C above optimum temperature) is generally referred as heat shock and can cause extensive damage on crop plants by affecting vital physiological and metabolic functions such as enhanced respiration, photoinhibition of photosystem II (PSII), increase in membrane fluidity, accumulation of reactive oxygen species (ROS), changes in carbohydrate partitioning, or protein denaturation (Fragkostefanakis et al., 2015; Fahad et al., 2017; Nadeem et al., 2018). However, exposure to moderately elevated temperatures (e.g., 5–10°C above optimum growth temperature) would require a longer exposure (i.e., multiple days) to obtain similar effects (Mesihovic et al., 2016). It is important to mention that the susceptibility of individual crops to a specific heat stress regime would also vary with the developmental stage of the plant. While plants at vegetative stage are able to maintain basic activities and to minimize the injuries derived from long-term moderate heat stress, reproductive development tends to be more affected under these conditions. Moreover, it has been observed in both monocot and dicot species that male gametophytes (pollen grains) are even more susceptible to damage from heat stress than their female counterparts in both long-term moderate and extreme heat stress. Therefore, the number and health of the reproductive organs will influence fruit set in heat stressed conditions, a critical phase for realizing yield potential (Mesihovic et al., 2016; Rieu et al., 2017).

Several solutions for providing crop thermotolerance include specialty crop inputs, selective plant breeding, or genetic modification approaches. The exogenous application of proline in heat stressed chickpea seedlings coupled an improved content of chlorophyll and antioxidant compounds with a significant improvement in the activities of enzymes of carbon fixation and sucrose metabolism (Kaushal et al., 2011). Other studies have shown how the foliar application of salicylic acid (SA) or phosphite in young plants reduced the adverse effects of extreme heat stress regimes through enhanced photosynthesis or accumulation of osmoprotectants (Khan et al., 2013; Xi et al., 2020). Breeding programs to obtain thermotolerant cultivars have focused on traits such as better photosynthetic rate, pollen viability, or fruit set under high temperatures. However, the development of new thermotolerant varieties through plant breeding is expensive and time-consuming (Chapman et al., 2012; Fahad et al., 2017). Another way to increasing yield under heat stress is based on the generation of genetically modified (GM) thermotolerant crops. Numerous plant species have shown increased thermotolerance through the enhancement of synthesis of heat shock proteins (HSPs), using various transgenic approaches (Grover et al., 2013; Gerszberg and Hnatuszko-Konka, 2017). HSPs are the first line of defense against heat stress damage acting as molecular chaperones in order to reduce or even prevent denaturation or aggregation of proteins and increasing the refolding of protein structure (Jacob et al., 2017). These evolutionarily conserved proteins affect a broad array of cellular processes and are grouped into five classes in plants, according to their molecular weight: HSP100, HSP90, HSP70, HSP60, and small heat-shock proteins (sHSPs; Reddy et al., 2016).

Plant biostimulants have been gaining increased attention during the last number of years, due to the growing interest of scientists, private industry, and growers in integrating these products into their armory of environmentally friendly tools that can assist in securing improved crop performance (Du Jardin, 2015; Yakhin et al., 2017; Rouphael and Colla, 2020). Globally, the biostimulant market is forecast to expand at a growth rate of 12.3% CAGR (Compound Annual Growth Rate) from 2019 to 2027 and it is expected to reach US$ 5.5 billion by 2027 (Transparency Market Research, 2019). After several years of negotiations among the European institutions, the new Fertilising Products Regulation (FPR) (EU) 2019/1009 was published in the Official Journal of the EU on 25th June 2019, recognizing plant biostimulants as a distinct category of agricultural inputs. Under the new regulation: "A plant biostimulant shall be a EU fertilizing product, the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant and the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, or (c) quality traits" (Regulation (EU) 2019/1009, 2019).

In the plant biostimulants field, the positive effects of seaweed extracts have been extensively demonstrated (Sangha et al., 2014),

turning them into the fastest growing product category in the global plant biostimulant market (Markets and Markets, 2019). However, it is important to note that seaweed extract biostimulants are not a homogenous category of products. Seaweed extracts vary depending on the family and species of seaweed used for manufacture (e.g., brown, green, or red), the source of the seaweed raw material and the process used for extraction (Craigie, 2011; Fletcher et al., 2017). The brown seaweed *Ascophyllum nodosum* has long been accepted by growers in the international market to have superior performance as compared to biostimulants made from other seaweeds. *Ascophyllum nodosum* extract (ANE) biostimulants have been shown to improve plant vigor, increase root development, enhance chlorophyll synthesis, promote earlier flowering, enhance fruit set and uniformity of fruit, reduce pod shatter, delay senescence, and enhance tolerance to abiotic stress (Sangha et al., 2014; Łangowski et al., 2019; Shukla et al., 2019). The impact of processing of the *A. nodosum* raw material on an ANE biostimulant product is also known to affect compositional and bioactivity-related parameters (Goñi et al., 2016, 2018). However, little attention has been paid to the identification of the mechanisms by which processing parameters of ANEs can affect their biostimulant performance in heat stressed crops.

After potato, tomato (*Lycopersicon esculentum*) is considered the most valuable vegetable crop grown globally. Although tomatoes normally grow in tropical, subtropical, and warm temperate climates, which facilitate longer growing seasons, losses of up to 70% can be seen in areas affected by summers with unusually high temperatures (Sato et al., 2002). Both extreme temperatures and prolonged periods of moderately elevated temperatures can impact different plant activities leading to reductions in fruit set or fruit yield (Mesihovic et al., 2016). Different tomato plant cultivars growing under chronic mild heat stress showed that pollen release, pollen viability, and anther morphology were major limiting factors for optimum fruit set (Sato et al., 2000; Müller et al., 2016; Xu et al., 2017). One of the main biochemical parameters that influenced pollen viability and development of young tomato fruits during heat stress periods was an optimal carbohydrate metabolism (Pressman et al., 2002; Firon et al., 2006; Li et al., 2012; Zhou et al., 2017). Furthermore, Fragkostefanakis et al. (2016) showed that heat stress response and thermotolerance in tomato developing pollen was linked to the accumulation of heat stress-induced chaperones, such as *HSP101.1*, *HSP70.9*, *HSP17.7C-Cl*, and other protective metabolites.

In this study, we performed a comparative analysis of two carbohydrate-rich biostimulant formulations derived from *A. nodosum*: C129, an ANE obtained at low temperatures, and the novel proprietary PSI-494 extracted under high temperatures and alkaline conditions through a targeted plant signal induction (PSI) approach to formulation development. We tested the efficacy of both ANEs in unstressed conditions as well as in mitigating long-term moderate heat stress in tomato during the reproductive stage. Evaluating specific phenotypical, physiological, biochemical, and molecular markers associated with enhanced thermotolerance, we revealed the distinct effect of ANEs obtained through different extraction methods and how it can be linked to their different molecular weight distribution profiles. Here, we show for the first time that the judicious application of specialized ANE biostimulants can target the negative effects of periods of high temperatures during the important reproductive stage and solve specific plant productivity challenges.

MATERIALS AND METHODS

Chemicals

The carbohydrate rich fraction of *A. nodosum*, crude enzymatic mixture, and the two ANEs; C129 and PSI-494 complex were provided by Brandon Bioscience (Tralee, Ireland). All chemical reagents and dextran standards were purchased from Sigma-Aldrich (Arklow, Ireland). The primers were purchased from Eurofins Genomics (Ebersberg, Germany).

Plant Material and Growth Conditions

Tomato seeds (*L. esculentum*, cv. Micro Tom) were purchased from Liscahane Nurseries (Tralee, Ireland). Seeds were surface sterilized with sodium hypochlorite for 1 min before being thoroughly rinsed with distilled water. Seed were set in plug trays using a growth medium composed of compost:vermiculite:perlite (5:1:1). On day 22 seedlings were transferred to 1 L pots [same growth medium as previous with the addition of 2 g calcium carbonate lime and 1 g of slow releaser fertilizer containing N/P_2O_5/K_2O (7/7/7, w/w/w)]. Plants were raised in a growth room at a temperature of 27/22 ± 2°C with 16 h of daylight and 8 h of night and 80 ± 5% relative humidity (RH) under a light intensity of 120 μmol m^{-2} s^{-1} in a complete randomized block design. Plants were irrigated with 1.5 L of water per tray twice a week in order to create equal soil moisture conditions in all pots. Temperature and relative moisture content were recorded regularly with a portable USB data logger (Log32TH, Dostmann electronic GmbH).

ANE Biostimulant Treatment Application and Heat Stress Conditions

Two formulations (C129 and PSI-494) obtained from a carbohydrate rich fraction of *A. nodosum* using two different extraction methods were applied to plants as ANE biostimulant treatments. The initial carbohydrate rich fraction was isolated using selective solvents according to Rioux et al. (2007). The C129 extract was obtained after treating the carbohydrate rich extract with a crude enzymatic mixture with carbohydrate depolymerizing activity at low temperature (<30°C). PSI-494 was produced using a proprietary extraction at high temperatures and alkaline conditions from the same carbohydrate rich fraction. Both ANE biostimulants possessed a very low macronutrient content with N:P:K values of 0.3–0.4:0.1–0.2:2–3% w/w. Prior to the application of heat stress, the ANE biostimulants were applied by foliar spray at a dilution of 0.106% (w/v) on 105-day-old plants with significant presence of flowers at early stages of pollen development (4–8 mm young buds). Water was applied as a control. After 3 days, plants of all three groups (control, C129 and PSI-494) were exposed to moderate heat stress for

14 days in a growth chamber (31/24°C with 16 h of light and 8 h of darkness and 80 ± 5% RH under a light intensity of 120 μmol m^{-2} s^{-1}). To minimize the influence of any positional effect, the relative position of the pots was changed every other day. Tomato pollination was aided using an electric toothbrush twice a week when plants began to flower. After the heat stress period, the plants were placed back in the growth room and ANE treatments were applied again as foliar spray at 0.106% (w/v). Control plants were sprayed with equal volume of distilled water. Recovery stage after heat stress was maintained for 1 week under unstressed conditions to obtain 129-day-old plants at fruit set stage. A two-spray application program before and after the stress period was based on current farmer practice for the use of ANEs and previously published by Goñi et al. (2018). Leaf and flower tissue were sampled in 122-day-old plants after being subjected to moderate heat stress for 14 days. Similar tomato plants were selected and grown under unstressed conditions for 122 days. ANE biostimulants and control treatments were applied by foliar spray as described above to evaluate growth promoting effects on non-heat stressed tomato plants. Leaf and flower sampling points for unstressed plants also corresponded to 122-day-old plants. All leaf and flower samples were collected 2 h after the end of the light period to avoid any influence of plant day-night cycle on soluble sugar profiles, snap-frozen in liquid nitrogen, ground and kept in −80°C until further analysis.

Chemical Composition and Structural Analysis of ANE Biostimulant Treatments

Dried ANE samples were placed inside a furnace at 600°C for 6 h in order to obtain and quantify the ash content. Total sugars were quantified according to Rioux et al. (2007). Total polyphenol content was determined spectrophotometrically following the method of Goñi et al. (2018). The content of unidentified organic components was calculated by difference to the total organic amount. The molecular weight (M_w) distribution of carbohydrates from different samples was analyzed using high performance size exclusion chromatography-refraction index detector (HPSEC-RID). The HPSEC Shimadzu system consisted of a system controller CBM-20A, a solvent delivery module LC-20 AD, an online degasser DGU-20A5, an autosampler SIL-20ACHT, a refraction index detector (Varian Prostar 350 RID), and an LC workstation. HPSEC analysis was performed using 4 PL aquagel-OH MIXED-H columns in tandem (8 μm, 300 × 7.5 mm; Agilent). The mobile phase (0.1 M NaAc/0.1 M Na_2SO_4 buffer pH 7.8) was used as isocratic elution at room temperature. The flow rate and injection volume were set to 1 ml min^{-1} and 40 μl, respectively. M_w values were calculated from the measured retention times through a calibration curve made with dextran standards.

Evaluation of Plant Height, Photosynthetic Performance, Total Flower and Fruit Number

Plant height, total number of flowers and photosynthetic performance were evaluated at the end of the heat stress period. The sampling point for unstressed plants corresponded to 122-day-old plants. Regarding the photosynthesis parameters, PQ-SPAD (relative chlorophyll content), Φ_{II} (quantum yield of PSII) and Φ_{NPQ} (quantum yield of non-photochemical exciton quenching) were evaluated using a MultispeQ device (Kuhlgert et al., 2016). Fruit set was evaluated at the end of the recovery stage in heat-stressed plants. The sampling point for unstressed plants corresponded to 129-day-old plants. A developing fruit was considered as a small, ripening fruiting body that had displaced the tomato flower which was containing it.

Evaluation of Pollen Viability

To conduct pollen viability analysis, one flower per plant at anthesis stage was collected by removal of the flower and bud using sterile forceps at the end of the heat stress period. The sampling point for unstressed plants corresponded to 122-day-old plants. Pollen viability was determined according to Paupière et al. (2017). Briefly, flowers were placed in a tube with 500 μl of germination solution [1 mM KNO_3, 3 mM $Ca(NO_3)_2$, 0.8 Mm $MgSO_4$, and 1.6 Mm H_3BO_3] and 20 μl of Alexander dye. Samples were left overnight at room temperature to allow for consistent staining of the pollen grains. Viable pollen was stained purple by the Alexander dye, while non-viable pollen was stained green. Pollen number was quantified using a Neubauer chamber hemocytometer. The recorded results were then transformed into the number of each type of pollen per flower and the results were expressed as percentage of viable pollen.

Sucrose, Glucose and Fructose Content in Plant Tissues

The levels of sucrose, glucose, and fructose were determined by HPAEC-PAD using a Carbopac PA-1 column and expressed as mg g^{-1} FW in leaf and flower tissue following the method of Goñi et al. (2018). These soluble sugars were measured in samples collected either at the end of the heat stress period or for 122-day-old unstressed plants. The measured results were expressed as difference of heat stressed samples (Untreated, C129, and PSI-494) with respect to the unstressed control.

RNA Extraction and RT-qPCR

Total RNA was isolated from about 50 mg of frozen ground flower material by Plant/Fungi Total RNA Purification Kit (Norgen Biotek, Canada) following the manufacturer's instructions. RNA was treated with RNase-Free DNase I Kit (Norgen Biotek, Canada) in order to remove efficiently genomic DNA contamination. RNA concentration and purity was measured in a μDrop™ Plate RNA using a Varioskan Flash instrument (Fisher Scientific). Expression analysis of *HSP101.1* (*Solyc03g115230*), *HSP70.9* (*Solyc11g020040*), and *HSP17.7C-Cl* (*Solyc06g076520*) genes was performed by RT-qPCR using a Roche LightCycler® 96 System (Roche, UK) and a LightCycler® RNA Master SYBR Green I one-step kit (Roche, UK) according to the manufacturer's instructions. The expression level of the tomato *ACTIN2* (*Solyc01g104770.2*) gene was used as the reference gene. $2^{-\Delta\Delta CT}$ was used to quantify normalized gene expression. The primers sequences used were as follows:

HSP101.1: forward 5'-ACCCGATCAGATTGCGGAAG-3' and reverse 5'-GAACCAGTTGGTTGCTGTGG-3'.
HSP70.9: forward 5'-GAGCTCAAGGATGCCATTTC-3' and reverse 5'-CAGATGATCCAGTTGTACCAG-3'.
HSP17.7C-CI: forward 5'-ATGGAGAGAAGCAGCGGTAA-3' and reverse 5'-ATGTCAATGGCCTTCACCTC-3'.
ACTIN2: forward 5'-TCTTGAAGCGTTTTAAAAGATGGC-3' and reverse 5'-TCACCAGCAAATCCAGCCTT-3'.

Statistical Analysis

Phenotypic assessment of plants was done in three independent plant trials, with six plants per treatment group and condition (18 independent biological replicates). The flower and leaf samples collected per independent plant trial were pooled for further analysis (three independent pooled biological samples for every plant tissue sample). Chemical and structural analysis of ANEs was performed on a minimum number of three biological replicates. Photosynthetic parameters were measured in one leaf at a central position for every plant (18 independent biological replicates) using three technical replicates per biological replicate. For biochemical and molecular analysis, at least three biological replicates of each treatment and condition were performed using the plant samples described above and three technical replicates per biological replicate were used. Statistics were evaluated with Sigma Plot 12 and Statgraphics Centurion XVI software. The differences in the chemical or structural analysis of ANEs were analyzed using *t*-test at $p \leq 0.05$. The effect of ANE treatments on plant soluble sugar content and *HSPs* gene expression were analyzed with the one-way analysis of variance (ANOVA) by Tukey's HSD test at $p \leq 0.05$. The rest of the plant data was compared by using two-way ANOVA, with Tukey's HSD test at $p \leq 0.05$. Where the interaction between the two factors condition (unstressed and heat stressed) and ANE treatment (AxB) was significant, data were subjected to one-way ANOVA, comparing all ANE treatments with each other within the same growth condition. Where AxB interaction was not significant, the effect of condition and ANE treatment was evaluated separately, comparing the respective means through *t*-test (condition) or one-way ANOVA Tukey's HSD test (ANE treatment) at $p \leq 0.05$. The application of all parametric tests was performed after checking the data normality (Shapiro-Wilk test) and equal variance assumptions. Unless stated otherwise, all data are expressed as average ± standard error (SE). Details of the individual sample size for each analysis are mentioned in the table and figure legends.

RESULTS

Compositional and Structural Analysis of ANE Biostimulants

The results presented in **Table 1** provide a compositional evaluation of the two ANE biostimulants by determining the levels of some key components, such as ash, total carbohydrates, and polyphenols. C129 and PSI-494 were primarily composed of carbohydrates and ash. Both ANE biostimulants differed by <2% in the amount of total carbohydrates ($p = 0.278$). The analysis of polyphenols, determined as phloroglucinol equivalents, indicated that both ANEs contained very low amounts of this component on a dry weight basis and there were not statistically significant differences between them ($p = 0.374$). As it can be observed in the HPSEC-RID chromatograms (**Figure 1**), the initial carbohydrate rich fraction used as substrate was composed of a homogenous 555.31 kDa peak which generated three carbohydrate peaks for both ANE biostimulants. These peaks were characterized as three groups of different molecular weights (**Table 2**). The C129 formulation, which was extracted gently at low temperatures was composed of a mix of molecules ranging between 2,881.47 and 1.28 kDa with a very high representation (97%) of molecules with an average M_w of 212.12 kDa. However, PSI-494 extracted at high temperatures using a proprietary formulation process was characterized as a product with lower M_w carbohydrates. Molecules ranging between 1.29 and 3.24 kDa were significantly more abundant than those observed in C129

TABLE 1 | Compositional analysis of two ANE biostimulant treatments.

Component % (w/w)[1]	ANE biostimulant treatment	
	C129	PSI-494
Ash[2]	32.10 ± 0.67	35.81 ± 0.87*
Total carbohydrates	64.81 ± 0.60	63.52 ± 0.55
Polyphenols	0.65 ± 0.08	0.55 ± 0.06
Other organics	2.44 ± 0.21	0.12 ± 0.03**

[1]*Data are the means ± SE (number biological replicates; n = 3).*
[2]*ANEs chemical compositional analysis is expressed with respect to their dry content.*
**Difference was significant at p ≤ 0.05 (t-test).*
***Difference was significant at p ≤ 0.001 (t-test).*

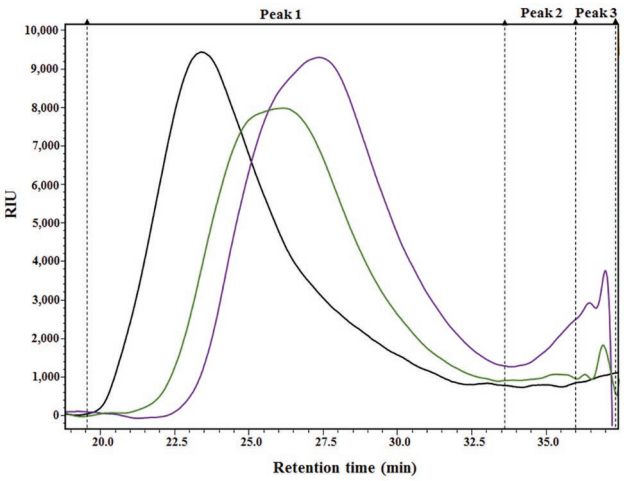


FIGURE 1 | HPSEC-RID analysis of the initial carbohydrate rich substrate and the generated *Ascophyllum nodosum* extract (ANE) biostimulants. Black chromatogram (AN substrate); green chromatogram (C129); purple chromatogram (PSI-494). The three main peaks were integrated and are shown in the chromatograms with dashed lines.

(8.82 vs. 1.76%) and the average M_w of the PSI-494's main peak was 1.8-fold smaller than that characterized in the ANE biostimulant obtained at low temperatures.

Effect of Heat Stress and ANEs on Height of Tomato Plants

The plant height was recorded from the start of the stem (at soil level) to the dorsal flowering body (highest point of the plant) in 122-day-old tomato plants. The two-way ANOVA test revealed that in conjunction both parameters (condition × ANE treatment) had no significant effect ($p = 0.698$; **Table 3**). The heat stressed plants showed an overall statistically significant decrease of plant height compared to the unstressed group (unstressed: 29.42 cm vs. heat stressed: 26.46 cm; $p = 0.014$; **Figure 2**). However, the effect of the different ANEs on this parameter was not statistically significant ($p = 0.650$) with respect to the control.

Effect of Heat Stress and ANEs on Reproductive Development Parameters of Tomato Plants

In order to evaluate how long-term moderate heat stress and ANE biostimulant treatments affected the reproductive stage of tomato plants, three different developmental parameters were evaluated (total flower number, pollen viability, and fruit number). As can be observed in **Table 3**, the two-way ANOVA test showed that the interaction between factors was not significant for the total flower number ($p = 0.754$). On the contrary, there was a significant increase in the number of flowers per plant in both treatments between unstressed (7.89) and heat stressed (12.55) plants ($p \leq 0.001$). When these differences were examined in detail in terms of the ANE treatment group, there were significant differences between the treated and untreated plants ($p = 0.024$). Interestingly, those plants treated with PSI-494 showed the highest absolute values of total flower number (11.98) compared to the control (8.95; **Figure 3A**).

Tomato pollen viability was compromised when plants were exposed to moderate heat stress for 14 days (31/24°C; **Figure 3B**). When a two-way ANOVA test was run, it was found that all three parameters (condition, ANE treatment, and condition × ANE treatment) were highly significant ($p \leq 0.001$; **Table 3**). Therefore, all data were subjected to one-way ANOVA, comparing all ANE treatments to each other under both growing conditions. One hundred twenty-two-day-old plants growing under unstressed conditions (27/22°C day/night) had pollen viability over 94%. Only the application of C129 led to a small but not statistically significant decrease with respect to control (−4.56%; $p = 0.196$). Although viable pollen was reduced by 80% in untreated plants growing under heat stress, our results also showed that C129 and PSI-494 significantly increased this parameter between 3.2 and 4.4 times compared to the control. However, no statistically significant differences were observed between both ANE treatments under heat stress conditions (**Figure 3B**).

Fruit number was quantified in 129-day-old tomato plants to determine how the effect of both heat stress damage during the recovery period and the second ANE application may impact on this yield related parameter (**Figure 3C**). When a two-way

TABLE 2 | Molecular weight distribution of two ANE biostimulant treatments expressed as the average M_w of the main peaks, the M_w corresponding to the interval of the whole peak or the relative peak area.

Treatment	# Peak	Peak properties[1]			
		Average M_w (kDa)	Start M_w (kDa)	End M_w (kDa)	Area (%)[2]
C129	1	212.12 ± 0.25	2,881.47 ± 2.34	3.11 ± 0.07	97.04 ± 0.56
	2	2.32 ± 0.07	3.11 ± 0.07	1.67 ± 0.03	1.29 ± 0.04
	3	1.45 ± 0.04	1.67 ± 0.03	1.28 ± 0.03	0.47 ± 0.02
PSI-494	1	117.67 ± 0.18	2,676.67 ± 2.15	3.24 ± 0.11	91.19 ± 0.45*
	2	1.87 ± 0.05	3.24 ± 0.11	1.68 ± 0.03	6.65 ± 0.07*
	3	1.44 ± 0.03	1.68 ± 0.03	1.29 ± 0.04	2.17 ± 0.03*

[1]Data are the means ± SE (number biological replicates; n = 4).
[2]The peak area was calculated in percentage over the total area of all peaks.
**Differences between the respective peak area values of both ANEs were significant at p ≤ 0.001 (t-test).*

TABLE 3 | Source of variance for height, reproductive, and photosynthetic parameters of tomato plants grown at two temperature conditions and treated with two ANE biostimulants.

Source of variance	Plant height	Flower number	Pollen viability	Fruit number	PQ-SPAD	Φ_{II}	Φ_{NPQ}
Condition (A)	*	***	***	*	ns	***	***
ANE treatment (B)	ns	*	***	***	ns	ns	ns
AxB	ns	ns	***	*	ns	*	*

*ns, non-significant; *significant at p ≤ 0.05, **significant at p ≤ 0.01, and ***significant at p ≤ 0.001, respectively.*

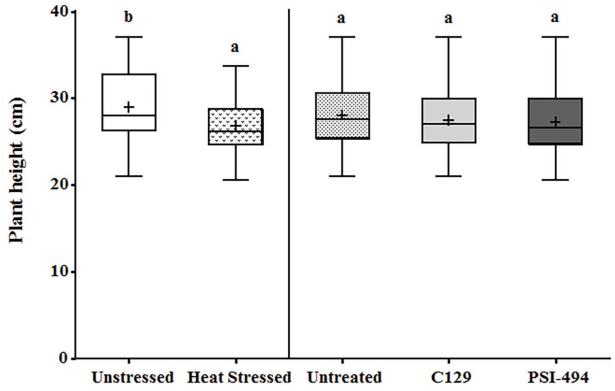


FIGURE 2 | Effect of heat stress and ANEs on height of tomato plants. Data were measured in 122-day-old plants and subjected to two-way ANOVA and Tukey's HSD test for evaluating the differences among means at $p \leq 0.05$. Since there was no significant AxB interaction, the effect of condition and ANE treatment was evaluated separately, comparing the respective means. Different letters indicate statistical differences for $p \leq 0.05$ based on *t*-test (condition) or one-way ANOVA Tukey's HSD test (ANE treatment). The vertical line is used to visually separate the evaluation of the effect of condition and ANE treatment. The horizontal line through the box and the cross represent the median and mean value, respectively. Number of biological replicates = 18.

ANOVA test was applied, it was found that there was a statistically significant interaction between condition and ANE treatment (p = 0.011; **Table 3**). Therefore, data of ANE treatments were examined using one-way ANOVA and results indicated that tomato plants grown under unstressed conditions and sprayed twice with C129 and PSI-494 increased their fruit number by 22 and 33%, respectively. However, this improvement in fruit set was not statistically significant (p = 0.289). PSI-494 did significantly increase fruit number by 86% compared to the untreated group in heat stressed plants ($p \leq 0.001$). This parameter did not show significant differences in stressed tomato plants treated with C129 (p = 0.455; **Figure 3C**).

Effect of Heat Stress and ANEs on Photosynthetic Parameters of Tomato Plants

Statistical analysis showed that the interaction between condition and treatment was not significant for the PQ-SPAD parameter (p = 0.579; **Table 3**). This parameter, which measures leaf chlorophyll content, was not significantly affected either by moderate heat stress (p = 0.893) or the application of both ANE treatments (p = 0.681; **Figure 4A**).

When Φ_{II} was analyzed using a two-way ANOVA test, it was found that two parameters (condition and condition × ANE treatment) showed statistically significant differences ($p \leq 0.001$ and p = 0.012, respectively; **Table 3**). While this photosynthetic parameter was more affected by long moderate heat stress than PQ-SPAD, it was only reduced by 2.25% compared to untreated unstressed plants. Although all treatments in both the unstressed and heat stressed groups had quantum yield values of PSII of over 0.750 (**Figure 4B**).

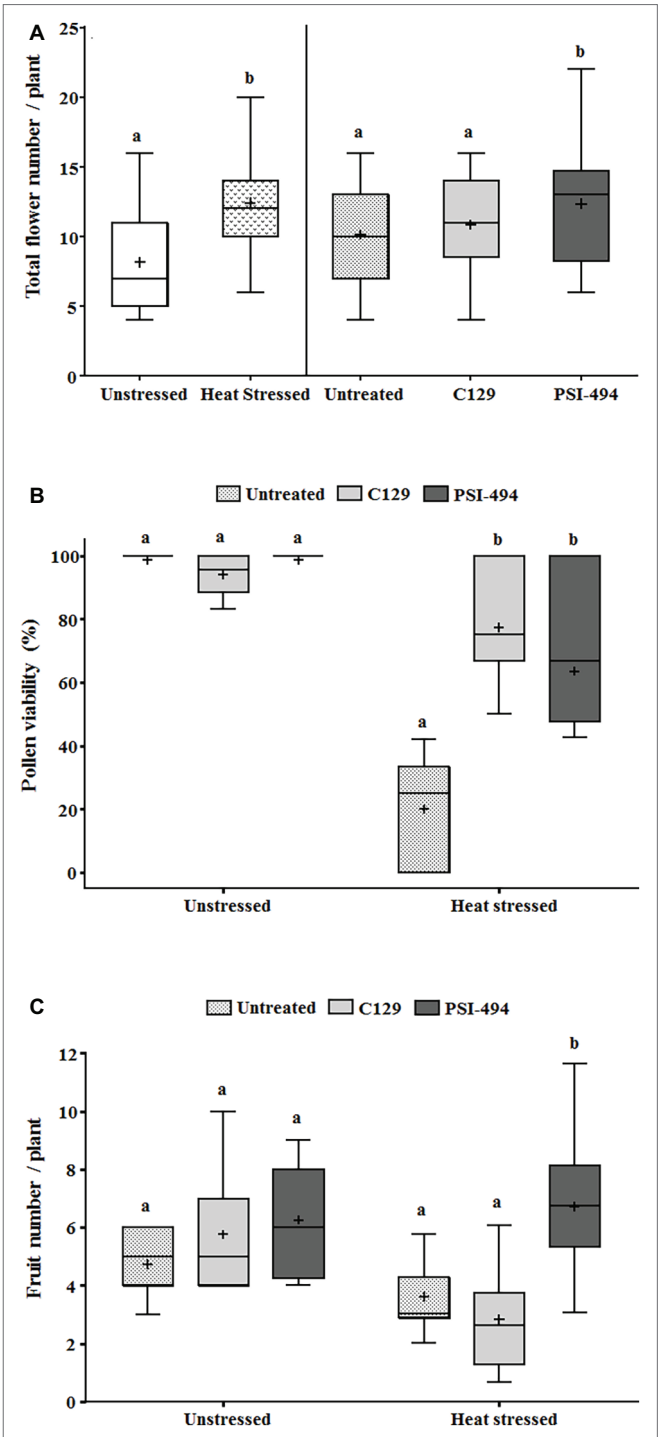


FIGURE 3 | Effect of heat stress and ANEs on reproductive parameters of tomato plants. **(A)** Total flower number; **(B)** pollen viability; and **(C)** fruit number. Total flower number and pollen viability were measured in 122-day-old plants and fruit number in 129-day-old plants. Data were subjected to two-way ANOVA and Tukey's HSD test for evaluating the differences among means at $p \leq 0.05$. Since there was no significant AxB interaction for total flower number, the effect of condition and ANE treatment was evaluated separately, comparing the respective means. Different letters indicate statistical differences for $p \leq 0.05$ based on *t*-test (condition) or one-way ANOVA Tukey's HSD test (ANE treatment). The vertical line is used to visually

(Continued)

FIGURE 3 | separate the evaluation of the effect of condition and ANE treatment. However, since interaction AxB was significant for pollen viability and fruit number, data were subjected to one-way ANOVA, comparing all ANE treatments with each other within the same growth condition (unstressed or heat stressed). In this case, different letters indicate statistical differences for $p \leq 0.05$ based on Tukey's HSD test. The horizontal line through the box and the cross represent the median and mean value, respectively. Number of biological replicates = 18.

The parameter Φ_{NPQ} was used to evaluate the effects of heat stress or ANE application on the efficiency of PSII in the energy dissipation in tomato chloroplast. A two-way ANOVA analysis found that both condition (heat stress) and condition × ANE treatment had a significant effect on Φ_{NPQ} ($p \leq 0.001$ and 0.039, respectively; **Table 3**). Under heat stress conditions, the small reduction of Φ_{II} was associated with an increase of Φ_{NPQ} by 21.69% compared to untreated unstressed conditions. However, when data of all treatments were analyzed using one-way ANOVA, similar values were observed in treated and untreated unstressed plants. Interestingly, this parameter was reduced by 9% in heat stressed plants sprayed with PSI-494 compared to control stressed conditions ($p = 0.034$; **Figure 4C**).

Effect of Heat Stress and ANEs on Soluble Sugar Content in Tomato Leaves and Flowers

The soluble sugar content in both leaf and floral tissues of 122-day-old plants was quantified by HPAEC-PAD. Our analysis revealed that the content of sucrose, glucose, and fructose in the leaf tissue of unstressed untreated plants was 1.06, 0.98, and 1.05 mg g^{-1} FW, respectively. **Figure 5A** shows that the content of these soluble sugars in leaf tissue decreases in untreated heat stressed plants compared to unstressed control plants. Interestingly, plants treated with PSI-494 showed a significantly lower decrease of sucrose content in foliar tissue relative to the unstressed healthy controls (−8%; $p = 0.047$) compared to that quantified in untreated stressed plants or plants treated with C129 (−38 and −18%, respectively). The foliar content of glucose and fructose was similar in untreated and ANE-treated heat stressed plants but was between 42 and 62% lower than that observed in leaf tissue of untreated unstressed plants (**Figure 5A**).

In the flower tissue of unstressed untreated tomato plants, fructose and glucose became the major soluble sugars (5.59 and 2.98 mg g^{-1} FW) and sucrose the minor sugar (1.47 mg g^{-1} FW). As it can be observed in **Figure 5B**, the content of sucrose, glucose, and fructose in untreated heat stressed flowers decreased by 22, 3, and 28%, respectively, with respect to that observed in the same tissue of unstressed control plants. However, heat stressed tomato plants treated with PSI-494 accumulated the highest content of soluble sugars in the flowers compared to the stressed control. The ANE biostimulant extracted at high temperatures was able to ameliorate the decrease of glucose and fructose (−1 and −17%, respectively). In addition, it induced a statistically significant accumulation of sucrose (11%; $p \leq 0.001$) with respect to those values measured in untreated unstressed plants. However, flowers of heat stressed

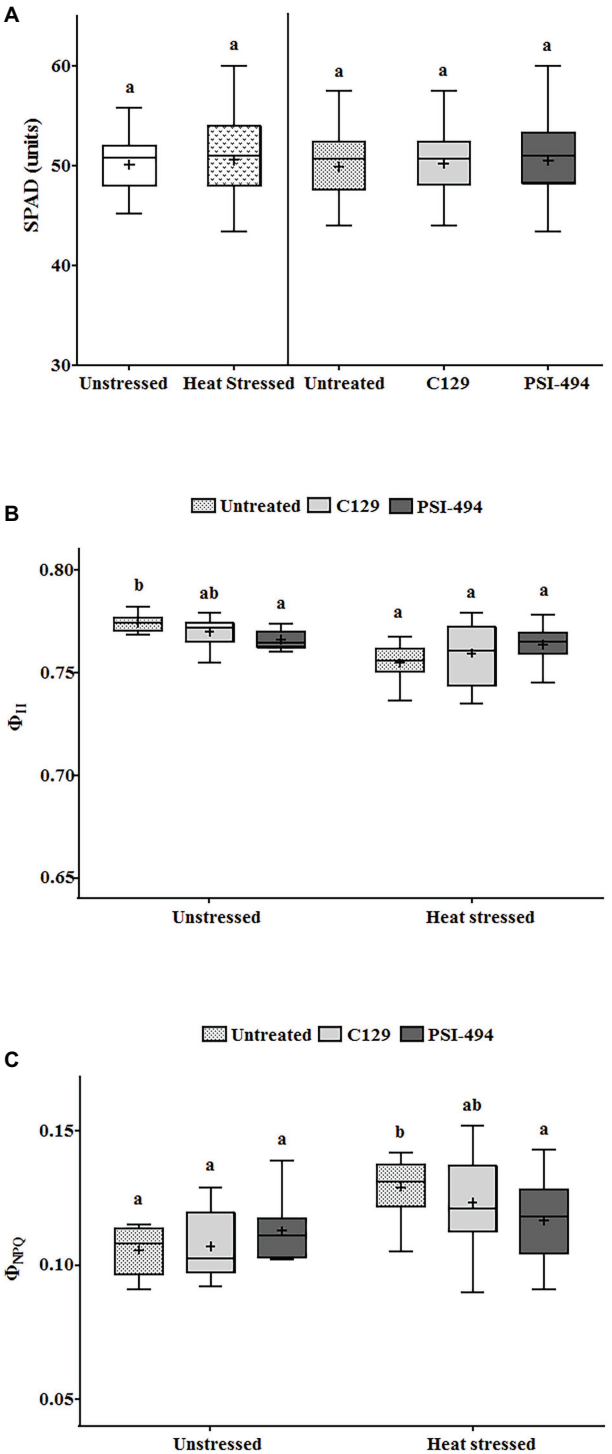


FIGURE 4 | Effect of heat stress and ANEs on photosynthetic parameters of tomato plants. **(A)** PQ-SPAD; **(B)** Φ_{II}; and **(C)** Φ_{NPQ}. Data were measured in 122-day-old plants and subjected to two-way ANOVA and Tukey's HSD test for evaluating the differences among means at $p \leq 0.05$. Since there was no significant AxB interaction for PQ-SPAD, the effect of condition and ANE treatment was evaluated separately, statistical differences for $p \leq 0.05$ based on *t*-test (condition) or one-way ANOVA

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FIGURE 4 | comparing the respective means. Different letters indicate Tukey's HSD test (ANE treatment). The vertical line was used to separate visually the evaluation of the effect of condition and ANE treatment. However, since interaction AxB was significant for Φ_{II} and Φ_{NPQ}, data were subjected to one-way ANOVA, comparing all ANE treatments with each other within the same growth condition (unstressed or heat stressed). In this case, different letters indicate statistical differences for $p \leq 0.05$ based on Tukey's HSD test. The horizontal line through the box and the cross represent the median and mean value, respectively. Number of biological replicates = 18.

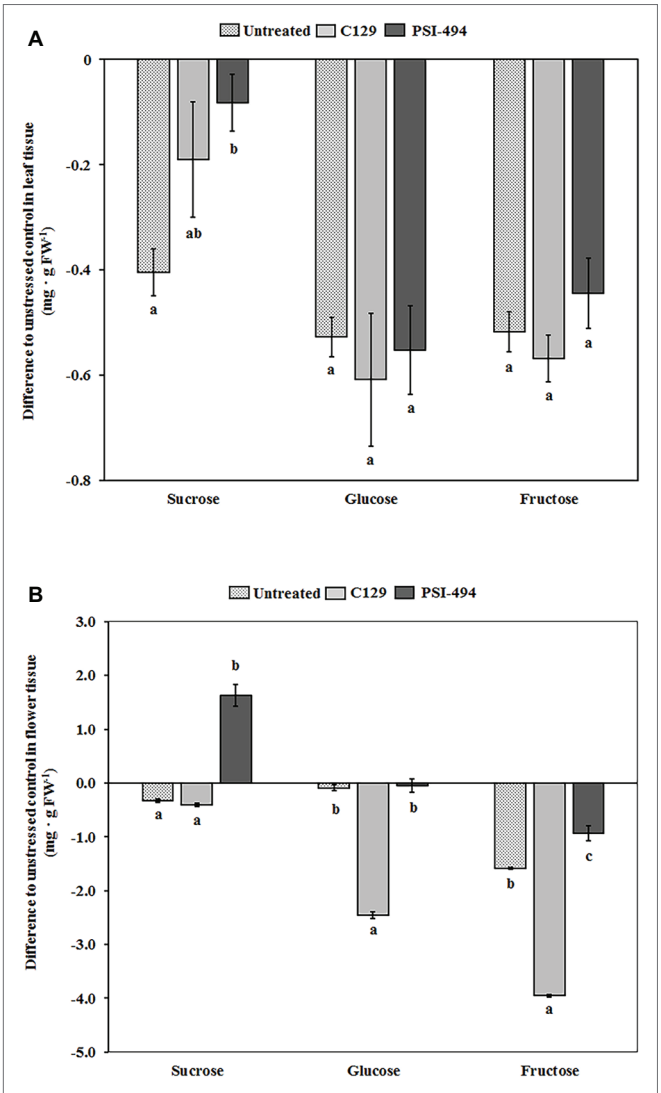


FIGURE 5 | Effect of heat stress and ANEs on endogenous soluble sugars of tomato plants. The levels of glucose, fructose, and sucrose were determined by HPAEC-PAD in **(A)** leaf tissue and **(B)** flower tissue of 122-day-old plants. Measured results were expressed as difference of the three heat stressed group samples (Untreated, C129, PSI-494) with respect to the unstressed control. The straight line at the "0" level represents the unstressed control and histograms represent the absolute variations of heat stressed plants. Different letters within the same soluble sugar indicate statistically significant differences between the treatments based on one-way ANOVA Tukey's HSD test at $p \leq 0.05$. Number of biological replicates = 4.

plants treated with C129 showed the lowest measured values, decreasing their content of endogenous sucrose, glucose, and fructose by 27, 82, and 71%, respectively, with respect to the unstressed control (**Figure 5B**).

Effect of Heat Stress and ANEs on Expression of *HSP* Genes in Tomato Flowers

In order to examine whether C129 and PSI-494 biostimulants affected the regulation of three stress-protective HSPs (*HSP101.1*, *HSP70.9*, and *HSP17.7C-Cl*) at transcriptional level in tomato flowers, relative changes in gene expression were analyzed by RT-qPCR in unstressed and heat stressed 122-day-old plants (**Figure 6**). When the unstressed group was examined, it was found that both ANE biostimulants decreased *HSP101.1* expression level between three and four times with respect to the control ($p \leq 0.001$; **Figure 6A**). However, no statistically significant differences were observed in the expression levels of *HSP70.9* and *HSP17.7C-Cl* between ANE-treated and -untreated unstressed plants (**Figures 6B,C**). Different effects were found when the expression of these three *HSPs* genes was examined in flower tissue grown under moderate heat stress. The application of the PSI-494 caused a significant upregulation within *HSP101* and *HSP70.9* expression levels by 2.05- and 1.68-fold with respect to the stressed control. *HSP17.7C-Cl* transcript level was 1.23 times higher in flowers of stressed plants treated with PSI-494 although this was not significant ($p = 0.160$). Conversely, the relative gene expression of the tested *HSPs* was similar or slightly downregulated in flowers of heat stressed plants treated with C129 vs. the control (**Figure 6**).

DISCUSSION

As part of the current global climate change, ambient temperatures are rising at a considerable rate and heat waves are becoming more frequent and severe. In many crop plants, including both monocots and dicots, elevated temperatures lead to reduced yield, which is alarming considering global food security (Change, 2014; Nadeem et al., 2018). Therefore, ensuring high yield under more unfavorable conditions is one of the greatest challenges of this century. Current knowledge shows that the plant heat stress response is highly complex, and heat tolerance should not be regarded as a single trait. Likewise, it has become clear that the focus on heat stress tolerance now has to be redirected from the vegetative to reproductive tissues due to their higher sensitivity to environmental fluctuations and their direct relationship with fruit production (Mesihovic et al., 2016; Rieu et al., 2017). Interestingly, although the utilization of plant biostimulants has proved popular for their ability to enhance abiotic stress tolerance (Van Oosten et al., 2017; Shukla et al., 2019), research literature describing the utilization of these crop inputs to provide heat stress tolerance is scarce. The available literature is mostly focused on plant species at vegetative stage (Kauffman et al., 2007; Zhang and Ervin, 2008; Botta, 2012). Therefore, it is important to expand the current

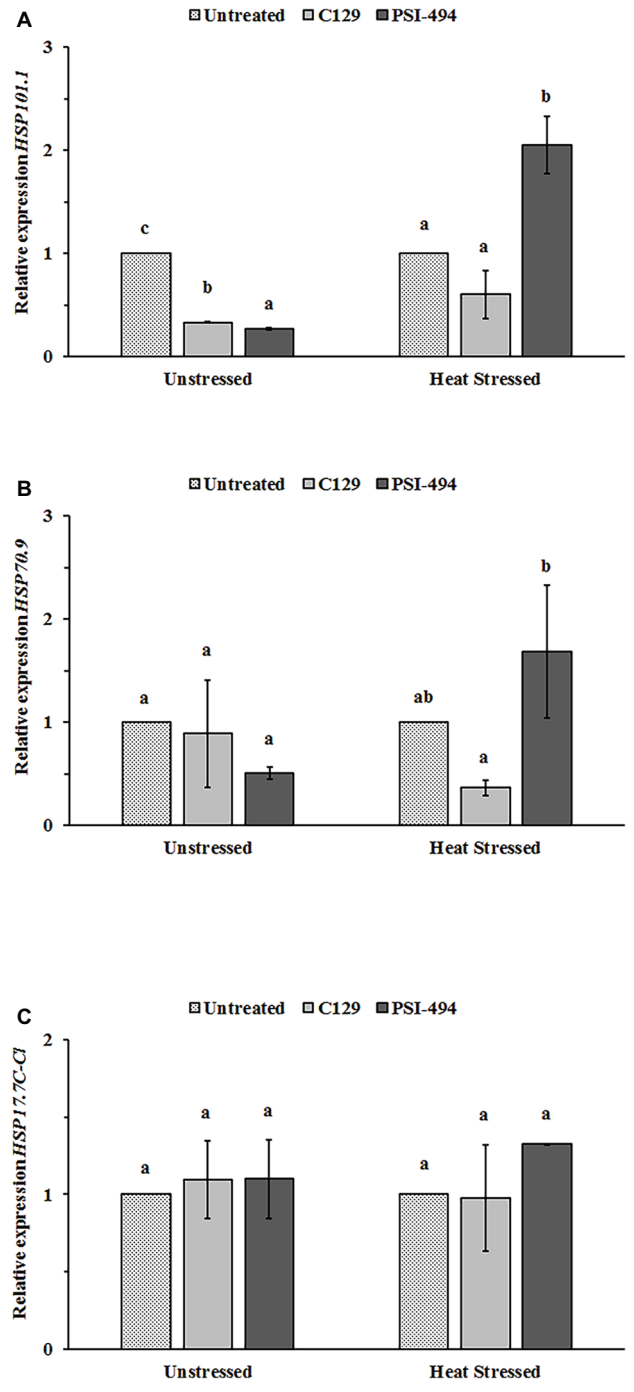


FIGURE 6 | Effect of heat stress and ANEs on *HSPs* gene expression in tomato flowers. **(A)** *HSP101.1*; **(B)** *HSP70.9*; and **(C)** *HSP17.7C-CI*. Data were measured in 122-day-old plants and expressed as the relative fold-change with respect to the *ACTIN2* (*ACT2*) gene expression levels. Different letters within the same growth condition indicate statistically significant differences between the treatments based on one-way ANOVA Tukey's HSD test at $p \leq 0.05$. Number of biological replicates = 3.

knowledge to other relevant crops during the reproductive phase to build credibility and acceptance in agricultural practice.

Processing Parameters of ANE Biostimulants Influence Thermotolerance in Tomato Plants

In this study, we found significant differences in the ability of two ANE biostimulants derived from the same carbohydrate rich fraction to induce tomato plant tolerance to moderate heat stress during the reproductive stage of the growth cycle. Two foliar applications of PSI-494, extracted under high temperatures and alkaline conditions, significantly enhanced the number of flowers, pollen viability, and fruit set compared to untreated control. However, the observed improvement in pollen viability in heat stressed plants treated with an ANE extracted at low temperatures (C129) did not translate to subsequent higher fruit set. Although the positive effects of seaweed biostimulants were initially correlated with phytohormone-like activity or the presence of compounds such as betaines (Craigie, 2011), growing evidence highlights seaweed carbohydrates as essential components in eliciting plant biostimulant activity (Goñi et al., 2020). According to the obtained chemical compositional data, more than 97% of C129 and PSI-494 corresponded to similar amounts of mineral content and carbohydrates. Therefore, as observed in a previous study on drought stress tolerance (Goñi et al., 2018), the results of the ANE biostimulant compositional analysis and heat stressed plant phenotype data were not correlated. However, processing conditions did have a significant role on one key structural parameter of carbohydrates related to M_w distribution. It was evident from the HPSEC-RID analysis that the proprietary extraction method used to generate PSI-494 was more successful in reducing the average M_w of carbohydrates extracted from the *A. nodosum* biomass. The most significant differences between PSI-494 and C129 were a smaller main carbohydrate peak and the higher relative abundance of secondary peaks. Previous studies have confirmed that low M_w polysaccharides and oligosaccharides from seaweeds were able to stimulate efficiently abiotic stress tolerance in several crop species (Liu et al., 2013; Wu et al., 2016; Singh et al., 2017; Li et al., 2018; Salachna et al., 2018). Our results would also suggest that there is a link between the lower molecular size of carbohydrates inside ANEs and enhanced heat stress tolerance in tomato plants at reproductive stage.

Impact of ANEs on Phenotypic and Physiological Markers of Heat Stress Tolerance

This research was focused on challenging flowering tomato plants with temperatures several degrees above their optimal conditions for anthesis and fruit development for multiple days (22–26°C; Luo, 2011). As opposed to heat shocks applied for short time periods (e.g., a few hours), this experimental design was considered to be more representative of naturally occurring stress conditions in the field. As moderate heat stress regimes might significantly affect the function of vegetative tissues and impair further reproductive cell functions, we also evaluated plant height and photosynthetic activity. However, the data presented demonstrate that moderate heat stress had little effect on overall tomato stem growth. In line with this, Zhou et al. (2017) found that mild heat stress conditions (36/28°C day/night) applied in two tomato

cultivars at anthesis stage for 7 days did not have significant effects on plant growth compared to unstressed conditions. Likewise, neither C129 nor PSI-494 had any statistically significant effect on plant height, suggesting that this vegetative trait may not be sensitive enough to describe the effects of ANE biostimulants.

Reduced fertility is a common problem associated with heat and has been found to be caused by high temperatures around meiosis (8–9 days before anthesis) and fertilization (2–3 days after anthesis) in various species (Mesihovic et al., 2016; Rieu et al., 2017). Therefore, the heat stress regime for 14 days was designed to study its effect not only on pollen development but also on the progamic phase and implications in respect to some heat sensitive reproductive traits in tomato. The results confirmed the harmful effects of moderate heat stress on pollen viability in tomato plants, which is in agreement with other studies (Sato et al., 2000; Pressman et al., 2002; Paupière et al., 2017; Xu et al., 2017). The application of ANE treatments did not have a substantial effect on plants grown under unstressed conditions (27/22°C day/night temperatures); however, there was a significant difference in the pollen viability between treated and untreated plants when heat stress was applied (31/24°C day/night temperatures). Plants treated with C129 and PSI-494 had higher pollen viability percentages than the untreated group after heat stress exposure, without significant differences in the efficacy of both ANE biostimulants. Although previous studies have highlighted the positive impact of seaweed extracts on different pollination parameters of high value crops such as grape or eggplant (Sabir, 2015; Pohl et al., 2019), no research to date has demonstrated the protective effect of ANEs on pollen under heat stress conditions. Furthermore, it has been previously described in different plant species that different stress types can stimulate precocious flowering and further seed production as an emergency response to highly unfavorable environmental conditions (Takeno, 2016). This stimulatory response would explain the increase in flowers in the heat stressed plants. Although promoted flowering has previously attributed to other commercial ANE biostimulants (Abubakar et al., 2012; Pohl et al., 2019), there was only a significant increase in flower number in tomato plants treated with PSI-494. These results highlight that despite the ANE biostimulants being manufactured from the same raw material their processing conditions can affect their ability to provide phenotypic and physiological benefits.

Fruit set interacts with other well-known heat sensitive traits determined before fertilization happened. For example, no positive correlations between either pollen viability or fruit set was found in ANE-treated plants under unstressed conditions, suggesting that male fertility was not a limiting factor for reproduction under optimal temperature growth conditions. Similar to the results of Xu et al. (2017) with several tomato cultivars, we also observed a clear positive correlation between fruit set and pollen viability in untreated plants under moderate heat stress. However, pollen viability values were not able to explain the measured differences in fruit set of ANE-treated plants subjected to long-term mild heat stress. Tomato plants treated with C129 decreased fruit number 1 week after heat stress with respect to control, PSI-494 stimulated a significant increase of this yield-related parameter. The fruit set value could be the result of the synergistic interaction between enhanced pollen viability and higher flower number observed after spraying PSI-494. The large differences observed in tomato plants treated with this ANE biostimulant suggest that other specific biochemical and molecular markers associated with enhanced thermotolerance could be also involved.

While heat stress can induce significant changes in photosynthetic apparatus of the plant (Mathur et al., 2014), chlorophyll spectrophotometric and fluorometric measurements only showed small changes after 14 days of moderate heat stress. Chlorophyll is usually the first port of call when analyzing plant health for both researchers and farmers as the "stay-green" trait often equates to healthiness visually, while leaf yellowing is associated to unhealthy. Unlike previous reports in a heat-sensitive tomato cultivar (Zhou et al., 2017), chlorophyll levels determined through PQ-SPAD parameter revealed maintenance of a high value in all plant groups regardless of the growth condition or ANE treatment applied. The fraction of light energy captured by PSII (Φ_{II}) is an effective parameter that provides information on the nature of photoinhibition under abiotic stress. Indeed, a decline in Φ_{II} would be due to the inactivation of PSII reaction centers aimed at photoprotection (Mathur et al., 2014; Kuhlgert et al., 2016). Heat stress and ANE applications had some statistically significant effects on Φ_{II}, indicating a minor modulation of PSII function. However, as observed before in other studies in tomato under moderate heat stress (Sato et al., 2000; Zhou et al., 2017), these differences in photosynthetic efficiency were not probably large enough to be the main factor related to the observed fruit set values. A decrease in Φ_{II} in untreated heat stressed plants was accompanied by a stimulation of Φ_{NPQ}, a sensitive parameter used for monitoring thermal dissipation of excess light energy absorbed by PSII (Tiezt et al., 2017). Conversely, stressed plants treated with PSI-494 maintained similar Φ_{NPQ} levels to those recorded in unstressed plants. Consequently, these results suggest that this ANE biostimulant treatment has the potential to increase the energy available for photochemistry, which is a desirable physiological trait to improve crop yields under chronic mild stressful conditions (Malnoë, 2018).

Impact of ANEs on Biochemical and Genetic Markers of Heat Stress Tolerance

It is important to highlight that it takes both pollination and fertilization to create a robust fruit set. If there is not an adequate amount of viable pollen, the male grain will not reach the stigma. However, timely pollination does not guarantee fruit set, as post-pollination processes such as pollen tube growth or fertilization are also heat sensitive (Peet et al., 1997; Erickson and Markhart, 2002). Therefore, we also evaluated whether the altered plant carbohydrate content observed in heat stressed plants was able to explain the stronger effect on fruit set provided by PSI-494. Sucrose is the primary end product of photosynthesis, which is translocated from source leaves to sink organs through phloem. Once it has reached those sinks, sucrose must be degraded into glucose and fructose (or their derivates) by sucrose synthase and invertase enzymes for various metabolic and biosynthetic processes (Ruan et al., 2010). Although untreated and ANE-treated plants had significantly lower sucrose content in leaf tissue after heat stress,

this decrease was significantly mitigated with the application of PSI-494. In agreement with the results observed in a heat tolerant tomato line (Li et al., 2012), it is likely that more sucrose would be available for partitioning to reproductive organs in stressed plants treated with PSI-494. Flowers and young fruits have a high energy demand throughout their development and rely heavily on the source-to-sink flow for the supply of carbon resources (Borghi and Fernie, 2017; Shen et al., 2019). As previously reported in different tomato heat sensitive cultivars, our data specifically suggest that carbohydrate metabolism in flower tissue was disturbed under long-term moderate heat stress (Pressman et al., 2002, 2006; Firon et al., 2006; Sato et al., 2006). While soluble sugar levels were reduced in untreated stressed plants, this was more obvious for those plants treated with the ANE biostimulant extracted at low temperatures (C129). However, an improved ability to maintain glucose and fructose content and even increase sucrose in flowers of plants treated with PSI-494 was also observed. These differences in the accumulation pattern of soluble sugars may be an important factor in explaining the results observed in the fruit set for the heat stressed tomato plants. A relationship between an appropriate carbohydrate metabolism in flowers and young fruits and increased fruit set has been exhaustively described in the bibliography for heat tolerant tomato varieties (Pressman et al., 2002; Firon et al., 2006; Li et al., 2012), which supports the potential of specialized ANEs to strengthen inherent thermotolerance mechanisms.

Monitoring the expression levels of *HSP* genes can give important information on the capacity of reproductive organs to activate protective mechanisms required for thermotolerance. By re-establishing protein homeostasis, the induction of such chaperones can not only have a temporary survival effect but can allow increased efficiency of the fertilization process (Fragkostefanakis et al., 2015; Reddy et al., 2016). This research analyzed the expression levels of three particular *HSP* genes in flower tissues: *HSP101.1*, *HSP70.9*, and *HSP17.7C-Cl*. In this regard, the recent work of Fragkostefanakis et al. (2016), showed the important role of these three HSPs in the mechanism of thermotolerance of tomato pollen. When all three *HSP* gene expression levels were examined for the heat stressed plants each ANE biostimulant had a varied effect. Tomato plants treated with PSI-494 caused a statistically significant upregulation within *HSP101.1* and *HSP70.9*, while C129 induced a downregulation of both genes in the flowers of heat stressed plants. These concurrent changes were interesting as it has been described how HSP100 isoforms are essential components of plant thermotolerance implicated in protein disaggregation, an activity that is complemented for refolding by cooperation with HSP70 isoforms (Seyffer et al., 2012; Fragkostefanakis et al., 2015; Mogk et al., 2015). Although the increase of *HSP17.7C-Cl* gene expression in flowers of stressed plants treated with PSI-494 was only statistically significant at 80% confidence interval, it is interesting to emphasize the potential biological significance of these results. As mentioned for HSP70, a collaborative mechanism between HSP101 and sHPSs for reverting irreversible aggregation of heat-sensitive proteins has also been reported (McLoughlin et al., 2016). Moreover, the overexpression of sHSP in tomato anthers and young fruits has been proposed as a significant contributor factor to heat tolerance (Giorno et al., 2010; Li et al., 2012). Therefore, the differential effect of ANEs on the expression levels of some relevant *HSP* genes in flower tissues may support a potential mode of action in the induction of thermotolerance.

Summary and Perspectives

Modern day agriculture is becoming more unpredictable due to climate change and the subsequent increase in abiotic stresses such as heat. ANE biostimulants can be a viable solution in creating more sustainable and environmentally acceptable agricultural practices. One of the current challenges is in acquiring acceptance among the agricultural community. This can only be achieved through communicating extensive research into defined mode of action and demonstrating the robustness of these crop inputs. Overall, our data indicate that treatment with one specialized ANE (PSI-494) could represent a potential tool for farmers to alleviate the damage of long periods of moderate heat stress at the reproductive stage leading to enhanced fruit set. Physicochemical characteristics of ANE biostimulants are derived from their processing conditions and appear to be related to their performance in enhancing fruit set during heat stress. This has been demonstrated by an increased flower number, improved pollen viability, enhanced carbohydrate metabolism, and *HSPs* gene expression in reproductive organs before fertilization.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

OG and SO'C secured funding and supervised the work. NC, OG, and SO'C conceived and designed the experiments. NC, OG, and ŁŁ performed the experiments. NC, OG, ŁŁ, and SO'C analyzed the data and wrote the article. All authors contributed to the article and approved the submitted version.

FUNDING

This research has been conducted with the assistance of funding from the M.Sc. by Research Programme from Institute of Technology Tralee. This funder provided support in the form of scholarship for the author NC.

ACKNOWLEDGMENTS

The authors would like to acknowledge the help of Thomas Higgins for his technical support with the characterization analysis of ANEs. In addition, we thank Brandon Bioscience for the gift of the ANE biostimulants (C129 and PSI-494) used in this study.

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Conflict of Interest: While Brandon Bioscience provided support in the form of salary for the author ŁŁ, this company did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fpls.2020.00976

Combining Molecular Weight Fractionation and Metabolomics to Elucidate the Bioactivity of Vegetal Protein Hydrolysates in Tomato Plants

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OPEN ACCESS

Edited by:
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Daniel Basilio Zandonadi, Federal University of Rio de Janeiro, Brazil

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *15 January 2020*
Accepted: *16 June 2020*
Published: *30 June 2020*

Citation:
Lucini L, Miras-Moreno B, Rouphael Y, Cardarelli M and Colla G (2020) Combining Molecular Weight Fractionation and Metabolomics to Elucidate the Bioactivity of Vegetal Protein Hydrolysates in Tomato Plants. Front. Plant Sci. 11:976. doi: 10.3389/fpls.2020.00976

The comprehension of the bioactive fractions involved in the biostimulant activity of plant derived protein hydrolysates (PH) is a complex task, but it can also lead to significant improvements in the production of more effective plant biostimulants. The aim of this work is to shed light onto the bioactivity of different PH dialysis fractions (PH1 < 0.5–1 kDa; PH2 > 0.5–1 kDa; PH3 < 8–10 kDa; PH4 > 8–10 kDa) of a commercial PH-based biostimulant through a combined *in vivo* bioassay and metabolomics approach. A first tomato rooting bioassay investigated the auxin-like activity of PH and its fractions, each of them at three nitrogen levels (3, 30, and 300 mg L^{-1} of N) in comparison with a negative control (water) and a positive control (indole-3-butyric acid, IBA). Thereafter, a second experiment was carried out where metabolomics was applied to elucidate the biochemical changes imposed by the PH and its best performing fraction (both at 300 mg L^{-1} of N) in comparison to water and IBA. Overall, both the PH and its fractions increased the root length of tomato cuttings, compared to negative control. Moreover, the highest root length was obtained in the treatment PH1 following foliar application. Metabolomics allowed highlighting a response to PH1 that involved changes at phytohormones and secondary metabolite level. Notably, such metabolic reprogramming supported the effect on rooting of tomato cuttings, being shared with the response induced by the positive control IBA. Taken together, the outcome of *in vivo* assays and metabolomics indicate an auxin-like activity of the selected PH1 fraction.

Keywords: dialysis fractionation, rooting assay, auxin-like activity, plant secondary metabolism, *Solanum lycopersicum* L.

INTRODUCTION

Nowadays, the improvement of sustainable agronomic practices to reduce the input of chemical inputs and to improve environmental aspects and quality of agricultural productions is becoming mandatory (Searchinger et al., 2013; De Pascale et al., 2017). With this regard, several technological innovations have been proposed, including the use of bio-based products such as plant biostimulants (Rouphael and Colla, 2018; Rouphael et al., 2018b; Xu and Geelen, 2018). Plant biostimulants include substances and/or microorganisms that are able to enhance plant growth, tolerance to abiotic stress, water, and nutrients use efficiency, rather than promote nutritional and functional quality of the products (Du Jardin, 2015; Rouphael et al., 2018b). Different classes of non-microbial and microbial products have been proposed among biostimulants, such as beneficial microorganisms (e.g. mycorrhiza or plant growth promoting rhizobacteria), humic substances, seaweed extracts, and protein hydrolysates (PH) (Calvo et al., 2014; Battacharyya et al., 2015; Canellas et al., 2015; Colla et al., 2015; Haplern et al., 2015; Rouphael et al., 2015; Ruzzi and Aroca, 2015; Colla et al., 2017a; Bitterlich et al., 2018). Among organic non-microbial plant biostimulants, humic acids and PH command half of the market share (Rouphael and Colla, 2018). These latter are a mixture of peptides and free amino acids resulting from the chemical or enzymatic partial hydrolysis of protein sources from either animal or vegetal origin (Colla et al., 2015; Colla et al., 2017b). The applications of PH-based biostimulants have been reported to enhance nutrient use efficiency and tolerance to abiotic stressors such as drought, extreme temperatures, and salinity (Calvo et al., 2014; Haplern et al., 2015; Lucini et al., 2015; Colla et al., 2017a; Colla et al., 2017b; Rouphael et al., 2017a; Rouphael et al., 2017b; Carillo et al., 2019a; Carillo et al., 2019b). In a recent review Colla and co-workers were able to summarize the main physiological and molecular mechanisms behind the biostimulant action of PH (Colla et al., 2017a). Direct and indirect mechanisms include: i) stimulation the C and N metabolism by triggering key enzymes, ii) increasing the antioxidant defense systems, iii) triggering hormone-like activities, and modulating the root system apparatus thus increasing nutrient uptake/assimilation and consequently boosting crop productivity.

Considering that several molecular mechanisms and biochemical processes have been related to PH activity to crops, it is clear that the biostimulant action is far beyond a mere supply of amino acids as nitrogen source. Besides representing an available source of nitrogen and carbon skeletons, the peptides in PH are supposed to exert a direct regulatory activity toward plants growth, known as hormone-like activity (Colla et al., 2014; Oh et al., 2018; Tejada et al., 2018). Signaling peptides are mainly short-amino acid chains (2–50 amino acids), having specific amino acid primary sequences and inducing biological effects at very low concentration (nM). Matsumiya and Kubo (2011) identified in PH obtained through enzymatic hydrolysis of soybean meal a signaling peptide having a 12 amino acid sequence; this peptide, the so-called "root hair promoting peptide", seems to stimulate key gene(s) that increase root number and length of root hair. Moreover, some amino acids can also exert a signaling role. As an example, L-glutamate was shown to inhibit primary root growth and increase root branching near the root apex when roots were exposed to low concentrations of the amino acid (Forde and Lea, 2007). Noteworthy, the information on the substances being actually responsible of the biostimulant activity of PH is still limited, and a synergic role of different components has been recently postulated (Paul et al., 2019a; Paul et al., 2019b). Nonetheless, different contributions of PH fractions can be postulated, with small molecules (including amino acids), oligopeptides, and polypeptides likely representing the fractions having a potential biostimulant activity.

The comprehension of the bioactive substances involved in the biostimulant activity is a complex task, but it can also lead to significant improvements in the field of plant biostimulants. Indeed, the comprehension of the components to which biological activity is related can assist the choice of the best sources for PH, the improvement of hydrolysis processes and PH manufacture in general and can support the definition of the best agronomic strategies. Taken together, these improvements might open the field toward new generation biostimulants (2.0). Given the complex composition of products from natural origin such as biostimulants, the understanding of the most active components can be achieved following fractionation through molecular weight cut-off. The results can assist companies to optimize the production process in order to maximize the amount of the most active fraction(s).

Taking into account that a PH is composed by several components differing for chemical structure and molecular weight, and considering that no information is available on the actual contribution of each specific fraction, the aim of this work is to shed light onto the biostimulant activity of the different components of Trainer®, a representative commercially available PH. With this purpose, molecular fractionation, *in vivo* bioassays and plant metabolomics were combined to investigate the contribution of low-molecular-weight components such as amino acids and peptides on the activity and mode(s) of action of a PH. Because the whole PH product and its fractions were tested up to doses corresponding to 300 mg of N L^{-1}, we cannot exclude that such high doses of PH and its fractions may act not only as biostimulants but also as sources of nitrogen. Besides this specific case, the approach proposed might find application in all cases where a biostimulant product is composed by a mixture of small molecules and high molecular weight macro-biomolecules.

MATERIALS AND METHODS

The PH and Its Fractionation

The legume-derived PH biostimulant Trainer® was a commercial product manufactured by Italpollina (Rivoli Veronese, Italy), and purchased from a commercial retailer. This PH is obtained by hydrolysis of proteins derived from legume seed flour that underwent enzymatic hydrolysis followed by separation of insoluble residual compounds by centrifugation and concentration. The product pH was 4.4. The product electrical

conductivity (EC) increases linearly with increasing PH concentration (C) in the water with the following relationship between EC (dS m^{-1}) and C (ml L^{-1}) in pure water:

$$EC = 0.1983\ C\ (R^2 = 0.996)$$

PH biostimulant Trainer® contains 5% of N (w/w) as free amino acids, and soluble peptides. The aminogram of the product was (%): alanine—Ala (1.2), arginine—Arg (1.8), aspartic acid—Asp (3.4), cysteine—Cys (0.3), glutamic acid—Glu (5.4), glycine—Gly (1.2), histidine—His (0.8), isoleucine—Ile (1.3), leucine—Leu (2.2), lysine—Lys (1.8), methionine—Met (0.4), phenylalanine—Phe (1.5), proline—Pro (1.5), threonine—Thr (1.1), tryptophan—Trp (0.3), tyrosine—Tyr (1.1), valine—Val (1.4) (Paul et al., 2019b). The macronutrient composition of Trainer® is as follows (%): P (0.09), K (0.41), Ca (0.07), and Mg (0.1). Trainer® also contains the following micronutrients (mg kg^{-1}): Fe (30.0), Mn (1.0), B (1.0), Zn (9.6), and Cu (9.0).

Ultrafiltration was carried out using a molecular weight cut-off (MWCO) cellulose acetate membrane (cellulose acetate, VWR, Milan, Italy) of 0.5–1 and 8–10 kDa, following manufacturer recommendations. The two MWCO were chosen to target the fractionation between small molecules and oligopeptides (around 9 amino acid residues—MWCO 0.5–1 kDa), and polypeptides (up to 90 amino acid residues—MWCO 8–10 kDa), respectively. Water was used for partition, and the product was allowed to diffuse for 24 h. At the end of partition, total N was measured in each fraction for both MWCO, through the Dumas' method using an elemental analyzer (Elemental vario MAX CN, Langenselbold, Germany). The nitrogen analysis showed low concentrations of N in all the PH-fractions in comparison to the whole product, due to the dilution that took place in the fractionation process. The total N content of each fraction was as follow (% w/w): 0.105 for PH1 (< 0.5–1 kDa); 0.861 for PH2 (> 0.5–1 kDa); 0.128 for PH3 (< 8–10 kDa); 0.384 for PH4 (> 8–10 kDa).

In Vivo Bioassays

Tomato rooting test bioassay was carried out to identify the auxin-like activity by estimating the ability of the whole product and its fractions to promote initiation of adventitious roots in tomato cuttings (Matsumiya and Kubo, 2011; Colla et al., 2014). Tomato (*Solanum lycopersicum* L. cv. Marmande, SAIS Sementi, Cesena, Italy) seeds were surface sterilized using commercial bleach with sodium hypochlorite at 2% for 20 min. After being raised with sterilized water, the tomato seeds were sown in a germination tray filled with vermiculite. The growth chamber had a 12 h photoperiod with a light intensity of 450 μmol m^{-2} s, air temperature of 24°C, and 65% relative humidity. After 25 d from sowing, 3-true leaf tomato cuttings were harvested. In the experiment 1, cuttings were immersed for 5 min in a solution (basal application) containing three rates of either the PH Trainer® or its fractions whereas distilled water and indole-3-butyric acid (IBA) were used as negative and positive control, respectively. Since the amino acids and peptides are organic nitrogenous compounds, a normalization of application rates for PH and its fractions was carried out in order to apply the same level of nitrogen for each dose level in experiment 1 (3, 30, or 300 mg L^{-1} of N). Product rates at each nitrogen level changed as follow (g L^{-1} for 3, 30, or 300 mg L^{-1} of N, respectively): PH1 (2.86, 28.57, 285.71), PH2 (0.35, 3.48, 34.84), PH3 (2.34, 23.44, 234.38), PH4 (0.78, 7.81, 78.12), and PH (0.06, 0.60, 6.00). IBA was applied at three rates as follow: 0.006, 0.06, and 0.6 g L^{-1}. In experiment 2, the following treatments were tested: water-treated control, foliar application of a water solution containing PH at 6 g L^{-1}, basal application of a water solution containing PH1 at 285.71 g L^{-1}, foliar application of a water solution containing PH1 at 285.71 g L^{-1}, and basal application of a solution containing IBA at 0.06 g L^{-1}. PH1 was selected as the most active fraction based on Experiment 1. In all treatments, the applied rate of PH or its fraction (PH1) was established in order to assure the same level of N (300 mg L^{-1} of N). Foliar application of PH or its fraction (PH1) was made by a quick dip of aerial part of cuttings into the solution. The cuttings of both experiments were planted in transparent plexiglas boxes containing 8 cm of wetted perlite. The boxes were closed to ensure a relative humidity close to saturation (100%). Treatments were arranged in a randomized complete block design with three replications. Each experimental unit consist of a box containing 30 cuttings. After 7 d from planting, the roots of cuttings were gently washed with distilled water, until the root systems were free from any perlite particles. The measurement of the total root length was made on 18 cuttings per treatment using a WinRHIZO Pro (Regent Instruments Inc., Canada), connected to a STD4800 scanner. In the second experiment additional 18 cuttings per treatment (6 cuttings per experimental unit) were sampled for metabolomics analysis. With this latter aim, cuttings were removed and gently washed with distilled water, the basal part was sampled (2.5 cm of basal part of cutting stems including roots) and immediately quenched by dipping in liquid nitrogen, then stored at −80°C until analyses.

Metabolomics

Samples of tomato cuttings gained from the *in vitro* assays were grinded with liquid nitrogen using pestle and mortar, and then extracted as previously reported (Rouphael et al., 2018a). Briefly, an aliquot (1.0 g) was extracted in 10 ml of 0.1% HCOOH in 80% aqueous methanol using an Ultra-Turrax (Ika T-25, Staufen, Germany). The extracts were centrifuged (12,000 × *g*) and metabolomic analysis was then carried out by UHPLC liquid chromatography quadrupole-time-of-flight mass spectrometry (UHPLC/QTOF-MS) as previously reported (Pretali et al., 2016). With this aim, a 1290 ultra-high-performance liquid chromatograph, a JetStream electrospray ionization source and a G6550 QTOF (all from Agilent technologies, Santa Clara, CA, USA) were used. Reverse phase chromatography was carried out on an Agilent Zorbax Eclipse-plus C18 column (100 × 2.1 mm, 1.8 μm) using a linear binary gradient elution (5%–95% methanol in water in 34 min, flow rate 220 μl/min). The mass spectrometer was operated in SCAN mode (100–1000 m/z) and positive polarity.

Mass (5 ppm difference in accuracy) and retention time (0.05 min as maximum shift allowed) alignment, as well as a filter-by-frequency post-processing were done in post-acquisition using

Agilent Profinder B.06 software. For filtering purposes, only the compounds annotated in at least 75% of replications within at least one treatment were retained. The combination of monoisotopic mass, isotopes ratio and spacing was used to annotate compounds. The reference database was PlantCyc 12.6 (Plant Metabolic Network, http://www.plantcyc.org; downloaded April 2018). According to COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi), our identification corresponded to Level 2 (putatively annotated compounds).

Statistical Analysis

In both bioassay experiments 1 and 2, ANOVA tests were conducted using the software package SPSS 10 for Windows (SAS Inc., Cary, NC). Duncan's multiple range test was performed at $p = 0.05$ on each of the significant variables measured.

Chemometric interpretation of the metabolomics dataset was carried out using Mass Profiler Professional B.12.06, as previously described (Salehi et al., 2018). Compounds abundance was Log2 transformed, normalized at the 75th percentile, and baseline against the median. Unsupervised hierarchical cluster analysis (HCA) was done to describe relatedness/distance of metabolomic signatures across treatments. With this aim, the heatmap based on fold-change values was used, similarity was set as "Euclidean" and the "Wards" linkage rule was chosen. The dataset was then loaded into SIMCA 13 (Umetrics, Malmo, Sweden), Pareto-scaled and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) supervised analysis was carried out. Outliers were preliminary investigated using Hotelling's T2 (95% and 99% confidence limits for suspect and strong outliers, respectively). CV-ANOVA ($p < 0.01$) and permutation testing (N = 300) were also applied to validate the model and to exclude overfitting. Goodness-of-fit R^2Y and goodness-of-prediction Q^2Y were calculated for the OPLS-DA model and finally, Variable Importance in Projection (VIP) analysis was used to select the most discriminant metabolites. The metabolites included in the dataset were subjected to fold-change analysis and ANOVA in a Volcano analysis, to describe the extent and direction of regulation following biostimulant treatments. Metabolites derived from Volcano analysis with their fold-change values were finally imported into PlantCyc pathway Tools software (Karp et al., 2010) to highlight the pathways and processes involved in plant response to treatments.

RESULTS

Biostimulant Action of PH and Its Fractions on Tomato Rooting

Overall, both the PH and its fractions increased the root length, compared to control (experiment 1; **Figure 1**). The highest root length (342.8 mm/plant) was obtained in the treatment PH1, corresponding to the small fraction (MWCO < 0.5–1 kDa) at dose 3, whereas the lowest value was observed in control treatment (170.9 mm/plant; **Figure 1**). In the experiment 2, PH1 and IBA were the most efficient treatments in promoting root growth in comparison with negative control (+83 and 64%

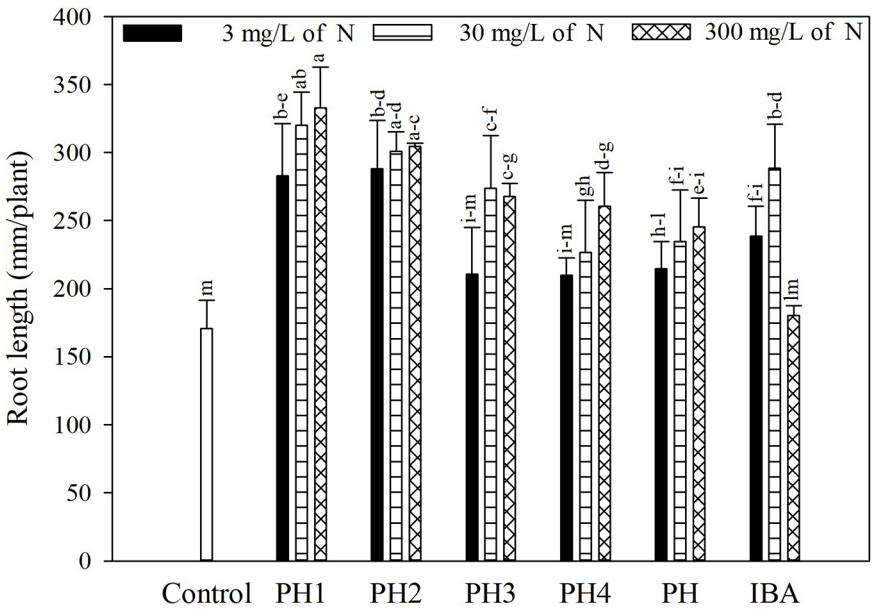


FIGURE 1 | Root length of tomato plants as affected by basal treatment of cuttings with solutions containing the protein hydrolysate Trainer® (PH) or one of its fractions (PH1 = fraction with molecular weight below 0.5–1 kDa; PH2 = fraction with molecular weight above 0.5–1 kDa; PH3 = fraction with molecular weight below 8–10 kDa; PH4 = fraction with molecular weight above 8–10 kDa), or indole-3-butyric acid (IBA) in the experiment 1. All products were applied at three doses. Different letters over bars indicate significant differences between treatments according to Duncan's multiple range test ($p = 0.05$).

for PH1 foliar and basal application, respectively; + 59% for IBA treatment). PH-treated cuttings showed intermediate values of root length (**Figure 2**).

Untargeted Metabolomics

A comprehensive analysis based on plant metabolomic profiling was performed. The untargeted metabolomic approach, using a hybrid quadrupole-time-of-flight mass spectrometer coupled to an UHPLC chromatographic system (UHPLC-ESI/QTOF-MS), was carried out to investigate the metabolic reprogramming induced by treatments and then to discern the pathways and processes elicitated by PH application.

In a preliminary step, the HCA allowed to group the samples according to their similarities/dissimilarities in an unsupervised manner. The fold-change (FC) of metabolites provided by UHPLC-ESI/QTOF-MS analysis were used to build the HCA heat-map and the relative clusters, as a first step of interpretation. The complete list of metabolites annotated, with individual intensities, is provided as supplementary material (**Supplementary Table 1**). HCA outcome (**Figure 3**) indicated that the changes in the metabolic profile could differentiate the effect of the different treatment on tomato plants. The clustering results showed that the samples were grouped in two principal clusters. The first cluster grouped samples from IBA treatment (positive control) and PH1 with foliar application, thus indicating a close relationship between the two. The second cluster included a sub-cluster grouping the (not fractioned) PH and PH1 with basal application, as well as another sub-cluster including the replicates from negative control. Therefore, this unsupervised analysis suggested that the treatments induced a change in metabolomic profile of tomato, and that such changes resulted from the combination of the material considered (PH *vs* PH1) and the mode of application (basal *vs* foliar).

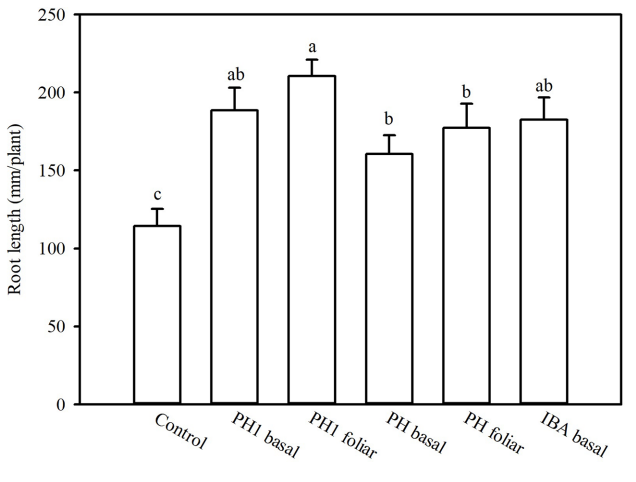


FIGURE 2 | Root length of tomato plants as affected by basal or foliar treatment of cuttings as carried out in the experiment 2. To this aim, solutions containing distilled water, protein hydrolysate (PH) Trainer® or its fraction with molecular weight below 0.5–1 kDa (PH1) or indole-3-butyric acid (IBA) have been tested. Different letters over bars indicate significant differences between treatments according to Duncan's multiple range test ($p = 0.05$).

To better understand the effect of each biostimulant and the differences between them, a supervised multivariate analysis was next performed considering the basal application of PH and PH1 together with the two controls (water and IBA). OPLS discriminant analysis allowed to effectively separate the treatments into the score plot hyperspace, by discerning predictive and orthogonal components of variance (**Figure 4**). All the treatment resulted well separated from each other, suggesting a different effect at molecular level. The model was validated, and the parameters indicated a good predictivity (R2Y=0.989; Q2Y= 0.8; CV-ANOVA $P = 4.28E^{-7}$). At the same time, permutation testing excluded model overfitting.

VIP analysis was therefore used to identify the metabolites mostly involved in the separation between treatments. Metabolites presenting a VIP score >1.4 were considered as discriminant and used for the discussion. These discriminant compounds identified by the supervised approach are provided in **Supplementary Table 2**. Among these metabolites, secondary metabolites were the most represented compounds suggesting that the treatment had a specific effect on the secondary pathways. In fact, hormones such as gibberellins and brassinosteroids were the principal discriminants isoprenoids. In addition, we underlined the presence of alkaloids and some glucosinolates, together with polyphenols as isoflavones and lignans.

A comprehensive overview of the metabolic processes involved in tomato plant response to the treatments was provided by the PlantCyc software. With this aim, the most significant compounds obtained from Volcano analysis (P-value<0.05; FC>1.5) were loaded into the Pathway Tools Omics Dashboard. This tool allowed to interpret the changes at molecular level and to link to a putative physiological process and, therefore, to expand the knowledge regarding the mode of action of the bioactive component(s) of the PH.

Figure 5 summarizes the differential metabolites, classified by categories based on their role in the biosynthesis pathways. As a broad overview, PH-fraction 1 showed a similar profile to positive control IBA, and different from PH. Secondary metabolism included the most intensely modulated categories of compounds, in response to PH and its fraction application (**Table 1**). Indeed, over 250 compounds included in secondary metabolism related pathways were affected by the treatments. Similarly, hormones and compounds belonging to cofactors, prosthetic groups, electron carrier's biosynthesis, and vitamins were identified as a general plant response to treatments. However, the intensity of the metabolite modulation and the carbon and nitrogen fluxes appeared to be distinct, based on the treatment. In this sense, PH induced an up accumulation in secondary metabolism and cofactors-related compounds, and a down accumulation of hormones. However, the fraction PH1 showed a behavior differing from the PH, that resulted in a down accumulation of several secondary metabolism biosynthetic pathways, in line with IBA treatment.

The biochemical processes including nitrogen-containing secondary metabolites, phenylpropanoids and terpenes were the

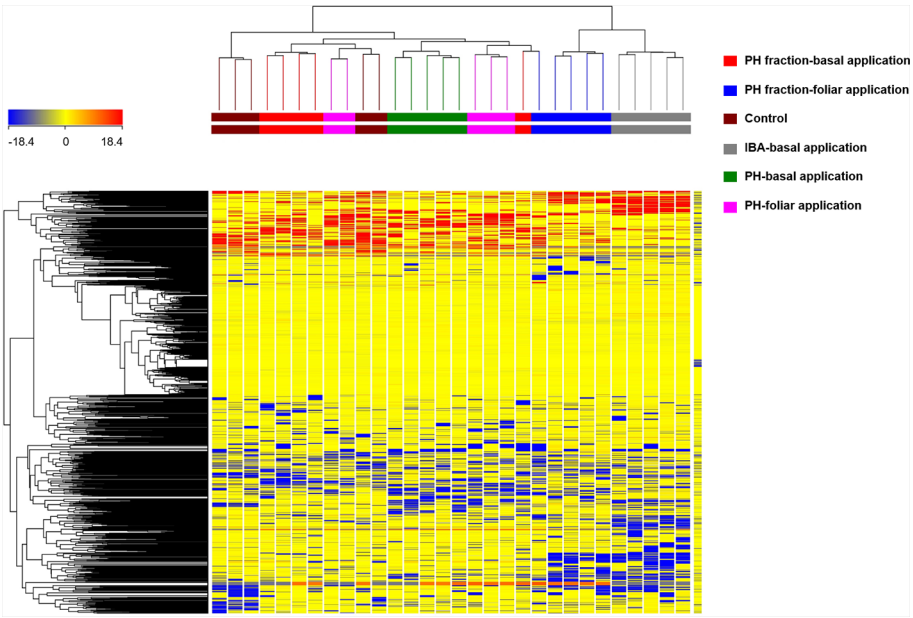


FIGURE 3 | Unsupervised hierarchical cluster analysis carried out from UHPLC-ESI/QTOF-MS metabolomic analysis of tomato plants treated with a protein hydrolysate (PH) or its fraction PH1 (MWCO < 0.5–1 kDa) either *via* basal or foliar application (Exp. 2). Indole-butyric acid (IBA) and water were used as positive and negative control, respectively. The fold-change based heat map from compounds' normalized intensities was used to build hierarchical clusters (linkage rule: Ward; distance: Euclidean).

most elicited by the biostimulant treatments, as confirmed from volcano plot ($p < 0.05$, FC > 1.5) analysis for differential metabolites (**Supplementary Table 3**). Flavonoids and alkaloids and, to a lesser degree glucosinolates, were particularly up-accumulated in PH treated-plants. It is worthy to note that compounds as N-feruloyltyramine, intermediate in the biosynthesis of suberin, and some lignans as (+)-secoisolariciresinol monoglucoside and (−)-pluviatolide, were up-accumulated in the presence of PH. The

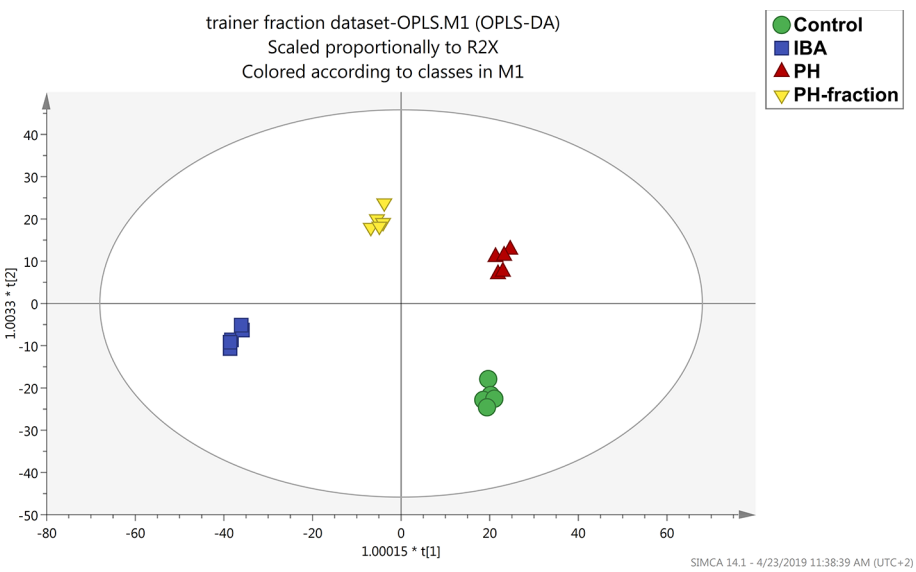


FIGURE 4 | Orthogonal projection to latent structures discriminant analysis (OPLS-DA) supervised modeling of tomato plants following basal application of a protein hydrolysate (PH) or its PH1 fraction (MWCO < 0.51 kDa) (Exp 2). The metabolomic dataset produced through UHPLC-ESI/QTOF-MS was Pareto scaled and then used for the multivariate OPLS-DA modeling. Indole-butyric acid (IBA) and water were used as positive and negative control, respectively.

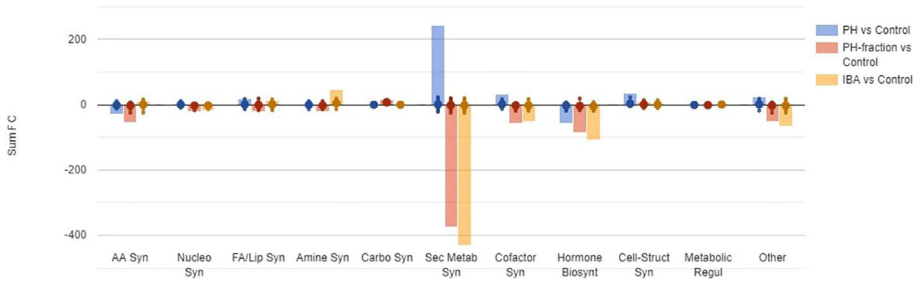


FIGURE 5 | A summary of biosynthesis processes involved in tomato plant response to foliar application of a protein hydrolysate (PH) or its PH1 fraction (MWCO < 0.5–1 kDa) (Exp. 2). The metabolomic dataset produced through UHPLC-ESI/QTOF-MS was subjected to volcano plot analysis (P<0.05, fold-change > 1.5) and differential metabolites loaded into PlantCyc Pathway Tool (https://www.plantcyc.org/). Indole-butyric acid (IBA) and water were used as positive and negative control, respectively. The x-axis represents each set of subcategories while the y-axis corresponds to the cumulative fold-change. AA Syn: amino acids biosynthesis; Nucleo Syn: nucleoside and nucleotide biosynthesis; FA/Lip Syn: fatty acid and lipid biosynthesis; Amine Syn: amine and polyamine biosynthesis; Carbo Syn: carbohydrate biosynthesis; Sec Metab Syn: secondary metabolite biosynthesis; Cofactor Syn: cofactor, prosthetic group, electron carrier, and vitamin biosynthesis; Hormone Biosynt: hormone biosynthesis; Cell-Struct Syn: cell structure biosynthesis; Metabolic Regul: metabolic regulator biosynthesis; Other: other biosynthesis.

defence compounds indole-3-carboxylate and psoralen, both considered phytoalexins, were found to be stimulated by PH.

On the contrary, the fraction PH1 showed a different effect on these compounds, since a general response to the treatment depressed the biosynthesis related to secondary metabolism and phytoalexins plant defence. Phenylpropanoids, predominantly flavonoids, terpenophenolics, as well as terpenoids, polyketides, and alkaloids were negatively affected by PH1 application. Despite the general decrease of defence compounds, metabolites as the fucocumarin psoralen or the phenylpropanoid ferulate were up accumulated. As recorded following PH application, the alkaloid isoalliin were highly increased. Interestingly, anthranilate, intermediate in the pathway of tryptophan and its related pathways, and the 3-hydroxycinnamic acid were down-accumulated in both PH-fraction 1 and IBA. The phytosiderophores mugineate and 3-epihydroxy-2'-deoxymugineate presented the same down-accumulation trend in both fraction PH1 and IBA.

Notably, also phytohormones presented a modulation as a consequence of the treatments (**Supplementary Figure 1**). Brassinosteroids, cytokinins, and jasmonates biosynthesis related compounds were down accumulated following application of PH. The pattern of the phytohormones in response to fraction PH1 and IBA application was very similar. Abscisic acid related compounds and cytokinins were down accumulated in the presence of both fraction PH1 and IBA. However, gibberellins were elicited by all treatments.

DISCUSSION

PHs are considered as plant biostimulants since they can have a positive effect on plants by increasing shoot and root biomass, rather than tolerance to abiotic stresses, thus promoting crop productivity (Colla et al., 2015). This positive effect could be linked to the

TABLE 1 | Summarized biosynthesis processes involved in plant response to foliar application of a protein hydrolysate (PH) or its fraction PH1 (MWCO < 0.5–1 kDa) (Expt. 2).

	Log FC PH			Log FC PH-fraction			Log FC IBA		
	No. compounds	Average	Sum FC	No. compounds	Average	Sum FC	No. compounds	Average	Sum FC
Amino acid biosynthesis	25	1.2	–29.9	25	–2.1	–53.7	25	0.4	9.2
Nucleosides and nucleotides biosynthesis	8	0.3	2.2	8	–2.6	–20.8	8	–2.3	–18.7
Fatty acid and lipid biosynthesis	24	0.8	18.9	24	–0.9	–21.7	24	0.5	11.7
Amines and polyamines biosynthesis	10	–0.4	–4.4	10	–2.2	–22.0	10	4.5	45.3
Carbohydrates biosynthesis	1	–0.1	–0.1	1	14.7	14.7	1	0.2	0.2
Secondary metabolites biosynthesis	252	0.9	221.2	254	–1.4	–344.1	252	–1.7	–425.1
Cofactors, prosthetic groups, electron carriers biosynthesis	27	1.3	33.8	27	–2.1	–57.0	27	–1.9	–52.3
Hormones biosynthesis	25	–2.3	–58.0	25	–3.4	–85.7	25	–4.3	–107.6
Cell structures biosynthesis	11	3.3	36.0	11	0.7	7.7	11	0.4	4.1
Metabolic regulators biosynthesis	3	–0.8	–2.3	3	–1.3	–3.9	3	0.5	1.5
Other biosynthesis	27	0.7	18.0	27	–3.1	–82.7	27	–3.1	–83.8

The metabolomic dataset produced through UHPLC-ESI/QTOF-MS was subjected to volcano plot analysis (P < 0.05, fold-change > 1.5) and differential metabolites loaded into PlantCyc Pathway Tool (https://www.plantcyc.org/). Indole-butyric acid (IBA) and water were used as positive and negative control, respectively. The average and summed Log fold-changes (Log FC) values, together with the number of compounds involved, is provided for each biosynthetic pathway and for each treatment.

interference of PH on nutrient uptake and Fe, C, and N metabolism (Colla et al., 2015; Paul et al., 2019a). The above findings can also be linked to the increased availability of nutrients for plant uptake resulting form the formation of metal complexes of amino acids and peptides in PHs. Similarly, humic substances improvement of plant iron nutrition as a consequence of metal complexation by humic substances (HS) extracted from different sources has been widely reported. In our study, OPLS-DA supervised multivariate modeling and the following VIP analysis indicated differences in the secondary metabolism and suggested that each treatment (i.e. the PH rather than its fraction) had a specific effect at metabolome level. The most active fraction of the PH (PH1, MWCO <0.5–1 kDa) was that including oligopeptides and amino acids. Santi et al. (2017) observed that PHs containing peptides and a low quantity of free amino acids presented a higher effect on root growth and micronutrient accumulation than free amino acid mixture. Similarly to HS (Zanin et al., 2019), the improvement of micronutrient uptake resulting from the PH applications can be related to the direct effects of PH on micronutrient-acquisition mechanisms and to the capability of amino acids and peptides to form stable complexes with metals. Moreover, small peptides have been postulated as key signaling molecules, since they could regulate various aspects of developmental processes in plants (Oh et al., 2018).

A general accumulation of nitrogen-containing compound was showed after PH application, in agreement with a previous study revealing that the PH enhances nitrogen uptake (Colla et al., 2014). In addition, probably due to the modulation of nitrogen metabolism, alkaloids presented a variation of their amount. The modulation of alkaloids is consistent with previous findings using a PH on lettuce (Lucini et al., 2015); these compounds are one of the largest groups of plant secondary metabolites containing nitrogen in their structure. Among other, the complex and partially understood roles ascribed to alkaloids in plant metabolism included the regulation of plant growth and the action as reservoir of nitrogen (Waller and Nowacki, 1978; Facchini, 2001). Several studies linked the nitrogen content and the bioavailability of N to the increase of alkaloids (Sreevalli et al., 2004; Banani et al., 2017). Moreover, it has been postulated that the increase of N assimilation due to the PH application could stimulate phenylpropanoids pathway (Colla et al., 2015), which could explain the changes observed in the phenylpropanoid metabolites following PH application. Noteworthy, several works reported that biostimulants are effective in modulating the profile of phenolic compounds in wine (Boselli et al., 2019; Salvi et al., 2019), tomato (Lucini et al., 2015), and pepper (Barrajón-Catalán et al., 2020). In plant, phenolics play a plethora of functions both in terms of physiology and development as well as regarding interactions with biotic and abiotic environments.

Besides the modulation of secondary metabolism, that was expected, it is important to point out that the PH and even more its fraction PH1 strongly affected the profile of phytohormones. Interestingly, PH1-induced metabolomic changes showed always the same trend as IBA-induced changes. This is coherent with the outcome of *in vivo* bioassays, where this fraction showed the highest auxin-like activity, similarly to IBA. The effect of the not fractionated PH was distinct from PH1, thereby indicating that fractionation enriched the product by the component having an auxin-like activity that promoted the growth of adventitious roots. The fractionation-related activity of biostimulant materials has been previously described for HS (Maggioni et al., 1987; Puglisi et al., 2009), even though the knowledge about the underlying mechanisms still remains partial and fragmentary (Calderin Garcia et al., 2016). With this regard Nardi et al. (2002) highlighted that the portions with low molecular weight (<3,500 Da) HS could easily reach the plasmalemma of plants, whereas high molecular weight fractions (>3,500 Da) could interact only with the cell wall. Similarly, Canellas et al. (2010) observed different hormone-like activities when testing different size fractions of a vermicompost humic substance. Although referring to a different biostimulant material, these different clues contribute to support the distinctive differences in metabolic signatures we observed between PH and its low molecular weight fraction PH1.

This latter PH1 fraction provided an auxin-like activity when applied to tomato. It is known that auxins play an important role in plant development, including rooting (Enders and Strader, 2015). Root growth is sustained by the apical meristem, a region near the root tip where the development program regulates cell division and elongation and is pivotally maintained by auxins, cytokinins, and gibberellins. In particular, auxins play a key role in root development, being involved in the positioning and formation of the meristem, and stimulation of mitotic activity (Muraro et al., 2016). In fact, auxins are reported to promote founder cells of both shoots and roots (Toyokura et al., 2019). Nevertheless, it is important to keep in mind that root development is the result of a rather complicated and still poorly understood coordinated multilayer interaction network between auxins, cytokinins, gibberellins, and ethylene (Liu et al., 2017). Cytokinins regulate the activity of the meristem antagonistically to auxins, negatively modulating the transport of auxins (Marhavý et al., 2011) and reducing mitotic activity *via* the promotion of cell differentiation (Dello Ioio et al., 2007). As far as gibberellins are considered, their involvement in root development is important during the early stages when they promote auxin transport and cell proliferation (Moubayidin et al., 2010). The hormonal regulation of root growth can be further expanded, since the gibberellin-related DELLA proteins are known to interact with jasmonate, ethylene, and brassinosteroids, (Liu et al., 2017), the latter acting antagonistically to auxins by inhibiting cell elongation in the root tip (Chaiwanon and Wang, 2015). Finally, abscisic acid coordinates auxins to determine root elongation and architecture, even under no stress conditions (Harris, 2015).

Notably, this complex regulation network can be linked to our results. The biostimulant fraction PH1 (*i.e.*, the fraction inducing the same metabolic modulation caused by IBA), determined a coordinated hormonal change that supports the auxin-like activity observed in the bioassays. The application of PH1 caused a down

accumulation of cytokinins (antagonists of auxins in root development), and a concurrent down accumulation of abscisic acid intermediates (inhibitor of root elongation). The increase in gibberellins observed following application of both PH and PH1, can have promoted auxins transport and cell proliferation while inhibiting brassinosteroids, these latter being antagonists of the auxins. Noteworthy, the biostimulants-induces increase in gibberellins has been previously linked to rooting of azalea cuttings, especially at the early stage of root development (Elmongy et al., 2018). Furthermore, the whole metabolite signature (mainly targeting secondary metabolism) was largely shared between IBA- and PH1-induced metabolomic reprogramming, thereby corroborating the auxin-like activity resulting from the changes in phytohormone profiles. Besides, we cannot exclude a direct contribution of the endogenously present auxin in determining to the shift in metabolic patterns we observed following application of PH1. The long-distance effects we observed in tomato plants are hard to be substantiated without specific labeling experiments. Bearing in mind that auxins are known to be transported from the shoot to the root apex (Muraro et al., 2016), we can speculate that this process might have supported the increased rooting when PH1 was foliarly applied. On the other hand, the ability of biostimulants to affect tissues other than those of application has been demonstrated by Kulikova et al. (2016) using a tritium-labeled humic acid.

It must be noted that also the non-fractionated PH modulated the hormone profile in tomato, with brassinosteroids, cytokinins, and jasmonates being down accumulated and gibberellins being accumulated following the treatment (**Figure 5**). Noteworthy, these changes are only partially shared with PH1 and to some extent are specifically induced by this treatment. This finding is in agreement with the fact that PH1 is a fraction of PH, where some components are enriched (small molecules, oligopeptides) and some other are depleted (higher molecular weight compounds).

In general, regardless the molecular mechanisms involved, small peptides have been confirmed as potentially important signaling compounds, as previously postulated for the product used in this work (Colla et al., 2014). Indeed, they could regulate various aspects of developmental processes in plants (Oh et al., 2018). Concerning root development, peptide hormones have been proposed as a key mechanism for cell–cell interactions in plants (Yamada and Sawa, 2013). These signaling peptides coordinate both development and responses to environment (Toyokura et al., 2019); despite their mechanism of action in the shoot is well known, their role in the root is relatively uncharacterized (Yamada and Sawa, 2013).

CONCLUSIONS

A combination of molecular fractionation by dialysis, *in vivo* bioassay for hormone-like activity, and metabolomics, has been successfully tested to identify the most active fraction of a vegetal derived-PH. Auxin-like activity was tested through a rooting assay, to preliminary screen the PH and its fractions, in comparison to the auxin IBA (positive control). This rooting experiment allowed identifying the fraction (PH1) having the highest auxin-like activity, to be tested further, in combination with MS-based metabolomics, to shed light onto the biochemical processes underlying the activity observed. The combination of fractionation (effective in reducing the complexity of a chemically diverse matrix like PH) with metabolomics was effective to depict the changes induced by the tested fraction at biochemical level. Such complementary contributions are effective to investigate the possible mode of action and the most bioactive components of a biostimulant product.

The smallest fraction of PH containing small molecules and oligopeptides (molecular weight < 1 kDa) was the most active in promoting the rooting of tomato cuttings. Moreover, metabolomics allowed to identify the mode of action of PH and its fraction (PH1) in comparison with the exogenously applied IBA. PH1 and IBA-treated cuttings showed a similar metabolomic signature (mainly affecting secondary metabolism and phytohormone profiles), thereby corroborating the auxin-like activity. Notably, *in vivo* bioassays were consistent with metabolomics, considering that PH1 was actually effective in promoting the growth of adventitious roots.

Therefore, provided that our work focused on auxin-like activity, PH1 was identified as the most active fraction of the PH. The results suggested that this approach is suitable to understand the biological activity retained by the different fractions in a complex biostimulant product. Noteworthy, the unfractionated PH was not devoid of biostimulant properties, suggesting that the choice of the best fraction(s) depends on the desired activity. Although the present work focused on auxin-like activity, it worth to consider that the fractions other than PH1 might be of interest, when different aims are to be targeted. The comprehension of active fractions can assist the manufacture of more effective biostimulants, where the yield of the desired fraction/s is optimized during the production process. With this regard, the present approach represents a good solution that can be applied in all cases where biostimulants are based on complex mixtures of bioactive substances differing in molecular weight, like for HS, PH, and/or algal extracts. Finally, future studies should also address the role of mineral nutrients in the biostimulant activity of PHs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LL and BM-M performed the molecular fractionation and metabolomic study, data analysis and interpretation, and wrote

many parts of the manuscript. YR, and MC performed the bioassays, data analysis and interpretation, and wrote many parts of the manuscript. GC and LL coordinated the whole project, provided the intellectual input, set up the experiments, contributed to the interpretation of the results, and corrected the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00976/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Phytohormone biosynthesis processes involved in plant response to foliar application of a protein hydrolysate (PH) or its fraction (MWCO < 0.5–1 kDa) (Exp. 2). The metabolomic dataset produced through UHPLC-ESI/QTOF-MS was subjected to volcano plot analysis (P<0.05, fold-change > 1.5) and differential metabolites loaded into PlantCyc Pathway Tool (https://www.plantcyc.org/). Indole-butyric acid (IBA) and water were used as positive and negative control, respectively. The x-axis represents each set of subcategories while the y-axis corresponds to the cumulative fold-change.

SUPPLEMENTARY TABLE 1 | Whole dataset produced from untargeted metabolomics carried out in tomato cuttings treated with either a protein hydrolysate (PH) or its fraction PH1 (Exp. 2). Compounds are presented with individual intensities, annotations and composite mass spectra (mass abundance combinations).

SUPPLEMENTARY TABLE 2 | VIP discriminant compounds as identified from OPLS-DA discriminant analysis of tomato cutting metabolomic profiles following either a protein hydrolysate (PH), PH1 or indole-3-butyric acid application (Exp. 2).

SUPPLEMENTARY TABLE 3 | Differential metabolites as derived from Volcano analysis (P-value<0.05, Bonferroni multiple testing correction; FC>1.5) in tomato cutting metabolomic profiles following either a protein hydrolysate (PH), PH1 or indole-3-butyric acid application (Exp. 2).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 July 2020
doi: 10.3389/fpls.2020.00953

An Enzymatically Hydrolyzed Animal Protein-Based Biostimulant (Pepton) Increases Salicylic Acid and Promotes Growth of Tomato Roots Under Temperature and Nutrient Stress

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OPEN ACCESS

Edited by:
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Antonio Ferrante,
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 24 March 2020
Accepted: 10 June 2020
Published: 01 July 2020

Citation:
Casadesús A, Pérez-Llorca M, Munné-Bosch S and Polo J (2020) An Enzymatically Hydrolyzed Animal Protein-Based Biostimulant (Pepton) Increases Salicylic Acid and Promotes Growth of Tomato Roots Under Temperature and Nutrient Stress. Front. Plant Sci. 11:953. doi: 10.3389/fpls.2020.00953

Biostimulants may be particularly interesting for application in agricultural and horticultural crops since they can exert a growth-promoting effect on roots. This may be important for promoting longitudinal and lateral root growth and therefore increasing belowground vegetative growth, which may in turn lead to improved aboveground vegetative growth and increased yields. Here, we examined the effects and mechanism of action of an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) on the root growth of tomato plants, with an emphasis on its possible role on chorismate-derived hormones (auxin, salicylic acid, and melatonin). Tomato plants growing in hydroponic systems were exposed to either nutrient stress conditions (experiment 1) or suboptimal temperatures (experiment 2) in a greenhouse, and the concentration of auxin, salicylic acid, and melatonin in roots were measured just prior and after the application of the biostimulant. Results showed that the application of Pepton exerted a growth-promoting effect on roots in plants growing under suboptimal conditions, which might be associated with enhanced salicylic acid levels in roots. The extent of effects of this enzymatically hydrolyzed animal protein-based biostimulant might strongly depend on the growth conditions and stage of root system development. It is concluded that an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) may exert a positive effect enhancing primary and lateral root growth of tomato plants growing under suboptimal conditions, by stimulating the biosynthesis of specific hormonal pathways, such as salicylic acid under stress.

Keywords: auxin, biostimulants, chorismate-derived hormones, melatonin, PIN proteins, salicylic acid, tomato plants

INTRODUCTION

Recently, much effort is being put worldwide to boost research focused on the environmentally friendly biostimulation of crop performance for improving plant production in the frame of sustainable farming management. Protein hydrolysates, and most particularly those obtained from recycling wastes of plant or animal origin, are good candidates to be used as plant biostimulants because of their high amino acids and soluble peptide concentrations (Sestili et al., 2018; Caruso et al., 2019). Among several protein hydrolysates used as biostimulants, enzymatically hydrolyzed animal protein-based biostimulants such as Pepton 85/16® (Pepton), represent a cost-effective approach to alleviate the negative effects of several stresses in horticultural crops (Polo et al., 2006; Polo and Mata, 2018; Casadesús et al., 2019). Furthermore, the application of Pepton at different levels (2 to 4 kg/ha) has been shown to improve linearly root length and several vegetative growth parameters thus increasing yield by 27% compared with the control treatment in cherry tomatoes under low stress ambient field conditions (Polo and Mata, 2018). However, still little is known about the possible effects exerted by this biostimulant on root growth and even less about its mechanism of action, although it seems that the positive effects of Pepton on horticultural crops may be exerted by the presence of amino acids, which can influence the physiology of plants both directly or indirectly, the latter through the stimulation of various metabolic processes, such as an increased production of growth-promoting hormones in aboveground vegetative tissues (Casadesús et al., 2019).

Auxins, salicylic acid, and melatonin are phytohormones involved in the signaling and regulation of many crucial processes in plants. Auxins have been widely described as growth and development regulators with multiple functions in plants, playing a key role in organ morphogenesis, apical dominance, adventitious rooting, and cell expansion, among other processes (see Taylor-Teeples et al., 2016). Salicylic acid is known to be involved in triggering the defense response against biotrophic and hemi-biotrophic pathogens (Loake and Grant, 2007) as well as having an important role in growth arrest under abiotic stress conditions (Carswell et al., 1989; Dong et al., 2014; Wani et al., 2017), although at low concentrations it can also promote lateral root formation (Pasternak et al., 2019). Finally, melatonin has not only been found to have auxin-like functions (Chen et al., 2009; Zuo et al., 2014; Wen et al., 2016) but it also has been suggested to act as a potential antioxidant in some specific plant organs and plant species (Arnao and Hernández-Ruiz, 2015) and a regulator of plant responses to pathogens (Chen et al., 2018). Interestingly, these three hormones share a common precursor—chorismate—thus a metabolic crosstalk occurs between them and a number of genes must be finely regulated to divert chorismate metabolism towards these compounds (**Figure 1**).

Indole-3-acetic acid (IAA), the major auxin bioactive form present in plants, can be produced from tryptophan or from

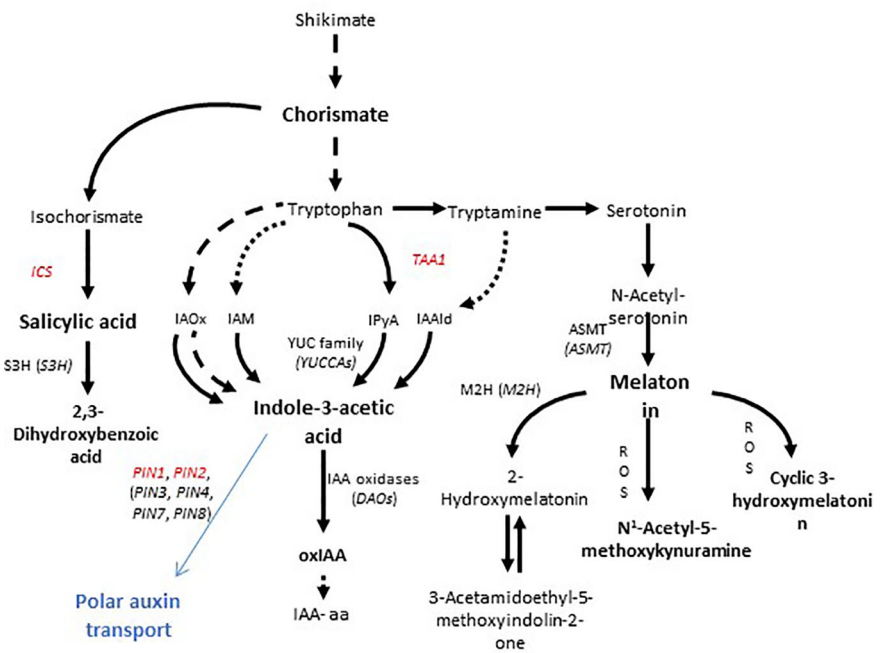


FIGURE 1 | Biosynthesis of chorismate-derived phytohormones and efflux transport of auxin in plants. Salicylic acid is derived from isochorismate, while auxin and melatonin come from tryptophan, which is in turn synthesized from chorismate. Isochorismate synthase (ICS), tryptophan amino transferase (TAA1), and N-acetyl-serotonin methyl transferase (ASMT) play crucial roles in the biosynthesis of salicylic acid, indole-3-acetic acid (auxin), and melatonin, respectively. PIN proteins show asymmetrical localizations on the membrane and are responsible for polar auxin transport (shown in blue). Genes, the expression of which was measured in the present study, are indicated in red.

tryptophan precursors in seemingly complex pathways that in all cases compete for the same chorismate precursor. Auxin promotes vegetative growth both above- and belowground by regulating cell expansion, cell differentiation, and organogenesis, while cell-cell polar transport mediated by PIN proteins regulated auxin distribution (Pacifici et al., 2015). Salicylic acid is a phenolic acid also derived from chrorismate (but not from tryptophan) that is not only involved in biotic stress response (mainly biotrophic pathogens) generally retarding plant growth and inducing pathogen-related genes (Qi et al., 2018), but also inducing adventitious root formation and improving root growth in particular under stress conditions (Armengot et al., 2014; Pasternak et al., 2019). Melatonin, another chorismate-derived compound, which competes for this common precursor with salicylic acid and auxin, and for tryptophan with auxin but not with salicylic acid, has also been shown to promote lateral root formation and development, by, among other mechanisms, modulating auxin response (Liang et al., 2017). In a previous study Pepton enhanced foliar levels of auxin, gibberellins and jasmonic acid in tomato leaves, whereas salicylic acid was not affected under conditions of water deficit stress (Casadesús et al., 2019).

The aim of this work was to assess to what extent an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) can promote root growth and, if so, to also establish a mechanism of action, with an emphasis on the endogenous contents of chorismate-derived hormones. This is very important not only to promote growth and yield in several horticultural crops, but also to better understand the mechanism of action of enzymatically hydrolyzed animal protein-based biostimulants.

MATERIALS AND METHODS

Growth Conditions, Treatments, and Samplings

Seeds of tomato (*Solanum lycopersicum*, var. "Ailsa Craig"), which were obtained from the Experimental Field Facilities of the University of Barcelona (Barcelona, NE Spain), were used for experiments. For experiment 1 (large roots), seeds were sown on May 23, 2019, in 1-L pots in a growth chamber (12 h light/12 h dark, at 21.9°C and 65% relative humidity). On June 11, 2019, seedlings were transferred to a closed hydroponic system prepared in an open-windows greenhouse situated at the Faculty of Biology of the University of Barcelona (Barcelona, NE Spain) with 12 plants for each container and 6 containers per treatment were established and filled with 21 L of half-strength Hoagland nutrient solution in (Hoagland and Arnon, 1938) distilled water (indicated on **Supplementary Table 1**). Treatments started after 2 weeks of growth in hydroponics, on June 25, 2019, when roots were sufficiently large to obtain high density of roots in the container to better produce nutrient deficiency by competition. Two treatments, control and Pepton, were imposed on plants at optimal temperature (24.6–25.51°C) in a background of nutrient deficiency (obtained by using half-strength Hoagland nutrient solution in a container with high plant density). Pepton was added and diluted on the nutrient solution at manufacturer's recommendation dose (Pepton at 1.06 g/container, calculated using plant density on the area of the study as equivalent of 4 kg/ha) the first day of the experiment. For experiment 2 (small roots), seeds were sown on December 20, 2019, in 1-L pots in a growth chamber (12 h light/12 h dark, at 21.9°C and 65% relative humidity). On January 17, 2020, seedlings were transferred to a closed hydroponic system prepared in the same greenhouse and in the same way as described before situated at the Faculty of Biology of the University of Barcelona (Barcelona, NE Spain) with 12 plants for each container and three containers per treatment were established and filled with 21 L of full-strength Hoagland nutrient solution (indicated on **Supplementary Table 1**). Treatments started after three days of growth in hydroponics, on January 20, 2020, when roots were still small. We applied two treatments, control and Pepton, in a background of low-temperature stress (water temperature of 11.0°C to 12.6°C obtained by conditioning the greenhouse to decrease the indoor temperatures). In both treatments, we applied the full-strength nutrient solution but in the treatment of Pepton, we added and diluted this biostimulant on the nutrient solution following manufacturer's recommendation dose (430 mg/container, calculated using plant density on the area of the study as equivalent of 4 kg/ha) the first day of the experiment. Samplings for both experiments were performed at days 1, 2, and 4 from the start of treatments at midday. At each time point, 1, 2, and 4 days from the start of treatments, the whole root system from 11 plants were collected in experiment 1 and from 5 plants in experiment 2, weighed and gently dried on a paper before being immediately frozen in liquid nitrogen and then kept at −80°C to be used for hormonal and gene expression analyses. **Table 1** contains the conductivity, pH, and temperature of the nutrient solution during experiments 1 and 2 for the different

TABLE 1 | pH, conductivity and temperature of the nutrient solution during experiment 1 (A) and experiment 2 (B).

(A) Experiment 1/nutrient deficiency (large roots).

Day	Treatment	Conductivity (μS/cm^2)	pH	Temperature (°C)
1	Control	815	5.90	25.5
1	Pepton	978	6.55	25.5
2	Control	619	6.64	24.8
2	Pepton	1032	6.78	24.6
4	Control	653	7.23	25.2
4	Pepton	824	6.89	25.1

(B) Experiment 2/suboptimal temperatures (small roots).

Day	Treatment	Conductivity (μS/cm^2)	pH	Temperature (°C)
1	Control	2546	6.67	11.0
1	Pepton	2516	6.69	11.0
2	Control	2543	6.82	11.7
2	Pepton	2520	6.90	11.7
4	Control	2547	6.76	12.6
4	Pepton	2510	6.84	12.6

testing time (1, 2, and 4 days).

Pepton Composition

Pepton is an animal protein enzymatic hydrolyzed product that contains high amounts of total organic matter (79%) and organic nitrogen (12%), with a carbon/nitrogen ratio of 3.6 (**Table 2**). The product is very rich in L-α amino acids (84.8%) and contains a high proportion of free amino acids (16.5%), iron (3000 mg/kg) and potassium (4.0%). The average molecular weight distribution of Pepton is around 2,000 to 3,000 Da, from which 66% of the peptides are considered short-chain amino acids (with less than 50 amino acids per chain) and 16% of peptides in Pepton are long-chain peptides (>50 amino acids). A complete chemical composition of this biostimulant can be found in Polo and Mata (2018). The manufacturing process of Pepton involved an enzymatic hydrolysis at high temperature (>50°C) at controlled conditions that hydrolyzed the animal protein. Following the hydrolysis process, the enzyme is inactivated by increasing the temperature and the final product is spray-dried at specific conditions that involved the treatment of >90°C throughout its substance. Final product is a hygienic, brown, granulated powder that meets all physical and microbiological requirements for being used as biostimulants in the agro industry.

TABLE 2 | Composition of the enzymatically hydrolyzed animal protein-based biostimulant (Pepton).

	Pepton
Total organic matter, %	79.0
Total nitrogen, %	13.0
Organic nitrogen, %	12.0
Ammonia nitrogen, %	1.0
Carbon/Nitrogen	3.8
Potassium oxide (K_2O), %	4.0
Phosphorous (P_2O_5), %	0.3
Calcium, mg/kg	300
Magnesium, mg/kg	500
Iron, mg/kg	3000
Amino acid composition	
Alanine, %	6.90
Arginine, %	3.22
Aspartic acid, %	9.93
Cysteine	<0.1
Glutamic acid, %	7.25
Glycine, %	4.06
Histidine, %	6.34
Isoleucine, %	0.15
Leucine, %	10.99
Lysine, %	7.19
Methionine; %	0.71
Phenylalanine, %	5.93
Proline, %	2.84
Serine, %	3.88
Threonine, %	2.47
Tryptophan, %	1.25
Tyrosine, %	1.92
Valine, %	6.79
Total amino acids, %	84.83
Free amino acids, %	16.52

Chorismate-Derived Hormones

Chorismate-derived hormones, including salicylic acid, auxin, and melatonin were determined by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) as described by Müller and Munné-Bosch (2011). Salicylic acid and IAA (auxin) were analyzed using negative ion mode while melatonin was measured using positive ion mode. Extracts were performed using 100 mg of well powered fresh root of each plant with a mixture of methanol and acetic acid (99:1, v/v) as a solvent. Deuterium-labeled plant hormones were added to the extract being the 250-μL the final volume and the mixture was vortexed and ultra-sonicated for 30 min (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, USA). Then the extract was vortexed again and centrifuged at 4°C for 10 min at 1300 rpm. The supernatant was collected, filtered using 0.22-μm hydrophobic PTFE Syringe Filters (Phenomenex, Torrance, CA, US). Two extractions were done and the final extract was injected into the HPLC/ESI-MS/MS system.

Estimation of Root Growth

Root growth of 11 plants for each treatment and time point was estimated by measuring the root weight of the whole root system on a dry matter basis for experiment 1 and those of five plants for the experiment 2. Dry weight was estimated after drying the tissue in the oven to constant weight at 80°C. In addition, root growth was also estimated by scanning the roots of five plants at the end of experiment 2 and using ImageJ software to calculate root area of each plant.

Gene Expression Analyses

Three individuals of each treatment were randomly selected at each time point for gene expression analyses. Approximately, 100 mg of fresh tissue of each individual plant were ground in liquid nitrogen until obtaining a fine powder. Then, total RNA from roots was extracted using the Spectrum Plant Total RNA Kit (Merck) according to manufacturer's instructions, including a DNase I digestion (On-Column DNase I Digestion Set, Merck). Yield and quality of an aliquot of denaturalized RNA for the qPCR analysis were tested with the Qubit fluorometer—using the Qubit™ RNA HS Assay Kit, Thermo Fisher Scientific—and the 2100 Bioanalyzer system (Agilent Technologies, California, USA), respectively. RNA was considered of high quality when RNA integrity number was equal or greater than five (Jeffries et al., 2014). Finally, RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

For specific primer design of *ICS2, TAA1*, and *PIN2* for *S. lycopersicum* var. Alisa Craig, coding sequences were acquired from the Sol Genomics Network (Ithaca, NY, USA) tomato database. Sequences were introduced in the FGENESH online program from Softberry portal (Mount Kisco, NY, USA), selecting the "generic *Tomato lycopersicum*" in the "organism" option. mRNA sequences were obtained and then introduced in Primer-BLAST from NCBI for primer design. Minimum size of the PCR product was set to 70 bp and maximum to 180 bp; minimum T_m was set to 60°C and maximum to 63°C; and, the

"organism" was changed to "green plants." Suggested sequences by NCBI of tomato were accepted, and then, the first pair of primers was selected after testing for formation of heterodimers with the OligoAnalyzer Tool from Integrated DNA Technologies (Coralville, IA, USA). Primers for the target gene *SlPIN1* and for the reference gene *EF1α* were directly selected from previous studies in tomato (Mascia et al., 2010; Gan et al., 2019, respectively). Primer sequences can be found in **Supplementary Table 2**.

Gene expression quantification was performed by RT-qPCR on cDNA using the Roche LightCycler® 480 Instrument II (Roche, Basel, Switzerland) and LightCycler® 480 SYBR Green I Master (Roche). The RT-qPCR reactions were set according to manufacturer's instructions with some modifications. The final volume was adjusted to 10µl, and the reactions started with 5 min of incubation at 95°C followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. The RT-qPCR reactions ended with 5 s at 95°C and 1 min at 65°C. For each sample, RT-qPCR was performed with *SlICS2, SlTAA1, SlPIN1*, and *SlPIN2* as target genes primers and with *SlEF1α* as reference gene primers. Cp values and dissociation curves were analyzed after RT-qPCR reactions with the LightCycler® 480 Software, Version 1.5 (Roche, Basel, Switzerland). All primers had an efficiency of *ca.* 2; therefore, relative expression levels were calculated with the $2^{-\Delta\Delta CT}$ method and normalized with the Cp values of each gene at control conditions (control treatment and time 1 day after application).

Statistical Analyses

To determine the effect of Pepton and time on root biomass, hormones and gene expression, a linear regression (*lm*) was used with "treatment/Pepton" and "time" as predictors. The *P* of the explanatory variables were estimated using conditional *F*-tests using the function Anova (*car* package). Differences were considered significant when $P < 0.05$. To meet normality and homoscedasticity of residues, which were visually checked as described by Zuur et al. (2009), data was either log or square root transformed before analyses. For Experiment 1 data, IAA and melatonin were square root transformed. For Experiment 2 data, root biomass was log transformed and salicylic acid and IAA were square root transformed. All statistical tests were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Root growth was stimulated by the application of this biostimulant during both experiments (**Figure 2**), being more evident after 4 days in experiment 2 (low temperature conditions, **Figure 2B**). A more detailed analysis of root growth imaging revealed that Pepton might have promoted lateral root growth during experiment 1 under nutrient deficiency condition (with longitudinal growth being limited by the depth of the hydroponic tanks, **Figure 3A**). However, the biostimulant promoted both longitudinal and lateral growth in a longer term (4 days) in smaller plants during experiment 2 (without any limitation on longitudinal root growth since tanks of the same depth were used for both experiments). Therefore, the highest differences in root growth were observed at 4 days for experiment 2 with values 2-fold higher in Pepton-treated plants than in control plants (**Figures 2** and **3B, C**).

The concentration of salicylic acid increased in Pepton-treated plants compared to control in all time points of experiment 1, using plants with large roots exposed to nutrient deficiency ($P < 0.001$, **Figure 4A**). In contrast, in experiment 2 (using plants with small roots exposed to suboptimal temperatures), the endogenous concentration of salicylic acid was similar between treatments, but on day 1 the salicylic acid concentration of Pepton-treated plants was higher than control plants ("Time x Treatment", $P = 0.046$) (**Figure 4B**).

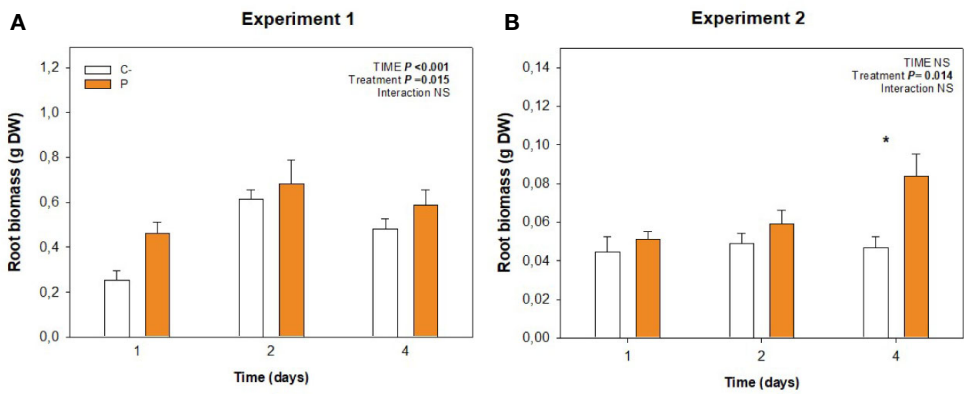


FIGURE 2 | Variations in root biomass in Pepton-treated tomato plants compared to controls. Data are the means ± SE of **(A)** n=11 individuals for experiment 1 and **(B)** n= 5 for experiment 2. Significant effects of "treatment" and "time" were tested using conditional *F* tests (linear model with "treatment" and "time" as explanatory variables, *P* values below 0.05 are shown in bold in the inlets). Experiment 1 was conducted under nutrient deficiency and large root system and experiment 2 under sub-optimal temperature and small root system. *P < 0.05.

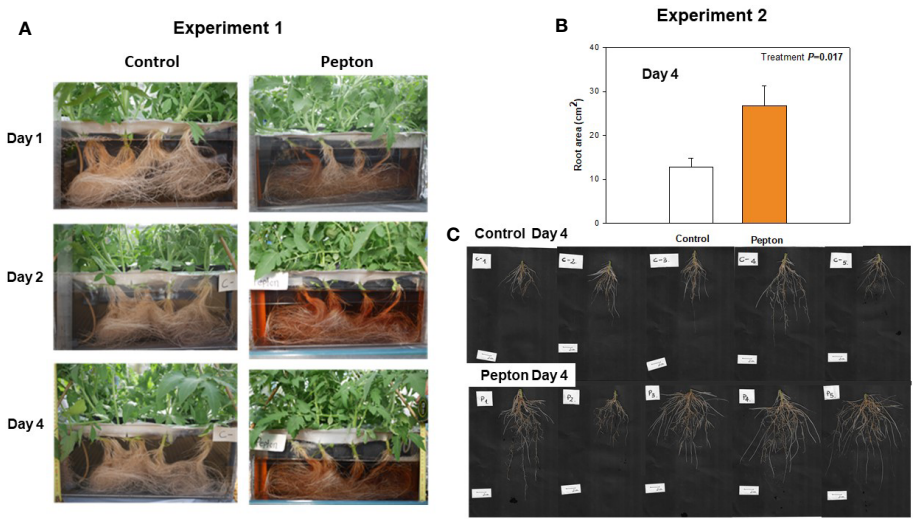


FIGURE 3 | Changes in root morphology and area in Pepton-treated tomato plants compared to controls. **(A)** Images correspond to day 4 for experiment 1. **(B)** Root area measured at day 4 for experiment 2. Data are the means ± SE of 5 individuals. Differences between treatments on root area were tested with a one-way ANOVA (*P* values are shown in bold in the inlet). **(C)** Images correspond to day 4 for experiment 2. Experiment 1 was conducted under nutrient deficiency and large root system and experiment 2 under sub-optimal temperature and small root system.

In both studies, melatonin concentration was not influenced by Pepton treatment (P > 0.10, **Figures 2C, D**). In case of auxin, in experiment 1 under nutrient deficiency conditions it seemed to occur a marginal increase in auxin concentration in Pepton-treated plants compared to the control ("Treatment" $P = 0.077$) (**Figure 4E**).

Gene expression analyses revealed that neither *ICS*, *TAA1* nor *PIN* expression was influenced by Pepton application during the two experiments (**Figures 5A, B**).

DISCUSSION

Enzymatically hydrolyzed animal protein-based biostimulants, such as Pepton, may be an alternative to protein hydrolysates of plant origin since they can promote circular economy and bridge the current gap between recycling animal waste products and agriculture. Considering that in developing countries, between 50 to 60% by weight of farm animals are not profitable for human consumption, finding sustainable applications that improve the use of high-quality source of organic material will reverse in better use of the limited resources of nature. Amino acids and peptides derived from protein hydrolysates, either from plant or animal origin, may improve plant performance through various mechanisms, including effects on hormonal signaling (Du Jardin, 2015; Yakhin et al., 2017; Casadesús et al., 2019; Zulfiqar et al., 2019). In the present study, it was shown that not only an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) can improve root growth in tomato plants, but that these effects may be mediated by chorismate-derived hormones, in particular by the involvement of salicylic acid or by sourcing aromatic amino acids as phenylalanine, the accumulation of which may also involve the production of salicylic acid.

Pepton Might Improve Lateral Root Growth Through Modulation of Salicylic Acid Contents

Recent studies suggest that biostimulants based on protein hydrolysates improve crop performance by stimulating carbon, nitrogen, and hormonal metabolism of plants including tomato (Colla et al., 2014; Colla et al., 2017; Casadesús et al., 2019). In the present study, Pepton treatment led to a significant effect on the endogenous concentrations of the chorismate-derived phytohormone salicylic acid.

Salicylic acid is a stress-related phytohormone, playing a crucial role in biotic stress responses, being the major hormone involved in systemic acquired resistance (Fu and Dong, 2013). Furthermore, salicylic acid is a typical growth inhibitor, as it occurs with other stress-related phytohormones, due to the commonly accepted trade-off between defense and growth (Van Butselaar and van den Ackerveken, 2020). However, phytohormones are involved in a large number of developmental processes and salicylic acid is not an exception. Indeed, salicylic acid is involved in signaling of lateral root initiation and growth interacting with auxin in a dose-dependent manner (Pasternak et al., 2019). Therefore, under nutrient deficiency, increased salicylic acid contents may represent a basic mechanism to explore new soil environments and face poor soils, in our case, probably fighting against the limits of the hydroponics container, so that this phytohormone can have an impact on crop performance and yield (Nibau et al., 2008).

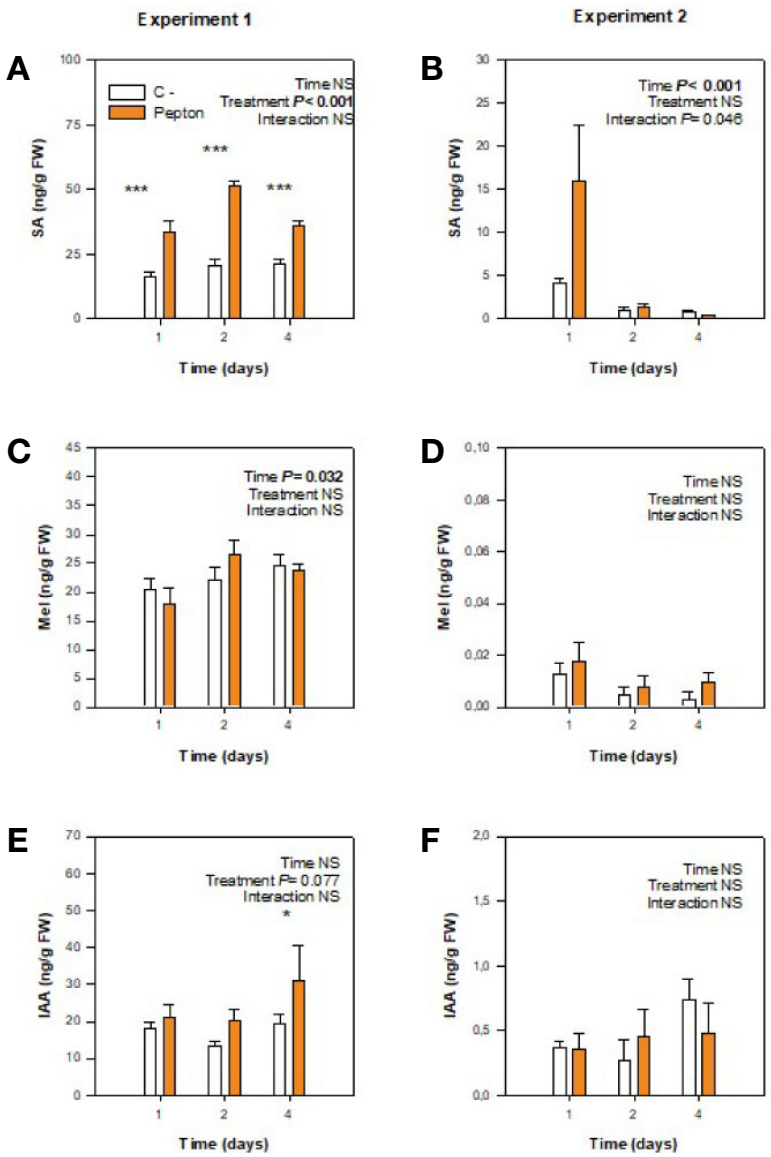


FIGURE 4 | Variations in the endogenous concentration of chorismate-derived phytohormones in roots of Pepton-treated tomato plants compared to controls. **(A, B)** salicylic acid (SA), **(C, D)** melatonin (Mel), **(E, F)** indole-3-acetic acid (IAA). Data are the means ± SE of n = 5 and 11 individuals for experiments 1 and 2, respectively. Significant effects of "treatment" and "time" were tested using conditional *F*-tests (linear model with "treatment" and "time" as explanatory variables, *P* values below 0.05 are shown in bold in the inlets). Experiment 1 was conducted under nutrient deficiency and large root system and experiment 2 under sub-optimal temperature and small root system.*P < 0.01; ***P < 0.001.

In previous studies, it has been observed an enhanced growth due to an auxin-like activity for protein hydrolysates products of plant origin (Colla et al., 2014; Ugolini et al., 2015; Elzaawely et al., 2016; Ertani et al., 2017), but Pepton did not contain nor seemed to largely influence auxin concentrations, thus other mechanisms might explain these positive effects on root growth.

On the one hand, the effects of Pepton might be related to a direct influence of amino acids, which can improve nitrogen metabolism and promote root growth. In fact, the abundance of nitrogen compounds can have a huge impact on root architecture and on lateral root formation (Nibau et al., 2008). On the other hand, Pepton effects might be related to an enhanced accumulation of salicylic acid concentrations, which might induce lateral root formation, in particular during experiment 1 (Shi et al., 2010; Armengot et al., 2014; Pasternak et al., 2019), in order to explore new soil and solve suboptimal nutrient environment situation. But, since melatonin and auxin concentrations did not increase in response to Pepton application in experiment 2, to understand the longitudinal root growth promotion observed, we evaluated the underlying mechanism through an examination of possible changes in polar auxin transport, an aspect that was investigated at the gene expression level.

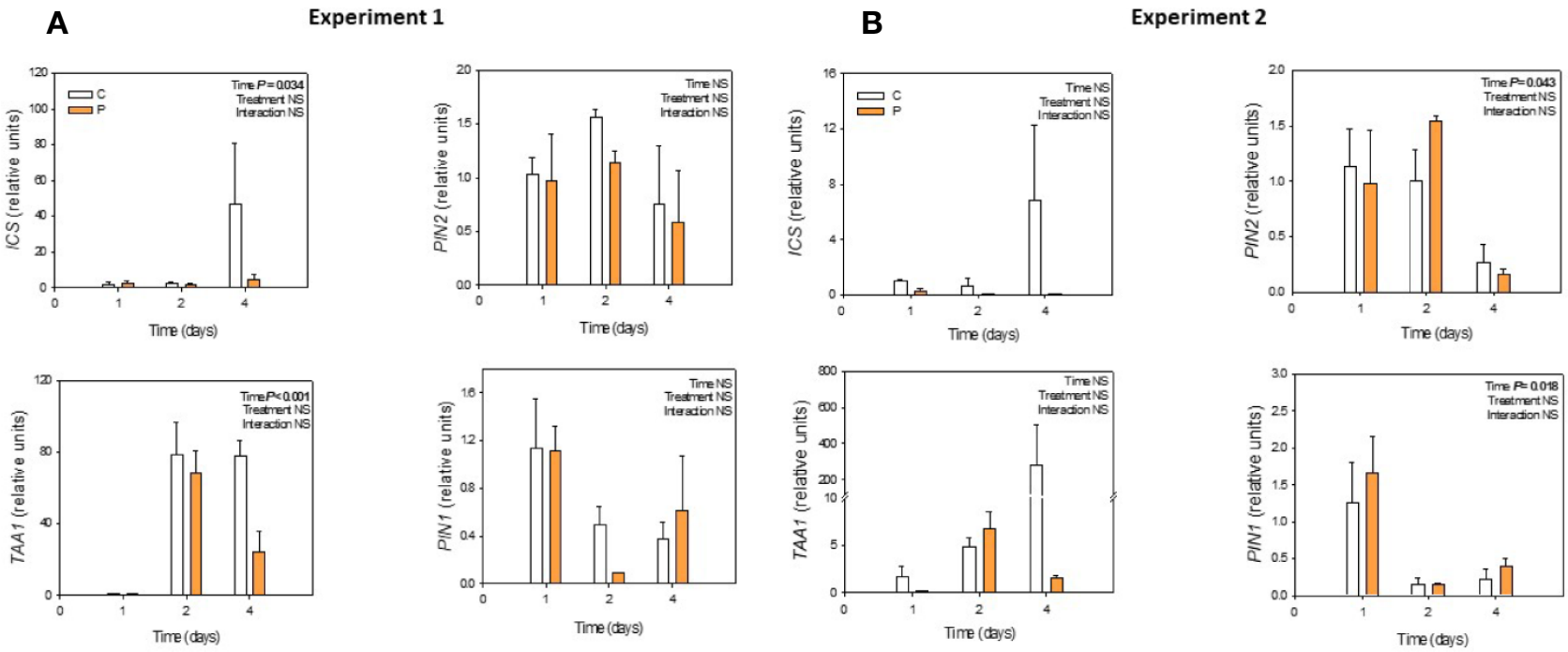


FIGURE 5 | Variations in the relative expression of the genes encoding for isochorismate synthase (*ICS*), tryptophan amino transferase (*TAA1*) and PIN proteins (*PIN1* and *PIN2*) in Pepton-treated tomato plants compared to controls. **(A)** Experiment 1 and **(B)** Experiment 2. Data are the means ± SE of n = 3 individuals. Significant effects of "treatment" and "time" were tested using conditional *F* tests (linear model with "treatment" and "time" as explanatory variables). No significant or marginal differences in any of the tested genes were observed ($P > 0.10$). Experiment 1 was conducted under nutrient deficiency and large root system and experiment 2 under sub-optimal temperature and small root system.

Using protein hydrolysates of plant origin, Ertani et al. (2017) demonstrated higher expression of several ethylene/jasmonates/abscisic acid responsive genes including wound-induced proteins and heat-shock proteins which are crucial in both biotic and abiotic stress response; however, to our knowledge no studies have investigated changes in gene expression upon the application of an enzymatically hydrolyzed animal protein-based biostimulant. We expected that the observed significant changes in salicylic acid concentration affected gene expression of at least some of the genes related with the synthesis of salicylic acid (*ICS*), or the synthesis of auxin (*TAA1*) or the auxin transport (*PINS*) in accordance with the mechanism of salicylic acid involvement in lateral root growth described by Pasternak et al. (2019). However, gene expression analyses revealed that neither *ICS*, *TAA1* nor *PIN* expression was influenced by Pepton application during the two experiments (**Figures 5A, B**). Despite we did not find statistical differences, probably because of high interindividual variability, data suggest a tendency of Pepton to downregulate *ICS* and *TAA1* expression at day 4 in both experiments. It has been shown a negative regulation of *ICS* expression by SA in *Arabidopsis thaliana* exposed to ozone (Ogawa et al., 2007) and auxin was also proposed to play a role in negative feedback regulation of *TAA1* in Arabidopsis (Suzuki et al., 2015), facts that could be a possible explanation for the results in experiment 1.

A Model Linking an Enzymatically Hydrolyzed Animal Protein-Based Biostimulant (Pepton) With Improved Root Growth

Overall, mechanisms of action of biostimulants are based on an enhancement of key physiological responses of plants to improve their development and yield. Regulation of gene expression, stimulation of amino acid biosynthesis, and increases of antioxidants, osmolytes, protein, or pigment contents have been hypothesized as mechanisms of action. In general, metabolic and hormonal effects, an improvement of nutrition efficiency, and physiological response to abiotic stress and to biotic stress have been reported (Yakhin et al., 2017). Casadesús et al. (2019) demonstrated positive effects of enzymatically hydrolyzed animal protein-based biostimulant (Pepton) on antioxidant system (plastochromanol-8), growth-promoting phytohormones auxins, cytokinins, and gibberellins, and defense-related phytohormones (jasmonic acid) in leaves of tomato plants under water stress, suggesting that this product acts by reducing the negative impact of stress and that the amino acid composition of the product (Phe, Tryp, and Tyr) may lead to the increases of both antioxidants and phytohormone observed since there are metabolic connections between Phe and Trp and IAA or between Tyr and tocochromanols. Our results are in agreement with this idea since under suboptimal conditions, reduced nutrient availability or low temperatures in experiments 1 and 2, respectively, Pepton exerted a positive effect on root growth, reducing the negative impact of stress. While in the leaves of plants under water stress Pepton affected auxin but not SA, in the roots of plants under nutrient deficiency Pepton affected mainly SA contents. This can be understood considering that plant responses to stresses are stress and organ specific so in each situation the plant stress response and the contribution of Pepton in alleviating negative impact of stress were differentially coupled. In the present study, it seemed that different mechanisms could be involved in the observed stimulation of root growth, both primary and lateral, by the biostimulant (**Figure 6**). First, as the product is a hydrolyzed protein-based product, it can be a source of amino acids, increasing their availability to the plant and this results in the stimulation of both primary and lateral growth observed in experiment 2 (in which roots had no restriction in longitudinal root growth, **Figure 6A**).

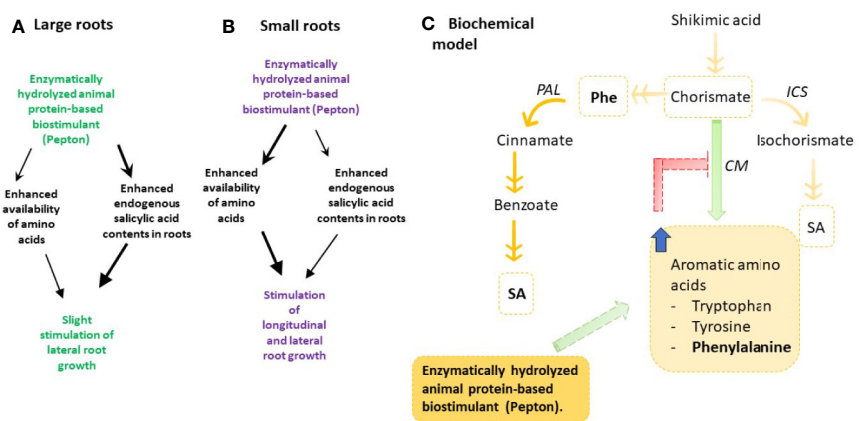


FIGURE 6 | Model proposed to explain the molecular mechanism of action of an enzymatically hydrolyzed animal protein-based biostimulant (Pepton). **(A)** Experiment 1, plants with large roots exposed to nutrient deficiency, **(B)** Experiment 2, plants with small roots exposed to suboptimal temperatures. Note that the hormonal pathway (salicylic acid increases) appeared to play a major role in experiment 1 than in experiment 2. **(C)** Integrated model proposed to explain the increased salicylic acid content. Note that an increased Phe availability provided by Pepton may stimulate diversion of the salicylic acid pathway through PAL instead of ICS. Orange arrows represent biosynthetic pathway steps, green and red arrows represent positive or negative relationships, respectively. Blue arrow represents an increase on the content of aromatic amino acids. Phe, phenylalanine; PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; CM, chorismate mutase.

These observed changes in root growth might be related to the effects of the biostimulant promoting nitrogen metabolism with possible changes in expression of nitrogen-related genes, as Santi et al. (2017) had reported using protein hydrolysates in maize seedlings. They reported that the presence of peptides rather than free amino acids was important to the effect on root growth highlighting a specific role of small peptides (1500-2000 Da) on the regulation of root growth. Peptides of the enzymatically hydrolyzed animal protein-based biostimulant (Pepton) are around 2000-3000 Da, from which 66% of them are considered short-chain peptides, thus they might exert a positive effect on regulation of root growth. However, this remains to be proved as we did not analyze the nitrogen metabolism in this study.

Second, when longitudinal root growth was physically restricted in experiment 1 and plants were exposed to nutrient deficiency, amino acids appeared not only to be used for nitrogen metabolism as proposed above but also to produce enhanced endogenous concentrations of salicylic acid ("Treatment" $P < 0.001$), with a marginal effect on auxin concentrations ("Treatment" $P = 0.077$) (**Figures 2A, E**) to induce lateral root growth as a response to the stressful conditions (**Figure 6B**). This enzymatically hydrolyzed animal protein-based biostimulant contains aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine (which are derived from chorismate). The possible increased availability of amino acids might lead to chorismate accumulation since the activity of chorismate mutase (which is the first enzyme in the production of phenylalanine from chorismate) is known to be sensitive to feedback inhibition by phenylalanine accumulation (Gilchrist and Kosuge, 1980). That supposed accumulation of chorismate and increased availability of phenylalanine, the substrate of phenylalanine ammonia lyase (PAL), could induce the production of salicylic acid by the PAL biosynthetic pathway (**Figure 6C**; Dempsey et al., 2011). This might particularly occur in experiment 1, with restriction in longitudinal root growth, thus in this regard, salicylic acid demand could be increased due to the stress conditions caused by nutrient starvation in addition to the longitudinal limitation in root growth. Therefore, the stimulation of root growth shown here by an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) could be explained by the supposed enhanced availability of amino acids used by the plant in two main different ways. In one way, it seemed that plants could use these extra amino acid availability directly for growth (in addition with possible small peptide regulation on root growth) when no growth restriction was present (experiment 2) and, additionally, to produce salicylic acid (probably through the PAL biosynthetic pathway) when stress conditions and restriction of longitudinal growth were present (experiment 1, **Figure 6**). The marginal increases in auxin observed during experiment 1 ("Treatment" $P = 0.077$) might also be facilitated by an enhanced availability of the auxin precursor, tryptophan (**Figure 1**).

CONCLUSION

It is concluded that an enzymatically hydrolyzed animal protein-based biostimulant (Pepton) exerts a positive effect on the root growth of tomato plants by stimulating salicylic acid accumulation and enhancing lateral root growth. In addition, we propose that the enhanced salicylic acid content might be explained by the presence of aromatic amino acids, in particular of Phe, in Pepton composition, which can divert salicylic acid production through PAL independent of chorismate. Further research is needed to explore the functional contribution of changed hormone levels versus nutritive effects of Pepton and other enzymatically hydrolyzed animal protein-based biostimulants on root development and growth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JP and SM-B conceived and designed the experiments with the help of AC. MP-L performed and wrote the statistical analyses. AC, and MP-L performed experiments. AC prepared figures. SM-B wrote the first draft of the manuscript with the help of AC, MP-L, and JP. JP and AC prepared the final manuscript with the help of SM-B. All authors contributed to the article and approved the submitted version.

FUNDING

This study was partially funded by the European Union Regional Development Fund within the framework of the ERDF Operational Program of Catalonia 2014-2020 with the reference RD17-1-0096 (Generalitat de Catalunya, Spain) and by APC Europe, S.L. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

The authors are deeply indebted to Centres Científics i Tecnològics from the University of Barcelona for their technical assistance, particularly to the personnel of Tècniques separatives and Unitat de Genòmica. We also thank Serveis de Camps Experimentals from the University of Barcelona for their help in growing the plants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00953/full#supplementary-material

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Conflict of Interest: JP is employed by APC Europe, S.L., Granollers, Spain. This does not alter our adherence to all the Frontiers Plant Science policies on sharing data and materials. APC Europe, S.L. manufacture and sells Pepton 85/16, the biostimulant used in this experiment.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 07 July 2020
doi: 10.3389/fpls.2020.00916

A PGPR-Produced Bacteriocin for Sustainable Agriculture: A Review of Thuricin 17 Characteristics and Applications

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A wide range of prokaryotes produce and excrete bacteriocins (proteins with antimicrobial activity) to reduce competition from closely related strains. Application of bacteriocins is of great importance in food industries, while little research has been focused on the agricultural potential of bacteriocins. A number of bacteriocin producing bacteria are members of the phytomicrobiome, and some strains are plant growth promoting rhizobacteria (PGPR). Thuricin 17 is a single small peptide with a molecular weight of 3.162 kDa, a subclass IId bacteriocin produced by *Bacillus thuringiensis* NEB17, isolated from soybean nodules. It is either cidal or static to a wide range of prokaryotes. In this way, it removes key competition from the niche space of the producer organism. *B. thuringiensis* NEB17 was isolated from soybean root nodules, and thus is a member of the phytomicrobiome. Interestingly, thuricin 17 is not active against a wide range of rhizobial strains involved in symbiotic nitrogen fixation with legumes or against other PGPR. In addition, it stimulates plant growth, particularly in the presence of abiotic stresses. The stresses it assists with include key ones associated with climate change (drought, high temperature, and soil salinity). Hence, in the presence of stress, it increases the size of the overall niche space, within plant roots, for *B. thuringiensis* NEB17. Through its anti-microbial activity, it could also enhance plant growth *via* control of specific plant pathogens. None of the isolated bacteriocins have been examined as broadly as thuricin 17 on plant growth promotion. Thus, this review focuses on the effect of thuricin 17 as a microbe to plant signal that assists crop plants in managing stress and making agricultural systems more climate change resilient.

Keywords: plant growth promoting rhizobacteria bacteriocins, *Bacillus thuringiensis* NEB17, anti-microbial activity, signal molecules, phytomicrobiome

OPEN ACCESS

Edited by:
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Reviewed by:
Claire Prigent-Combaret, UMR5557 Ecologie Microbienne (LEM), France
Francisco Javier López-Baena, Universidad de Sevilla, Spain

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *31 March 2020*
Accepted: *04 June 2020*
Published: *07 July 2020*

Citation:
Nazari M and Smith DL (2020) A PGPR-Produced Bacteriocin for Sustainable Agriculture: A Review of Thuricin 17 Characteristics and Applications. Front. Plant Sci. 11:916. doi: 10.3389/fpls.2020.00916

INTRODUCTION

Microbes produce antimicrobial substances to compete with each other for nutritional resources and niche space. These excreted microbial substances comprise a range of types: broad-spectrum non-ribosomal antibiotics, metabolic products (organic acids), lytic agents (lysozymes), and bacteriocins (Riley, 1998). Bacteriocins are ribosomally synthesized antibacterial peptides secreted

by bacteria (Arnison et al., 2013). They are distinct for antibiotics in that they inhibit organisms closely related to the producer strains, active at very low concentrations and are formed in the ribosome (Mak, 2018). Bacteriocins are bactericidal and/or bacteriostatic – inhibiting growth of target organisms – depending on identity, growth conditions, growth stage of the target strain, and on bacteriocin concentration (Nes et al., 2006). Most bacteria, Gram-negative or Gram-positive produce at least one type of bacteriocin; archaea may produce bacteriocin-like antimicrobials known as archaeocins (Riley and Wertz, 2002; Cotter et al., 2005). The "bacteriocin" concept was introduced in 1953. The proteinaceous nature of bacteriocins means they can be degraded in the digestion system of animals, allowing them to be used as natural preservatives in foods (Cleveland et al., 2001). Most research around bacteriocins has been conducted on lactic acid bacteria (LAB), known for their biopreservative potential in the food industry, and often produced by "generally recognized as safe" (GRAS) microbial strains (O'sullivan et al., 2002). While bacteriocins can be effective biocontrol agents in the food industry and medicines, less effort has been focused on their potential for agricultural application. Simultaneously, there is the need to reduce the negative effects of chemical fertilizers, herbicides, and pesticides on the environment, with a view to achieving environmentally sustainable agriculture. The main objective of this review is to summarize the characteristics of thuricin 17 and provide knowledge regarding its efficacy in plant growth promotion and resistance to abiotic stresses. We attempt to illuminate the promising possibility of bacteriocins as biostimulant agents for the agriculture sector.

BIOSYNTHESIS AND MODE OF ACTION OF BACTERIOCINS

Most bacteriocins are synthesized as biologically inactive peptides with an N-terminal leader peptide, holding the molecule in an inactive configuration. The N-terminal sequence plays a major role in interactions with the excretory apparatus and is also recognized by enzymes responsible for modifications, in the case of post-translationally modified bacteriocins (Cotter et al., 2005). However, a growing number of newly identified bacteriocins lack leader sequences and are active immediately after translation (Fujita et al., 2007). Specific immune proteins, encoded in the genome, are required for expression of bacteriocins, allowing the producer bacterial cell to resist the bacteriocin action (Rameshkumar et al., 2016). Production of bacteriocins and immune proteins is often mediated by quorum-sensing mechanisms, which may also be induced by environmental stressors (Nes and Eijsink, 1999). The killing mechanism for most bacteriocins is pore formation in cell membranes and enzyme activity, particularly nucleases against DNA, rRNA, and tRNA (Bizani et al., 2005; Chavan and Riley, 2007; Gillor and Ghazaryan, 2007). Bacteriocins are strongly cationic peptides that easily bind to the membrane bilayer of negatively charged phospholipids. The interaction between bacterial target membranes and the hydrophobic elements of bacteriocins produces non-specific ionic channels; pore formation causes leakage of intracellular components, such as ions, ATP, and small proteins, collectively leading to cell death (Bharti et al., 2015; **Figure 1**). Modes of bacteriocin action are related to the peptide's primary structure. Since classification of bacteriocins is based on structure, bacteriocins belonging to the same class have similar modes of action (Iwatani et al., 2011).

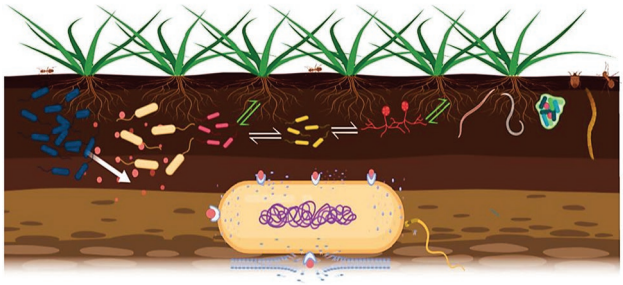


FIGURE 1 | Signal exchanges in the phytomicrobiome are indicated; white arrows for microbe-to-microbe signals and green arrows for plant-to-microbe and microbe-to-plant signals. Bacteriocins can act as microbe-to-microbe and microbe-to-plant signal molecules as well. Producer strain (blue color) excretes bacteriocin against closely related strains. The bacteriocins bind to the transport/receptor proteins in the outer membrane of the target cell and pass through it by various mechanisms, then form pores that result in efflux of proteins, ATP, and ions, causing cell death.

BACTERIOCIN CLASSIFICATION

Bacteriocins excreted by Gram-negative bacteria typically fall into four categories based on their size; to be specific: colicins, colicin-like bacteriocins, microcins, and phage tail-like bacteriocins (Chavan and Riley, 2007). Bacteriocins of Gram-positive bacteria are more discussed in more detail as our target bacteriocin, thuricin 17 is produced by a Gram-positive bacterium. These bacteriocins are grouped into four classes, based on their genetic and biochemical characteristics or the presence of post-translational modifications, molecular weight, heat stability, proteolytic enzyme stability, presence of disulfide or monosulfide bonds, and cidal method: (1) Class I are small post-transitionally modified bacteriocins (<5 kDa, 19–37 amino acids) containing the unusual amino acids lanthionine and methyllanthionine (hence the name lantibiotics) that have negative or no net charge and target indispensable catalytic enzymes of vulnerable species (Deegan et al., 2006), and they are heat stable peptides and target the skeleton of the cell wall (Sahl and Bierbaum, 1998; González-Martínez et al., 2003). This class is divided to subclasses *Ia* and *Ib*: the first being positively charged bacteriocins that kill by pore formation. Nisin, a member of this class, is the only bacteriocin regarded as safe for human consumption (McAuliffe et al., 2001; Gharsallaoui et al., 2016). The second subclass includes bacteriocins with rigid, globular structures acting by inhibition of catalytic enzymes required for peptidoglycan synthesis in target cells (Ditu et al., 2014). (2) Class II bacteriocins are heat-stable with molecular weights less than 10 kDa, and

they are non-modified and distinguished by a hydrophilic N-terminal sequence (Heng et al., 2007). This class can be subclassified into four groups: subclass *IIa*, is the largest subclass, which includes antilisterial bacteriocins such as pediocin PA-1, with large potential in food preservation as well as medical use (Rodríguez et al., 2002; Fimland et al., 2005). Subclass *IIb* consists of multi-component bacteriocins requiring at least two different peptides, further subdivided into synergistic (S) and enhancing (E) inhibitory agents (Marciset et al., 1997; Flynn et al., 2002; Garneau et al., 2002). Subclass *IIc* are circular bacteriocins requiring cysteine residues for activity (Joerger and Klaenhammer, 1986). Subclass *IId* is comprised of one-peptide, linear bacteriocins possessing specific cidal methods related to their diversity of fundamental structures (Iwatani et al., 2011; Cotter et al., 2013). The bacteriocin focused on in this review, thuricin 17, is categorized in the latter subclass: a small single peptide with a molecular weight of 3.162 kDa and sharing N-terminal homology (DWTXWSXL) with bacteriocin F4 synthesized by *Bacillus thuringiensis* spp. kurstaki strain BUPM4 (Kamoun et al., 2005). The similar amino acid sequence of these two bacteriocins indicates a possible specific role of bacteriocins of this sequence and similar modes of action (Gray et al., 2006b). (3) Class III includes large peptides (>30 kDa), which are divided into heat-labile lytic bacteriocins, lysing the bacteria cell wall in an enzymatic manner, and heat-labile non-lytic bacteriocins that disturb glucose transfer or metabolism (Joerger and Klaenhammer, 1986). (4) Class IV are complex circular bacteriocins with lipid or carbohydrate moieties making them susceptible to glycolytic or lipolytic enzymes (Lewus and Montville, 1991; Wirawan et al., 2007).

RHIZOSPHERE MICROBIOME BACTERIOCINS

Of the various microbial populations present in the rhizosphere, bacteria are the most abundant microorganisms (Kaymak, 2010). Bacteriocin producer strains can be present in the rhizosphere, and some strains are plant growth promoting bacteria (PGPR; Subramanian and Smith, 2015). A diverse group of signal molecules (microbe-to-plant, plant-to-microbe, and microbe-to-microbe) are exchanged in the rhizosphere (**Figure 1**) and govern the establishment of successful plant-microbe relationships (Smith et al., 2015). Plant-associated bacteria use bacteriocins as non-self-propagating suppressive agents causing hostility between closely related strains (Tagg et al., 1976), and bacteriocinogenic activity has been detected in nearly all rhizobial species (Triplett and Sadowsky, 1992) and plays a significant role in the phytomicrobiome. A narrow body of studies has taken shape around ecological impacts of bacteriocins in natural environments, with native strains. For example, production of multiple R-tailocins by *Pseudomonas chlororaphis* 30-84 is considered to be a competitive approach that contributes to the persistence of the producer strain in the wheat rhizosphere microbiome, as compared to bulk soil, perhaps there could be more bacterial interaction in the rhizoplane due to the greater population and nutrient availability (Dorosky et al., 2018). However, most research only focus on pairwise interactions of a bacteriocin producer and a target strain in a culture assay. The capacity for bacteriocin excretion by PGPR is reported, such as *Pseudomonas fluorescens* SF39a, isolated from the wheat rhizosphere secreting bacteriocins that inhibits the growth of the phytopathogenic *Pseudomonas* and *Xanthomonas* strains (Godino et al., 2016). "Rhizobiocins" are bacteriocins synthesized by rhizobia (Schwinghamer, 1975) such as production of bacteriocin-like substances from *Bradyrhizobium japonicum* and other slow-growing rhizobia (Gross and Vidaver, 1978), some rhizobial strains associated with *Medicago* and *Rhizobium leguminosarum* bv. *viciae* (Wilson et al., 1998; Hafeez et al., 2005). It has also been reported that *R. leguminosarum* strains possess symbiotic plasmid pRL1J, which contains essential nodulation and nitrogen fixation genes as well as determinants for secretion of small, medium, or large bacteriocins (Schwinghamer and Brockwell, 1978; Hirsch, 1979; Hirsch et al., 1980). Interestingly, some bacteriocins play a significant role in nodulation competitiveness against specific strains. For example, molecular features and biological characteristics of rhizobiocin, produced by *R. leguminosarum* 248, provide nodulation competitive advantage over specific strains, either recently isolated or wild types ones (Oresnik et al., 1999). *Bacillus* strains produce antimicrobial substances, including peptides and lipopeptides, antimicrobials, and bacteriocins. The small bacteriocin cerin 7, produced by *Bacillus cereus*, was the first reported bacteriocin-like compound from a *Bacillus* species (Oscáriz et al., 1999). Many bacteriocins occur in the rhizosphere, for instance cerein8A from *B. cereus* (Bizani et al., 2005), Bac-GM17 from *Bacillus clausii* GM17 (Mouloud et al., 2013), H4, IH7, and Bac14B from *Bacillus subtilis* (Compaoré et al., 2013), that have potential for agricultural application. Because of a wide range of proteins it excretes, *B. thuringiensis* is the most studied among *Bacillus* species; it can be easily separated from closely related species by its ability to produce natural insecticides against diptera, coleoptera, and lepidoptera larvae (Schnepf et al., 1998; Palma et al., 2014). Until now, synthesis of 18 bacteriocins from *B. thuringiensis* have been reported (Mojgani, 2017), such as thuricinS, thuricin7, entomocin110, morricin269, and tochicin (Cherif et al., 2001, 2008; Chehimi et al., 2007; De la Fuente-Salcido et al., 2008). However, bacteriocin production by *Bacillus* PGPR is poorly understood and none have been studied for plant growth promotion as extensively as thuricin 17, discovered in our laboratory (Smith et al., 2008) and produced by *B. thuringiensis* NEB17 (BtNEB17), a non-symbiotic endophytic bacterium isolated from soybean root nodules. Co-inoculation of this strain with *B. japonicum* 532C promoted soybean root nodulation, plant growth, and yield (Bai et al., 2003). Subsequently, a compound was isolated from the growth medium in which BtNEB17 was cultivated, and named thuricin 17 (Gray et al., 2006a).

CHARACTERISTICS OF THURICIN 17: A NOVEL BACTERIOCIN FROM CLASS *IId*

Thuricin 17 is synthesized during mid-exponential growth and continues through to the stationary phase, thus it would seem to be a secondary metabolite. The nucleotide sequence of the gene region encoding thuricin 17 indicated that there are three

copies of the gene synthesizing this bacteriocin. There have been, over time, changes in the nucleotide sequences of the three genes, but all the changes are at the third codon position and code for redundancies, so that the genes all code for the same protein, suggesting constraints on evolution of the genes (Lee et al., 2009). The dual function (bacteriocin and microbe-to-plant growth promoting molecular signal) nature of this protein might be the constraint: it both inhibits a range of bacteria and triggers plant growth (Lee et al., 2009). To understand the antimicrobial activity of thuricin 17, a range of *Bacillus* and *non-Bacillus* species were studied; results indicated no inhibitory effect on nodulating rhizobia and other PGPR strains (Gray, 2005). However, thuricin 17 acts as inhibitor to *Escherichia coli*, a unique finding regarding this peptide since it is uncommon for Gram-positive bacteria to inhibit Gram-negative bacteria (Gray et al., 2006a). Thuricin 17 is highly resistant to denaturation between −20 and 100°C, and is biologically stable across a pH range of 1.0–9.25 (Gray et al., 2006a). Bacteriocins can have a cysteine residue in the C terminus, among the last three positions, which can permit formation of a disulfide bridge, allowing folding of the peptide into a cluster (Oscáriz and Pisabarro, 2001). The presence of four cysteine residues in thuricin 17 allows for the possible formation of two disulfide bridges. This might be the reason for stability of this peptide to extreme temperatures and pH levels (Gray et al., 2006b). The mode of action of the bacterial peptide is both bactericidal and bacteriostatic (Gray, 2005). *B. cereus* ATCC 14579 has been observed to manifest a static effect whereby *B. thuringiensis* spp. *thuringiensis* Bt 1627 was able to recover and showed delayed growth, suggesting that the mechanism involved either degradation of a lethal peptide or that there had been a shift in gene expression to allow resumption of growth (Gray et al., 2006a).

POTENTIAL ROLE OF THURICIN 17 AS A PLANT BIOSTIMULANT

Bacteriocin excretion provides producer strains with an advantage, through significant reduction of direct competitor populations, allowing improved performance and survival of the producer strain. PGPR producing bacteriocins benefit from this competitive ability to inhibit closely related strains and thus clearing the niche space for themselves (Riley and Wertz, 2002). A bacteriocin that also promotes plant growth and development through mechanisms such as a decrease in the population of root associated plant-bacterial pathogens, would result in more vigorous plants (Subramanian and Smith, 2015). However, another advantage that extracellular PGPR (ePGPR) could provide is exemplified by the bacteriocin producing *B. thuringiensis* NEB17 which was shown to have no harmful effects on nodulating rhizobia and a range of other known PGPR, such as *Serratia proteomaculans* 1-102, 2-68, *Pseudomonas putida*, and other *Bacillus* species such as, *Bacillus licheniformis* Alfa-Rhiz and *B. subtilis* NEB 5 and NEB4 (Gray, 2005). Perhaps, because various species of PGPR occupy different niches in the rhizosphere, less interspecies competition occurs among them, such as rhizobial PGPR occupying the interior of nodule cells (Rachwał et al., 2016), versus *Bacillus* PGPR present in the nodule cortex (Spratt, 1919). Thus, bacteriocins may target PGPR types, which are likely to compete with the producer strain most directly, and often these are closely related strains with similar physiologies and requirements. In this way, the bacteriocin expands available niche space for the producer strain by eliminating potential competitors.

Positive correlations, indicating potential positive interactions, between ePGPR bacteriocin production and nodulation by intracellular PGPR (iPGPR) indicate another mechanism of plant growth promotion (Prudent et al., 2015). Co-inoculation of *B. thuringiensis* NEB17 with *B. japonicum*, isolated from soybean root nodules, enhanced soybean nodulation (Bai et al., 2003). Thuricin 17, produced by *B. thuringiensis* NEB17, increases plant growth through direct and indirect mechanisms (**Table 1**). Indirect mechanisms of action for this "signal" molecule include induction of plant disease resistance (Mabood et al., 2014) and inhibition of susceptible pathogenic strains by binding to receptors or the cell membrane/wall, leading to an increase in ecological niche space for producer strains or nodulation of associated plants (Gray and Smith, 2005).

TABLE 1 | Examples of thuricin 17 application to promote plant growth and resistance to abiotic stresses.

Crop	Growth condition	Thuricin 17 mode of action	Reference
Arabidopsis thaliana	Salt stress	More than two fold changes in activation of some important carbon, energy, and antioxidant metabolism pathway proteins including PEP-carboxylase, Rubisco-oxygenase, and pyruvate kinase, leading to mitigation of stressful conditions	(Subramanian et al., 2016b)
A. thaliana	Salt stress	Increased levels of IAA (85%) and SA (42%) and decreased gibberellins, cytokinins and jasmonate, causing amelioration to salt stress	(Subramanian, 2014)
Corn (*Zea mays*)	Non-stressful	Enhanced leaf area and dry weight at 3 leaf stage	(Lee et al., 2009)
Canola [*Brassica napus* (L.)]	Stressful cold temperature and salinity	Promoted dry biomass and root development	(Schwinghamer et al., 2016a)
Soybean (*Glycine max*)	Non-stressful	Induced defense system: enhanced production of liginification-related enzymes and their isoforms, peroxidase and superoxide dismutase enzymes (antioxidative enzymes)	(Jung et al., 2008, 2011)
Soybean	Non-stressful	Provided competitive advantage to the nodulating stain when thuricin 17 was applied as root-drench on inoculated plants with *Bradyrhizobium japonicum* 532C so nodule number, root and shoot total biomass increased; foliar application also enhanced leaf area, leaf greenness, and shoot N concentration	(Lee et al., 2009)
Soybean	Water stress	Enhanced abscisic acid (ABA) levels in leaves and roots leading to root elongation which increased water and nutrient uptake	(Prudent et al., 2015)

Treatment with thuricin 17 enhanced production of phenolics, phenylalanine ammonia lyase activity (lignification-related enzymes), and also the levels of peroxidase and superoxide dismutase enzymes (antioxidative enzymes) in 2-week old soybean plants, indicates that it provoked defense system responses (Jung et al., 2008, 2011). Direct stimulation takes place when this compound binds to receptors in leaf or root tissues, and acts as a pseudo-stress signal leading to triggering of various metabolic pathways, resulting in enhancement of photosynthetic rates. Although thuricin 17 is quite stressful to some prokaryotes, it may induce a pseudo-stress response in plants (Gray, 2005). Generally, plants elevate photosynthetic rates under pathogen or insect challenge, to compensate for decreased photosynthesis in damaged tissues (Nowak and Caldwell, 1984). In the case of thuricin 17, the response has been induced without any stress to counteract, resulting in a net increase in growth (Gray and Smith, 2005). When thuricin 17 was root-drench-applied nodule number root, shoot, and total biomass of soybean was increased; foliar application also enhanced leaf area, leaf greenness, and shoot N concentration (Lee et al., 2009). Similarly, leaf area and dry weight of corn and soybean seedlings were enhanced by thuricin 17 treatment, indicating that this signal molecule is effective on both C3 dicot and C4 monocot species (Lee et al., 2009).

Research on thuricin 17 has demonstrated its promising role as a plant growth promoter under stressful conditions. As an example, thuricin 17 treated soybean plants showed a reduced impact by water deficit stress; application of thuricin 17 to soybean roots plus inoculation with N_2-fixing *B. japonicum* increased root and nodule biomass by 37 and 55%, respectively and also increased leaf area, photosynthetic rate, and abscisic acid levels in roots under water deficit stress (Prudent et al., 2015). Canola [*Brassica napus* (L.)] showed a positive response to thuricin 17 treatment, which caused reconfiguration of leaf arrangement plus enhanced biomass production and root development in peat pellets and plant culture vessel growth systems, under stressful temperatures and salinity conditions (Schwinghamer et al., 2016a). Only canola seeds treated with thuricin 17 developed roots under very stressfully low temperature (10/4°C) and salt stress conditions (Schwinghamer et al., 2016a). Likewise, plants treated with thuricin 17 produced one more leaf per plant than the control treatment and other biostimulant treatments at 30/30°C, which is very stressful for a temperate zone crop such as canola (Schwinghamer et al., 2016b). Rosettes of *Arabidopsis thaliana* Col-0 treated with thuricin 17 had decreased levels of cytokinins, gibberellins, jasmonic acid, and abscisic acid at 24 h after treatment with thuricin 17, and increased levels of indole-3-acetic acid (IAA; 85%) and salicylic acid (SA; 42%) compared to controls (Subramanian, 2014).

A proteomic study indicated more rapid and efficient mobilization of carbon, nitrogen, and storage proteins of soybean seeds treated with thuricin 17, resulting in enhanced germination under salt stress (Subramanian et al., 2016a). Treatment of 3-week old *A. thaliana* plants with thuricin 17 resulted in alteration of carbon and energy metabolism pathways under unstressed and salt stress conditions: PEP carboxylase, rubisco-oxygenase, pyruvate kinase, and proteins of the light harvesting complex, energy and antioxidant pathways were all increased by thuricin 17 treatment, mitigating salt stress effects (Subramanian et al., 2016b). Collectively, these findings highlight the role of thuricin 17 as a microbe-to-plant signal stimulating plant growth, particularly under conditions of environmental stress. Thuricin 17 is the only bacteriocin examined in such depth. Currently, we are conducting studies to discover full mode of action of thuricin 17, and its role in mitigation of either abiotic or biotic stress; for the latter as biocontrol agents, we are still unsure as only *in vitro* antagonism experiments have been conducted and results are still unpublished. To be a successful biocontrol agent, the bacteriocin needs to be examined in plant, to compete with phytopathogens. If the results are promising, thuricin 17 would be of great interest for commercial application.

CONCLUSIONS

Global demand for agricultural produce is on the rise and the productivity of crops must be increased, even in the face of developing climate change conditions. Biomolecules produced by PGPR are of great interest in this capacity. Overall, thuricin 17 acts as a signal molecule to promote plant growth and development, particularly under stressful conditions, through a range of mechanisms; changes in carbon, energy, and antioxidant metabolism pathway protein activities, induction of synthesis of enzymes related to plant defense systems, increases in photosynthetic rate, stimulated production of some phytohormones such as IAA, SA, and ABA, and modification of the root system to better uptake of water and nutrients. The potential role of PGPR excreted compounds that are both bacteriocins and plant growth promoters presents inspiring possibilities and research opportunities. They stimulate plant growth, in part through alleviation of abiotic stress effects and could allow more sustainable management in agriculture, plus increased resilience to climate change conditions. More studies should be conducted to elaborate the biocontrol potential/impact of the compound, examining the ecological role of thuricin 17 in the natural environment and working to discover the thuricin 17 receptor, to determine its action on signaling pathways within plant cells.

AUTHOR CONTRIBUTIONS

MN assembled the literature and developed a first version of the manuscript. DS provided the initial perspective and provided editorial and conceptual input as the manuscript development progressed. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported through funding supplied by Agriculture and Agri-Food Canada, through the Eastern Canadian Oil-seed Development Alliance.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 July 2020
doi: 10.3389/fpls.2020.00852

Phaeophyceaean (Brown Algal) Extracts Activate Plant Defense Systems in *Arabidopsis thaliana* Challenged With *Phytophthora cinnamomi*

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Seaweed extracts are important sources of plant biostimulants that boost agricultural productivity to meet current world demand. The ability of seaweed extracts based on either of the Phaeophyceaean species *Ascophyllum nodosum* or *Durvillaea potatorum* to enhance plant growth or suppress plant disease have recently been shown. However, very limited information is available on the mechanisms of suppression of plant disease by such extracts. In addition, there is no information on the ability of a combination of extracts from *A. nodosum* and *D. potatorum* to suppress a plant pathogen or to induce plant defense. The present study has explored the transcriptome, using RNA-seq, of *Arabidopsis thaliana* following treatment with extracts from the two species, or a mixture of both, prior to inoculation with the root pathogen *Phytophthora cinnamomi*. Following inoculation, five time points (0–24 h post-inoculation) that represented early stages in the interaction of the pathogen with its host were assessed for each treatment and compared with their respective water controls. Wide scale transcriptome reprogramming occurred predominantly related to phytohormone biosynthesis and signaling, changes in metabolic processes and cell wall biosynthesis, there was a broad induction of proteolysis pathways, a respiratory burst and numerous defense-related responses were induced. The induction by each seaweed extract of defense-related genes coincident with the time of inoculation showed that the plants were primed for defense prior to infection. Each seaweed extract acted differently in inducing plant defense-related genes. However, major systemic acquired resistance (SAR)-related genes as well as salicylic acid-regulated marker genes (*PR1*, *PR5*, and *NPR1*) and auxin associated genes were found to be commonly up-regulated compared with the controls following treatment with each seaweed extract. Moreover, each seaweed extract suppressed *P. cinnamomi* growth within the roots of inoculated *A. thaliana* by the early induction of defense pathways and likely through ROS-based signaling pathways

OPEN ACCESS

Edited by:
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University of Padua, Italy

Reviewed by:
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Nanjing Agricultural University, China
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Cape Breton University, Canada

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *31 March 2020*
Accepted: *27 May 2020*
Published: *07 July 2020*

Citation:
Islam MT, Gan HM, Ziemann M, Hussain HI, Arioli T and Cahill D (2020) Phaeophyceaean (Brown Algal) Extracts Activate Plant Defense Systems in Arabidopsis thaliana Challenged With Phytophthora cinnamomi. Front. Plant Sci. 11:852. doi: 10.3389/fpls.2020.00852

that were linked to production of ROS. Collectively, the RNA-seq transcriptome analysis revealed the induction by seaweed extracts of suites of genes that are associated with direct or indirect plant defense in addition to responses that require cellular energy to maintain plant growth during biotic stress.

Keywords: seaweed, *Ascophyllum nodosum*, *Durvillaea potatorum*, RNA-Seq, *Phytophthora cinnamomi*, *Arabidopsis thaliana*

INTRODUCTION

Plants have evolved marvelous interactive and adaptive systems to grow in challenging and changing environments, including the activation of plant defense response systems. Plants are continually exposed to adverse conditions in their environment whether they be under cultivation or as part of a natural system. Adverse growing conditions can lead to compromised plant growth, reproduction and productivity, and can be abiotic and biotic factors that may occur simultaneously. Abiotic factors are those such as drought and soil salinity while biotic factors include insect herbivory and disease caused by various pathogens. Employing new ways to activate plant-defense-response systems to counteract adverse factors could be transformative for agriculture and for enhancing biodiverse landscapes.

In this regard plant biostimulants, such as those made from seaweed extracts, are unique. Plant biostimulants are defined by a biological mode of action that utilizes plant mechanisms to provide their benefits such as enhanced tolerance to stresses, enhanced nutrient use and productivity (Brown and Saa, 2015). In Europe, the general principles used to justify plant biostimulant claims highlight that their effect is independent of nutrient content (Ricci et al., 2019). Plant biostimulants are used at low rates of application which differentiates their mode of action from synthetic nutritional fertilizers. The low dosage range is consistent with plant biostimulants having properties that accentuate plant response systems for better plant growth and improved tolerances. Many published studies have shown that biostimulants provide a multitude of plant growth benefits such as improved tolerances to abiotic and biotic stresses (Khan et al., 2009; Calvo et al., 2014). To achieve such a wide range of plant benefits across diverse plant families, implies that the molecular mechanisms underlying the plant responses are conserved, complex and pleotropic in character. Despite these insights their modes of action remain elusive.

Seaweed extracts are used successfully to improve agricultural productivity (Calvo et al., 2014; Arioli et al., 2015). A greater understanding of their biological modes of action will further enhance productivity gains in the future. There are a range of commercial seaweed-based products which are available off-the-shelf for commercial and home garden care and the majority of these claim that their use promotes plant growth, improves soil quality and/or enhances resistance against biotic and abiotic stress (Arioli et al., 2015). The effect of several of these products on abiotic and biotic stress mitigation and their mechanism of action has been explored in various studies (Khan et al., 2009; Shukla et al., 2019).

Our hypothesis was that plants treated with seaweed extract would increase their tolerances to subsequent stresses through the activation of a combination of plant defense responses. We envisaged this type of mode of action could be extended by combining different types of seaweed extracts.

The conditioning of plants to stress is an important feature for enhancing crop resilience and reducing productivity losses due to abiotic and biotic stresses (Kerchev et al., 2019). Plant conditioning is based on the molecular activation and priming of plant molecular defense systems so enhanced plant tolerance is exhibited upon subsequent stress occurrences (Martinez-Medina et al., 2016). Importantly, pre-treatment of plants using seaweed extracts is a practical approach to proactively initiate the conditioning phenomenon and was incorporated in our experimental design.

This study used a unique combination of approaches for new insights into the effect of different types of seaweed extracts on the activation of plant defense systems. Here, we used three different seaweed extracts, different plant response time points for assessments, a plant pre-treatment approach (to apply the different seaweed extracts) and the well-studied model system of *Arabidopsis thaliana* with the root pathogen *Phytophthora cinnamomi*. Three seaweed-derived extracts, namely extracts from the brown algae *Durvillaea potatorum* (native to the southern hemisphere) and *Ascophyllum nodosum* (native to the northern hemisphere), were used either separately or in a mixture, to treat Arabidopsis plants and then to compare activation of their plant defense systems. The model system of *Arabidopsis thaliana* with the root pathogen *Phytophthora cinnamomi* was utilized to trigger and synchronize the abiotic attack.

Phytophthora cinnamomi is an oomycete pathogen with an extremely wide host range. It is a notoriously aggressive forest pathogen and is considered a major threat to natural ecosystems in Australia (Cahill et al., 2008; Hardham and Blackman, 2018; Costa et al., 2020). *P. cinnamomi* is also a serious threat to horticultural, ornamental and nursery industries and, for example, causes one of the most damaging diseases of avocado. The pathogen infects the feeder roots and often the trunk of larger species causing disease that leads to branch die-back, loss of production and eventual death (Reeksting et al., 2016).

To characterize the plant defense response systems upon pathogen inoculation we used molecular and cellular techniques. High throughput RNA-sequencing was used at key plant response time points. This approach complimented the excellent transcriptomics reports on the action of biostimulant extracts (Nair et al., 2012; GonñI et al., 2016; Jithesh et al., 2019; Omidbakhshfard et al., 2020), particularly with respect to

the ability of biostimulants to alter the outcome of root pathogen infection. To further confirm the defense transcriptome induction, Reactive Oxygen Species (ROS) were investigated by staining for hydrogen peroxide in the seaweed extract treated plants at 12 h post-inoculation (hpi) with *P. cinnamomi*. Microscopic analysis was performed to confirm that the pathogen had infected the inner root cell layers, and the extent of infection quantified using quantitative PCR. A plate assay was used to confirm that the seaweed extracts had no direct effect on pathogen growth.

We report that the extracts from two different brown seaweeds and their combination, activated plant defense responses upon pathogen-induced stress. Plants treated with each extract had different but overlapping transcriptomic gene expression profiles, and showed higher ROS levels that coincided with the activation of plant defense.

MATERIALS AND METHODS

Arabidopsis thaliana Growth Conditions

Seeds of *Arabidopsis thaliana* ecotype Ler (LEHLE, TX, United States[1]) were surface-sterilized within a 1.5 mL microcentrifuge tube that contained 50% v/v ethanol (Chem-supply, Australia), 5% of H_2O_2 30% solution (Sigma-Aldrich, Australia) for 5 min and subsequently rinsed three times in sterile distilled water (sdH_2O) and suspended in 0.2% (w/v) water agar. The seed suspension was stored in the dark at 4°C for 2−3 days. The stratified seeds were then seeded into Petri dishes (9-cm-diameter) containing Murashige and Skoog basal medium 0.44% (w/v) (Sigma-Aldrich, Australia) supplemented with 3% sucrose (w/v) (Chem-supply, Australia) and 0.8% (w/v) bacteriological agar and adjusted to pH 5.7 with 1 M potassium phosphate dibasic or potassium phosphate monobasic (Sigma-Aldrich, Australia). Seeds were evenly distributed in Petri dishes by placing individual seeds on the agar surface with a 1000 μL pipette tip, 120 seeds per plate. Petri dishes were transferred to a plant growth chamber (Thermoline Scientific, Australia) under cool white fluorescent lights (100 μmol photons m^{-1} s^{-1}) with a 16:8 h (day: night cycle) at 21 ± 2°C for 14 days. Plants of uniform size were then selected for further use in experiments.

Plant Growth and Treatment With Seaweed Extracts

Plants were grown in a sand-based tube system that used commercial propagation sand (Bunnings, Australia) that was autoclaved and sterilized before adding to the tubes. The tubes used were 5 mL plastic disposable pipette tubes (Axygen, Australia) with a piece of cotton wool (Woolworths, Australia) inserted into the narrow end to form a plug that held the sand in place. Each tube was filled with sand to within 5 mm of the top and then 1 mL of diluted (1:400) seaweed extract or water as the control, was added at the top of the tube to just moisten the sand. The seaweeds used in this study are different, so the extracts are not identical, therefore we standardized the testing approach. The 1:400 dilution of the extracts were chosen because of (i) greenhouse and field studies demonstrating the efficacy for this dilution (Mattner et al., 2013, 2018), and (ii) by testing for root growth efficacy using the dilution as described previously (Arioli et al., 2015). For alignment with our previous greenhouse and field studies and the root growth testing, each of the seaweed extracts where standardized to 16% (w/w) soluble solids before preparing the 1 in 400 dilution for each seaweed extract. Three seaweed extracts designated as "AN" (an alkaline hydrolysis product from *Ascophyllum nodosum*), "DP" (an alkaline hydrolysis product from *Durvillaea potatorum*) and "AN/DP" (an alkaline hydrolysis product from both *A. nodosum* and *D. potatorum, Seasol*^{TM}) were used in this study. Single plants of *A. thaliana* seedlings were gently removed from the MS plate and the roots carefully placed within a 10 mm deep hole made by pushing the narrow end of another 5 mL tube into the sand. Following placement of the seedling roots within the hole a further 1 mL of diluted seaweed extract (1:400) or water was added to enclose the root system by the sand. Tubes were then placed in a plastic rack and transferred to the plant growth chamber under the conditions described in section "*Arabidopsis thaliana* Growth Conditions." Each day, and up until 6 days after placing the seedlings in the growth chamber, 700 μL of seaweed extract (1:400) was added to each tube or distilled water for the control.

Infection With *Phytophthora cinnamomi* Zoospores

Zoospores of *P. cinnamomi* were produced according to Islam et al. (2017) and the zoospore density adjusted to 1×10^5 zoospores/mL. Inoculation of the roots of plants grown in tubes took place on day seven whereby 700 μL of the zoospore suspension was carefully dispensed by pipette against the side wall of the plant growth tube just above the sand surface. The inoculated plants (8 plants/replicate/treatment) were then harvested at 0 h (i.e., immediately) and then at 3, 6, 12 and 24 h post-inoculation (hpi). To remove individual plants from a growth tube whilst avoiding damage to the root system a tube was briefly submerged in distilled water held within a container and the tube gently tapped to remove sand and the whole plant. The intact plant was then immediately placed with its roots submerged in water within a square plastic culture dish (10 × 10 cm) and the roots agitated gently to remove residual sand particles. Whole plants were gently and briefly dried on absorbent paper and frozen in liquid nitrogen followed by storage at −80°C. To confirm that roots had been inoculated, roots of eight plants from each treatment were sampled at 24 hpi and placed on PARPH medium (Islam et al., 2017) within 9cm-in-diameter Petri plates and examined for typical *P. cinnamomi* hyphal growth after 72 h incubation at 24°C in the dark. Images of whole root systems were also captured using a digital camera at 7 days after transferring the plants into the sand system and root length measured on individual plants with the aid of imageJ

[1]www.arabidopsis.com

software. Final root growth data represent the mean of three biological replicates (each replicate contained 8 plants) from two independent repeats.

Gene Expression Analysis by Semi-Quantitative PCR

The plants were grown and treated with seaweed extracts as described in section "*Arabidopsis thaliana* Growth Conditions" and "Plant Growth and Treatment With Seaweed Extracts," respectively. Then the plants were inoculated and harvested (8 plants for each time point for each treatment) at 0 h and then every 3 h until 9 hpi, as described in section "Infection With *Phytophthora cinnamomi* Zoospores." Total RNA was isolated from plant tissues using a TRIzol®-based RNA extraction system (Life Technologies, United States) according to the manufacturer's instructions. The quantity of RNA was measured using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, United States) and the ratio (>1.8) of sample absorbance at 260/280 was used to determine the purity of samples. All samples extracted were of high yield and purity. The isolated RNA samples were then treated with DNAse-1 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions to remove any residual gDNA. Then the Tetro cDNA synthesis kit (Bioline, Australia) was used to synthesize cDNA from isolated RNA according to the manufacturer's instructions.

Semi-Quantitative RT-PCR Conditions

The expression of genes involved in SA and JA mediated pathogen resistance pathways (*PR1*, *PR5*, *NPR1*, *PDF1.2*, and *THI2.1*) described by Lemarié et al. (2015) were examined using semi-quantitative PCR. The *actin* and *EF-1 alpha* genes were used as internal controls. The primer sequences of *NPR1*, *PDF1.2*, *THI2.1*, and *Actin* were as described by Eshraghi et al. (2011) and the primers for *PR1*, *PR5*, and *EF-1 alpha* were designed using primer3plus (**Supplementary Table 1**). PCR reactions were performed with GoTaq green master mix (Promega, United States) and each reaction contained 2 μL of cDNA and 0.5 μM of the respective primers. PCR cycles consisted of an initial denaturation step of 3 min at 95°C, followed by repetitions (28−36 cycles, depending on the primer set) of the following three steps: a 30 s denaturation step at 95°C, 30 s annealing step ranging between 54°C and 60°C, 1 min elongation step at 72°C and a final extension step at 72° for 5 min. Initial reactions were performed to determine the annealing temperature of each primer set and the appropriate cycle number of the PCR reaction. PCR products were analyzed on a 1% agarose gel with 0.5 × TBE buffer and visualized using gel red staining with a gel doc system. The final gel images are representative of two biological replicates from two experimental repeats.

Examination of the Host Transcriptome Using RNA-Seq

Plant Growth, Seaweed Extract Treatments, and Plant Inoculation

Arabidopsis thaliana plants were grown as described in section "*Arabidopsis thaliana* Growth Conditions" and seaweed extracts were applied as described in section "Plant Growth and Treatment With Seaweed Extracts." The plants were inoculated as described in section "Infection With *Phytophthora cinnamomi* Zoospores" and harvested at 0, 3, 6, 12, and 24 hpi (8 plants at each time point for each treatment). Control plants, treated with water alone, were also harvested at these times as described in section "Plant Growth and Treatment With Seaweed Extracts." The harvested plants were immediately frozen in liquid nitrogen and kept in a −80°C freezer until RNA was extracted. This experiment contained three biological replicates at each time point for each treatment (5 time points of four treatments including the control resulted in 60 samples for analysis).

RNA Extraction and cDNA Library Preparation

The total RNA was extracted from whole plants using a commercial kit (RNeasy Plant Mini Kit, Qiagen, Valencia, CA, United States) following the manufacturer's instructions. RNA concentration and integrity was then determined first using spectrophotometry (NanoDrop ND-1000 spectrophotometer, Thermo Fisher Scientific, United States) with absorbance ratio of A260/280 nm and A260/230 nm. RNA integrity was also confirmed with the 4200 TapeStation system (Agilent Technologies, Santa Clara, CA, United States). Only RNA samples with an A260/280 nm ratio between 2.0 and 2.1 and RNA integrity number (RIN) greater than 7 (Sigurgeirsson et al., 2014) were used for further analysis. DNA libraries were constructed from total RNA of control and inoculated samples using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® according to the manufacturer's instructions (New England BioLabs, United States). The quality of the libraries was assessed by using a 4200 TapeStation 6000 system and their quantities were measured by Qubit dsDNA BR assay kit (Thermo Fisher Scientific, United States). The library was sequenced using the NovaSeq 6000 platform (Illumina) in a paired-end 150 bp run.

Processing of Sequenced Reads

The sequencer-generated raw reads were pre-processed and mapped to the reference genome using CLC Genomics Workbench (version 8.5.1, CLC Bio, Arhus, Denmark). During pre-processing of RNA-Seq data, adapter sequences, reads with >10% of unknown bases, low quality reads (sequences with more than 50% bases with quality value ≤ 5) and ambiguous bases were removed to obtain high quality reads for further analysis. High quality reads were mapped to the reference *A. thaliana* genome using the default parameters of the Workbench software to generate normalized gene expression values in the form of reads per kilobase of transcript per million mapped reads (RPKM). Proportion-based statistical analysis of differentially expressed reads was performed using default parameters to identify differentially expressed genes (DEGs) between water treated and each seaweed treated sample harvested at different hours post-infection by *P. cinnamomi*. DEGs were then filtered based on a FDR-corrected *P*-value of <0.05 and a fold change of ≥1.5 for up-regulated DEGs and ≤−1.5 for downregulated genes. The Illumina RNA-Seq datasets analyzed for this study have been

deposited in the SRA database with the accession number of PRJNA609590[2].

Functional Classification of Up-Regulated DEGs

Gene ontology and KEGG

The Blast2go 5 PRO (B2G) program was used to perform GO functional classification into biological process, molecular functions and cellular components to analyze the up-regulated gene function distribution at a macro level. Further, the B2G program was also used for Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations for up-regulated DEGs by searching against the KEGG database[3].

Hierarchical clustering and heatmap visualization

For each treatment type, 30 genes with the largest sum of absolute *t*-test Z-scores were selected. With R version 3.6.2 (R Core Team, 2019), the heatmap.2 function was used to display Z-scores across the five time points. For multidimensional scaling analysis, the uniquely mapped read counts for each gene in each sample underwent library size normalization and distance estimation using the cmd scale function. Hierarchical clustering was performed using the hclust function with Spearman correlation values and a tree cut parameter of 0.67.

Pathway analysis

The Gaussian based *t*-test Z statistic (CLC Bio) was used to rank genes from most up-regulated to most down-regulated prior to Gene Set Enrichment Analysis (GSEA) with gene sets obtained from the Reactome and MapMan databases (Thimm et al., 2004; Naithani et al., 2016). Enrichment analysis was performed using the FGSEA R package version 1.12.0 (Korotkevich et al., 2019) with a significance threshold of FDR adjusted *p*-Values less than 0.05.

Quantitative PCR to Validate RNA-Seq Expression

The Tetro cDNA synthesis kit (Bioline) was used to synthesize cDNA from previously isolated RNA that was used for RNA-seq analysis. Briefly, 1 μg RNA was mixed with 1 μL random hexamer, 1 μL 10 mM dNTP mix (final concentration 0.5 mM), 1 μL ribosafe RNase inhibitor (final concentration 0.5 u/μL), 4 μL 5 × RT buffer, 1 μL tetro reverse transcriptase (final concentration 10 u/μL), and DEPC-treated water up to a total of 20 μL. Then the mix was incubated in the PCR machine according to the following order: initial incubation 25°C for 10 min followed by 45°C for 30 min and then the reaction terminated at 85°C for 5 min.

The primers of all tested genes were designed using primer3plus software (**Supplementary Table 2**) and annealing temperature of each primer pair was selected using gradient qPCR. The resulting qPCR product was analyzed via gel-electrophoresis to check that the correct gene product was obtained based on the primer design. Moreover, PCR efficiency of all genes was determined by a standard curve analysis of cDNA samples using a series of 10-fold dilutions of cDNA to determine the gene-specific PCR amplification efficiency for each primer pair used in RT-qPCR experiments. The real time PCR amplifications were carried out using SYBR Green detection chemistry. cDNAs were run in triplicate on 96 well reaction plates with the CFX Connect real time PCR (Bio-Rad, United States). 10 μL of reaction mixture containing 5 μL of iTaq^{TM} universal SYBR Green Mix (Bio-Rad, United States), 0.4 μL of each 10 μM of primer and 2 μL of diluted cDNA as template and 2.2 μL RNase/DNase free sterile water (Sigma-Aldrich, Australia). The following amplification program was used in all RT-qPCR reactions: 95°C for 3 min, 40 cycles of 95°C for 10 s and annealing temperature (54−60°C) for 30 s at optimized temperatures for specific candidate genes. The specificity of each amplification reaction was verified by a melting curve analysis after 40 cycles. No template controls (NTC) were included for each primer pair to avoid possible contamination of assay reagents. Three biological replicates were used for each time point and each reaction was run in triplicate for each target and reference gene. All samples were run in parallel with actin reference genes (*ACT2* and *ACT8*) to normalize cDNA loading. The relative expression values for each target gene were calculated against reference genes using the following equation according to Livak and Schmittgen (2001): $\Delta\Delta CT$ = (*CT*, Target – *CT*, reference gene) Time X − (*CT*, Target – *CT*, reference gene) Time 0.

Microscopic Examination of *P. cinnamomi* Infection of Roots of *A. thaliana*

To monitor the root infection process following inoculation with motile zoospores, the plants were grown with seaweed extracts or water as a control and inoculated with *P. cinnamomi* as described in section "*Arabidopsis thaliana* Growth Conditions," "Plant Growth and Treatment With Seaweed Extracts," and "Infection With *Phytophthora cinnamomi* Zoospores." Then the plants were harvested at 12, 24, 48, and 72 hpi for each extract treatment and the roots removed and stained using a trypan blue staining protocol (Wang et al., 2011). Briefly, harvested roots were transferred into 2 mL Eppendorf tubes containing diluted trypan blue solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 10 mg of trypan blue). The tubes were incubated in a heated water bath and boiled for 4 min. After cooling to room temperature, the samples were de-stained by replacing the staining solution with chloral hydrate solution (5 g chloral hydrate/2 mL water) for 24 h. The samples were finally mounted in 50% glycerol and viewed with a light microscope (Zeiss, Göttingen, Germany) using bright field illumination. Images were captured with a digital camera (Zeiss, Göttingen, Germany) mounted on the microscope. The final images are representative of three biological replicates (each with at least 5 plants) at each time point for each treatment.

[2]https://www.ncbi.nlm.nih.gov/sra/PRJNA609590

[3]http://www.genome.jp/kegg

P. cinnamomi Quantification Using qPCR

To quantify the amount of *P. cinnamomi* in *A. thaliana* roots following inoculation plants were first grown with seaweed extracts (AN, DP, and AN/DP) for 6 days and inoculated with *P. cinnamomi* as described in section "*Arabidopsis thaliana* Growth Conditions," "Plant Growth and Treatment With Seaweed Extracts," and "Infection With *Phytophthora cinnamomi* Zoospores." Then, the plant roots were harvested at 12, 24, 48, 72, and 96 h post-inoculation (hpi). The pathogen was quantified from harvested roots according to Engelbrecht et al. (2013). Briefly, DNA from a *P. cinnamomi* culture was extracted using PrepMan Ultra Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol and kept at −20°C until further use. DNA from root samples was extracted using the CTAB based method (**Supplementary Method 1**). The amount of plant genomic DNA present within the sample was quantified first by real-time PCR using primers (**Supplementary Method 1**) amplifying the A. *thaliana* a*ctin* gene. A normal one-step real-time PCR was conducted for the plant a*ctin* gene. The amount of plant DNA was calculated using a standard curve developed from a series of known concentrations of *A. thaliana* genomic DNA. The amount of *P. cinnamomi* DNA present within *A. thaliana* root samples was quantified using a nested real-time PCR method. LPV3 primers (Engelbrecht et al., 2013) were used in the outer first round of PCR, then LPV3N primers were used for the second round nested PCR to bind within the outer PCR product (**Supplementary Method 1**). The amount of pathogen DNA was calculated based on a standard curve developed from a series of known concentrations of *P. cinnamomi* DNA. Finally the quantity of *P. cinnamomi* was determined as ng of *P. cinnamomi* DNA/100 ng of *A. thaliana* DNA. The final amount determined represents the mean of three biological replicates from two independent experimental repeats.

Histochemical Localization of Hydrogen Peroxide (H_2O_2)

For hydrogen peroxide (H_2O_2) detection, the plants were grown and inoculated as described in section "*Arabidopsis thaliana* Growth Conditions," "Plant Growth and Treatment With Seaweed Extracts," and "Infection With *Phytophthora cinnamomi* Zoospores". At 12 hpi, the whole plants were harvested and were placed in a 2 mL micro centrifuge tube with 1 mL diaminobenzidine (DAB) (1 mg/ mL). The seedlings were incubated in the dark at room temperature for 3 h for H_2O_2 detection (Thordal-Christensen et al., 1997;Venus and Oelmüller, 2013). Samples were then transferred to and incubated in a decoloring solution (EtOH: lactic acid: glycerol = 1:1:1) at 80°C for 20 min (Venus and Oelmüller, 2013). Seedling roots were then visualized using a light microscope (Zeiss, Göttingen, Germany) with bright field illumination and images captured with a digital camera mounted on the microscope. The optical density of the colored precipitate was measured using imageJ software. The final images were representative of three biological replicates each with 5 plants.

RESULTS

The Influence of Seaweed Extracts on *A. thaliana* Root Growth and Infection by *P. cinnamomi*

Arabidopsis thaliana Ler seedlings were grown in a sand growth system (distilled water as a control and 1:400 seaweed extract as a treatment) and root lengths were measured 7 days after the commencement of treatment. All three seaweed extracts (AN, DP, and AN/DP) significantly enhanced root growth compared with the controls (**Supplementary Figure 1**). Moreover, root growth was observed to be significantly higher in extract-treated plants at the time of inoculation and the difference in root growth rate was maintained up to 96 hpi (Data not shown).

The quantitative measurement of the amount of *P. cinnamomi* in *A. thaliana* roots grown with seaweed extracts showed that overall there was less pathogen growth compared with the water control at 24, 48, and 72 hpi (**Figure 1A**). However, at 12 hpi, the amount of *P. cinnamomi* was higher in all three seaweed extract-treated roots compared to the water control. For the AN and DP seaweed extract- treated roots about the same amount of pathogen was found at 96 hpi, as was found in the water treated controls. Notably the combined extract (AN/DP) showed a plateauing of the amount of *P. cinnamomi* from 24 h onward to a level that was sustained well below that of the controls (**Figure 1A**).

Analysis of SA and JA Related Gene Expression

The expression of SA and JA-related genes, *PR1*, *PR5*, *NPR1*, *PDF1.2* and *THI2.1* was analyzed using semi-quantitative PCR and the *Actin* gene was used as an internal control to confirm even loading of DNA and reaction efficiencies of all cDNA samples prepared. A differential expression pattern was found for each resistance-related gene in all three seaweed extract treatments. Results showed that *Actin* expressed equally in all tested cDNA samples indicating the quality of cDNA and equal loading on the gel (**Supplementary Figure 2**). A higher expression of *PR1* was found in AN-treated plants at 3 hpi and the expression increased at 6 and 9 hpi. A similar trend was found for the AN/DP treatment. However, consistently higher expression from 3 hpi was observed for the DP treatment. A similar expression pattern (up-regulated at 3 hpi) was recorded for *PR5*. Moreover, the expression of *NPR1* was found to be consistently induced in all three treatments. The expression of *PDF1.2* was found to be higher only in those plants treated with DP and AN/DP. In addition, the expression of *THI2.1* was up-regulated at 3 hpi in plants treated with AN, and at 6 and 9 hpi in those plants treated with DP and AN/DP (**Supplementary Figure 2**).

Transcriptome Analysis of Plants Treated With Seaweed Extracts and Then Infected With *P. cinnamomi*

Confirmation of Pathogen Infection in Roots

Microscopic examination of *A. thaliana* roots grown with seaweed extracts and inoculated with *P. cinnamomi* revealed the

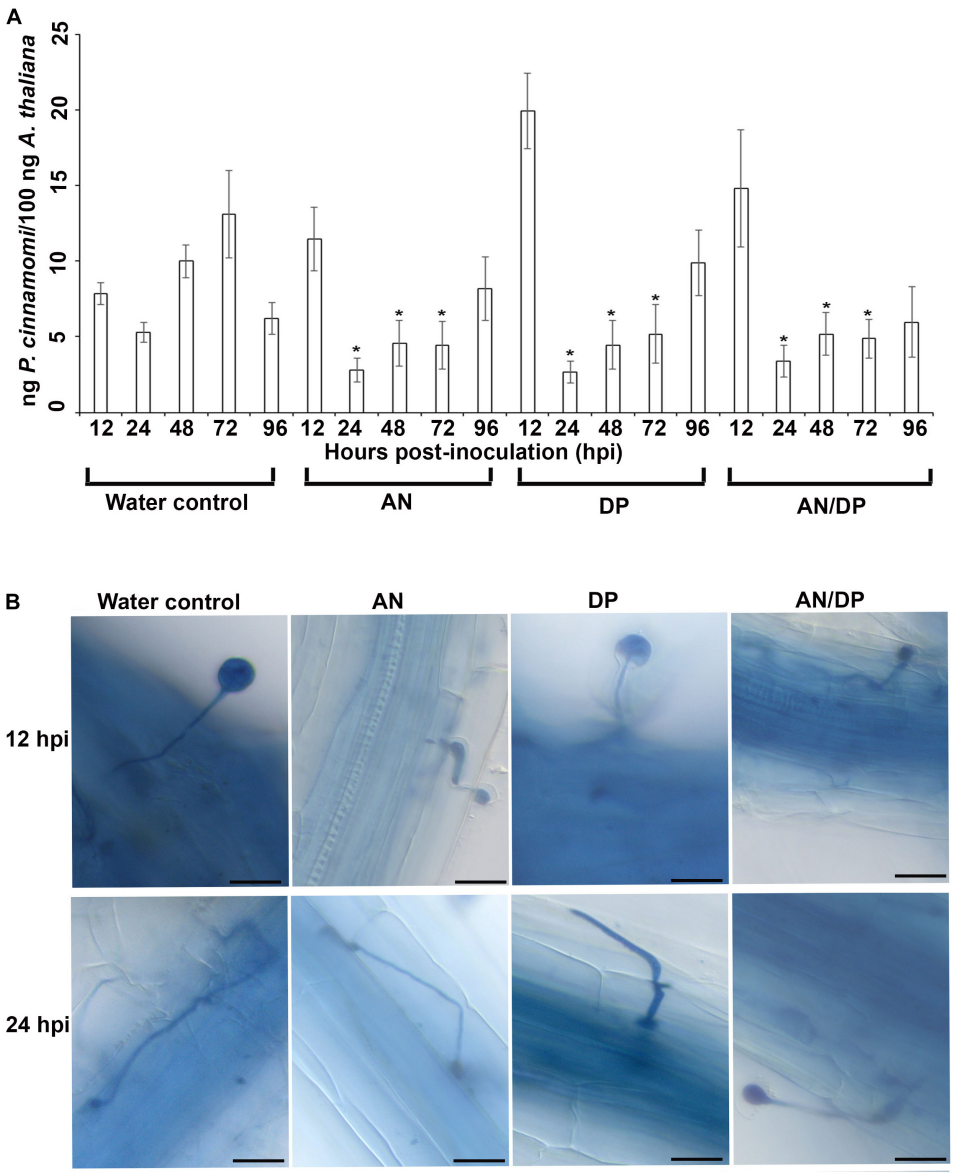


FIGURE 1 | Effect of seaweed extracts on *P. cinnamomi* infection in roots of *A. thaliana*. Plants were grown in a sand culture system with either seaweed extracts (AN, DP, and AN/DP) or water as a control for 6 days and then inoculated with *P. cinnamomi* on day 7. **(A)** Nested real time PCR quantification of *P. cinnamomi* DNA. Plant roots were harvested from 12 to 96 hpi. Data presented are from two experimental repeats each with three biological replicates. Error bars represent the standard error of means. *significant difference for amount of pathogen in different treatment compared to water control at $P = 0.05$ according to Duncan's multiple range test. **(B)** Whole roots of *A. thaliana* infected with *P. cinnamomi* zoospores following treatment of roots with seaweed extracts (AN, DP, or AN/DP). Images were captured at 12 and 24 hpi. Scale bar = 20 μM. Each image is representative of three biological replicates.

different patterns of penetration and establishment of infection (**Figure 1B**). This microscopic analysis confirmed that the system established and optimized for this study was one in which the plants were successfully inoculated and that the pathogen grew both on the root surface and within the root.

Overview of RNA-Seq Data and Mapping to the *A. thaliana* Reference Genome

The Nova-Seq platform generated 43−155 million reads (average length 151 bp, paired end reads) and these reads were processed through CLC genomics workbench to remove adapters and ambiguous reads from the samples. A read refers to the sequence of a cluster that is obtained after the end of the sequencing process which is ultimately the sequence of a section of a unique fragment. After trimming, more than 98% of reads were recovered as high quality reads to proceed for mapping to the reference genome (**Table 1**).

On average, more than 87% of the total reads were mapped to the reference *A. thaliana* genome. The reads not mapped to the reference genome were expected to be pathogen reads as

TABLE 1 | RNA-Seq read statistics before mapping and after quality selection and trimming.

Sample id	Total reads	Total nucleotides	Total reads after trimming	Percentage of reads after trimming
Water control				
H-0	54,233,072	7,492,370,850	53,448,230	98.22
H-3	51,132,182	8,447,132,374	50,478,514	98.70
H-6	107,420,061	16,220,429,261	105,873,930	98.64
H-12	51,528,171	6,934,219,584	51,256,437	99.27
H-24	52,952,768	7,824,829,664	51,259,041	96.95
AN treatment				
AN-0	66,404,596	10,027,093,996	65,450,105	98.62
AN-3	137,378,291	20,744,121,891	135,219,054	98.24
AN-6	152,472,398	23,023,332,098	149,964,428	98.49
AN-12	60,409,115	9,121,776,415	59,615,072	98.68
AN-24	155,067,084	23,415,129,684	153,620,985	99.33
DP treatment				
DP-0	47,324,764	7,146,039,364	47,164,165	99.64
DP-3	48,885,610	7,377,197,110	48,824,421	99.93
DP-6	45,391,111	6,854,057,711	45,343,642	99.89
DP-12	44,783,657	6,762,331,553	44,719,573	99.87
DP-24	46,672,822	7,047,596,122	46,627,376	99.90
AN/DP treatment				
AN/DP-0	43,800,423	6,613,863,823	43,421,661	99.23
AN/DP-3	49,173,252	7,425,161,052	49,052,075	99.80
AN/DP-6	48,539,686	7,328,092,586	48,140,503	99.20
AN/DP-12	78,530,707	11,858,136,807	78,425,815	99.87
AN/DP-24	65,967,493	9,961,091,393	78,425,815	99.72

Data are presented as the average of three biological replicates.

the samples were inoculated with *P. cinnamomi*. In addition, there were few reads that were mapped as broken pairs (**Supplementary Table 3**).

Overview of Differentially Expressed Genes (DEGs)

Differentially expressed genes (DEGs) were identified based on their expression value normalized through Reads Per Kilobase per Million mapped reads (RPKM) according to a previous study (Kasirajan et al., 2018). RPKM estimates the gene expression level of a gene normalized for both transcript length and library sequencing depth, allowing a direct comparison of expression levels within and between samples. The following parameters were considered to filter the DEGs of each treatment: FDR corrected *P*-value $\leq$ 0.05, fold change $\geq$ 1.5 for up-regulated genes, fold change $\leq$ 1.5 for down-regulated genes, each sample was compared with the respective water control. The highest number of up-regulated genes was found in the AN-treated samples harvested at 12 hpi followed by the AN/DP-treated samples harvested at 24 hpi. The highest number of down-regulated genes was found in the AN/DP-treated samples harvested at 3 hpi followed by those harvested at 6 hpi (**Supplementary Table 4**).

Functional Analysis of Up-Regulated DEGs

Comparison of number of DEGs between treatments and time points

Venn diagram analysis revealed that most of the DEGs from each of the three treatments were uniquely expressed at a specific time point (**Figures 2A–C**). For example, a total of 882 DEGs were found to be expressed in the AN treatment harvested at 12 hpi and among them 711 DEGs were uniquely expressed at this time point. Whereas there were 56 DEGs that were common to the 6 hpi time point, 37 DEGs were commonly expressed at the 24 hpi time point, 19 DEGs were commonly expressed at the 3 hpi time point and 13 DEGs at the 0 hpi time point.

Figure 2D shows that a total of 3,704 unique DEGs (expressed at least at one time point, filtered according to the criteria mentioned above) were found in the three treatments. Out of them, 926, 704 and 1027 were expressed in the AN, DP and AN/DP treatments, respectively. Moreover, 349 DEGs were commonly expressed in the AN & DP treatments whereas 277 DEGs were common to the AN & AN/DP treatments and 158 DEGs were common to the DP & AN/DP treatments. In addition, 263 DEGs were commonly up-regulated across all three treatments.

Gene ontology (GO)

The functional classification of up-regulated DEGs was analyzed using Gene Ontology (GO) and classified into three broad categories: molecular function, biological process and cellular component. These broad categories are very useful for identifying the key changes brought about by the treatments.

In the **molecular function** GO category, protein-binding and metal ion-binding were highly represented for all three treatments (**Figures 3–5**). Most importantly, the categories associated with plant defense pathways such as hydrolase

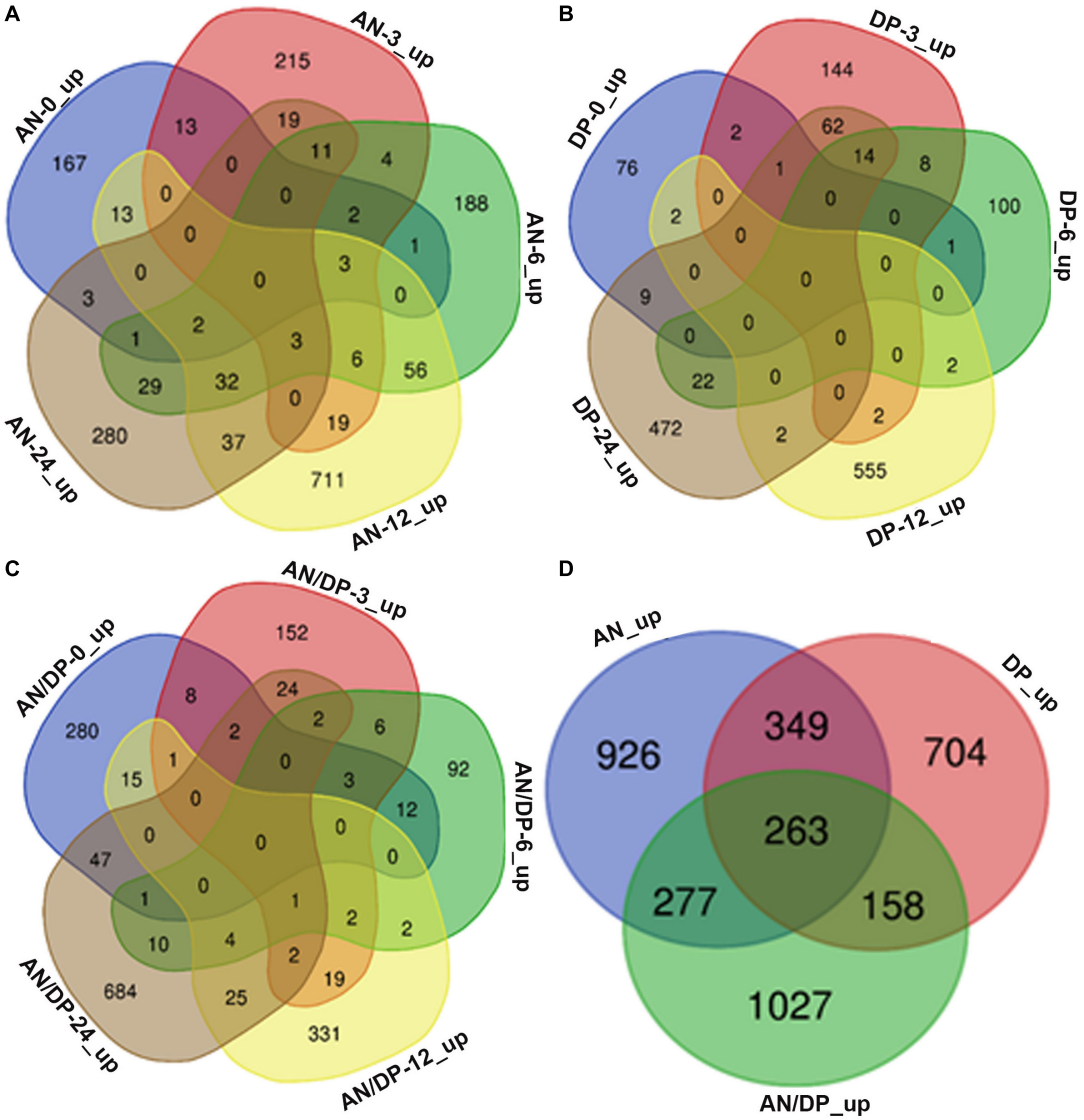


FIGURE 2 | Venn diagrams that show commonalities and differences among up-regulated DEGs at five time points following treatment of *A. thaliana* with **(A)** AN, **(B)** DP, **(C)** AN/DP, and **(D)** up-regulated DEGs (at at least one time point) for each of the three treatments.

activity, kinase activity, protein serine/threonine kinase activity, transcription factor binding, receptor serine/threonine kinase binding and terpene synthase activity were enriched in the analysis. For example, protein kinases play a central role in signaling in pathogen recognition and the subsequent activation of plant defense mechanisms (Romeis, 2001). Moreover, the genes identified as having hydrolase activity are likely to be involved in hydrolyzing the pathogen cell wall (Serrazina et al., 2015). The highest number of genes that were represented in the different molecular function categories was found at the 12 hpi time point for the AN treatment whereas for the other two treatments it was at the 24 hpi time point (**Figures 3–5**).

Most of the up-regulated transcripts in all three seaweed extract-treated *A. thaliana* plants fell into the **biological process** categories of cell wall organization, oxidation-reduction process and phosphorylation (**Figures 3–5**). In terms of the most important categories related to plant defense pathway processes the following were identified: defense response, hydrogen peroxide catabolic process, response to salicylic acid, response to auxin, innate immune response, response to abscisic acid, auxin activated signaling pathway, ethylene-activated signaling pathway and response to jasmonic acid, all were enriched in the biological process category. Classical defense phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and more recently, growth-related phytohormones, such as auxins, cytokinins (CKs), brassinosteroids (BRs), abscisic acid (ABA), and gibberellins (GAs) have all been shown to modulate plant immune defenses (Han and Kahmann, 2019).

Interestingly, in a comparison between the three treatments, an up-regulation of the expression of genes related to systemic

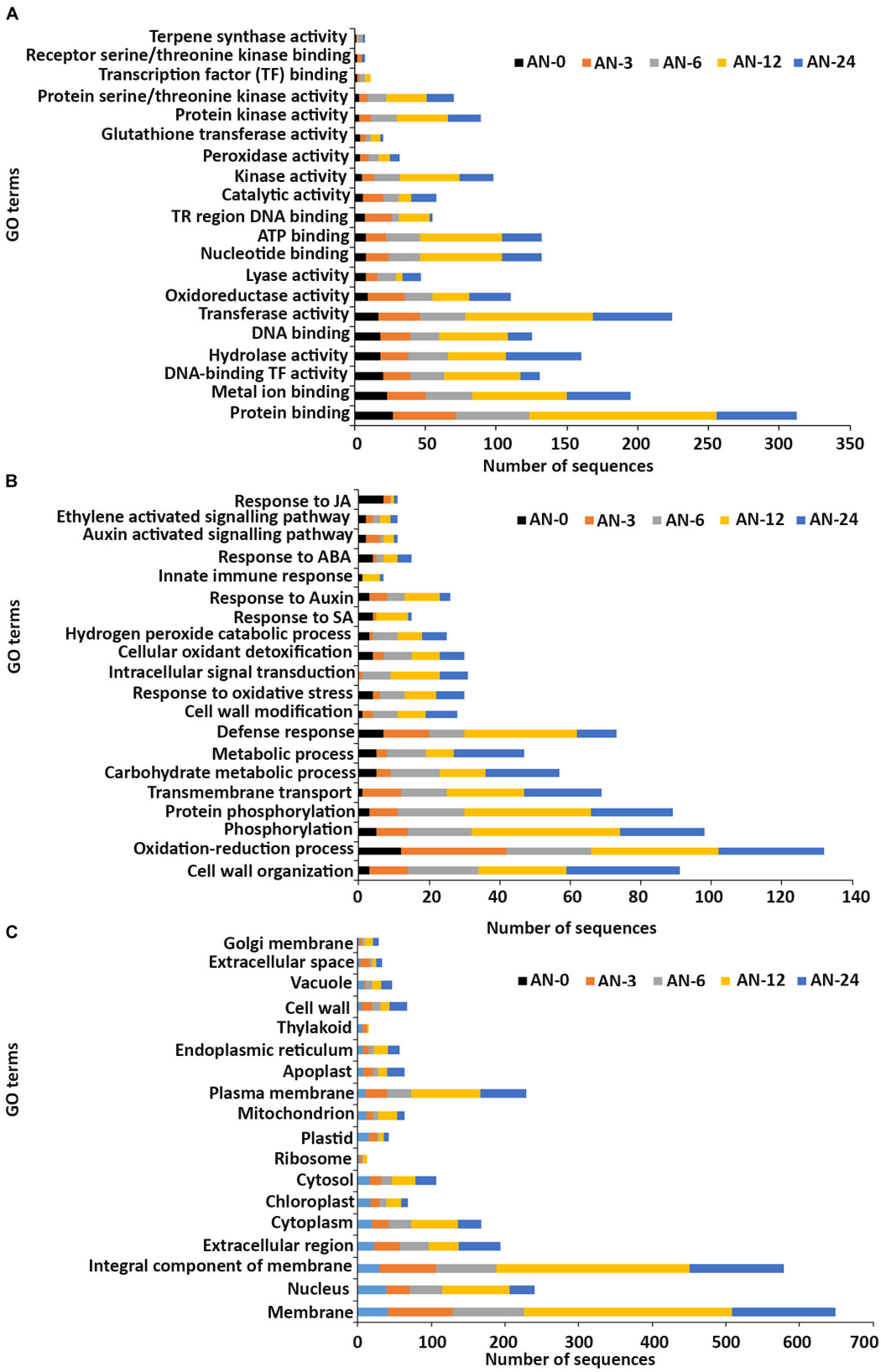


FIGURE 3 | Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) from the AN treatment. The DEGs were categorized into panels **(A)** Molecular function, **(B)** Biological process, and **(C)** Cellular component.

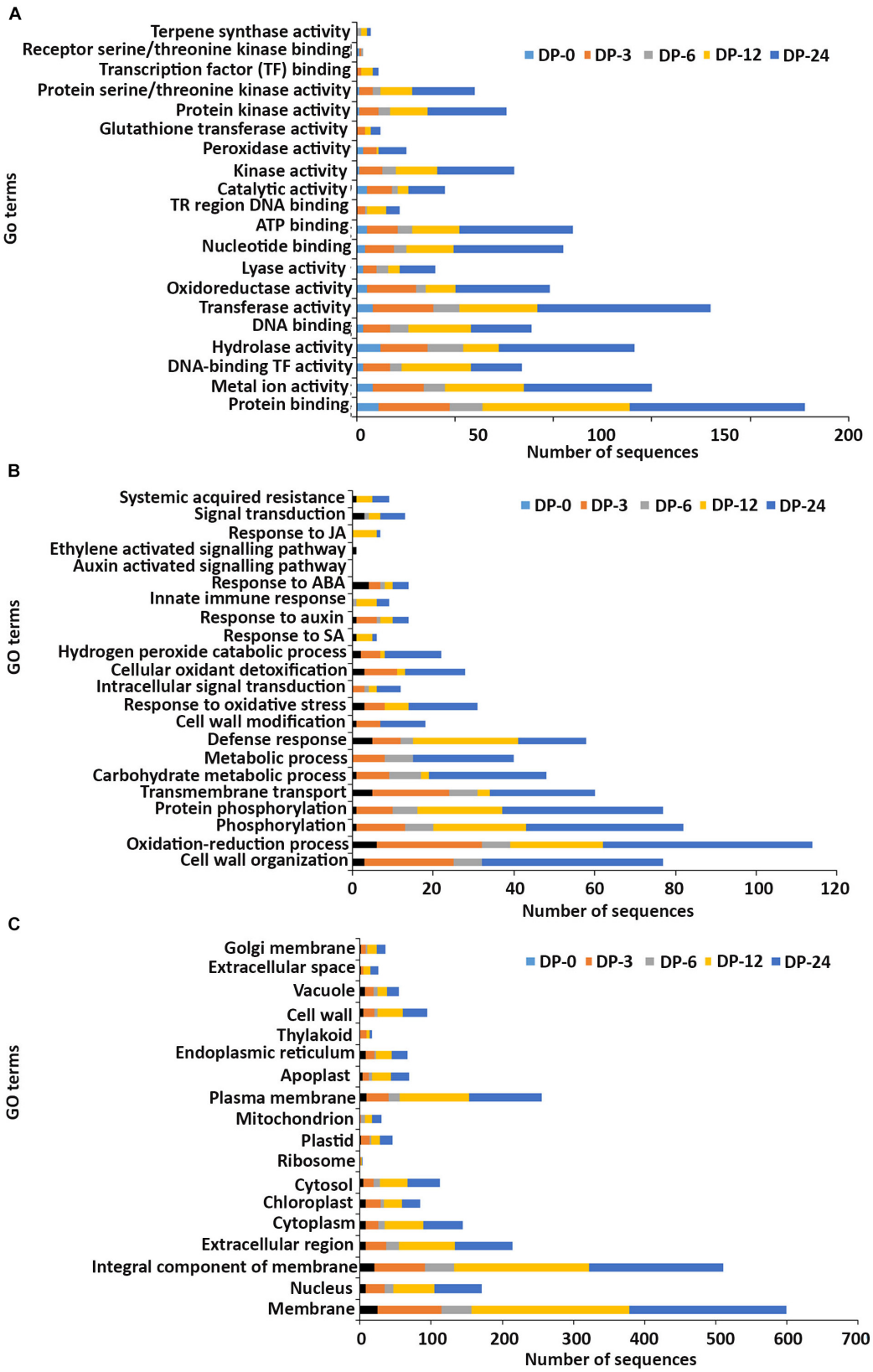


FIGURE 4 | Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) from the DP treatment. The DEGs were categorized into panels **(A)** Molecular function, **(B)** Biological process, and **(C)** Cellular component.

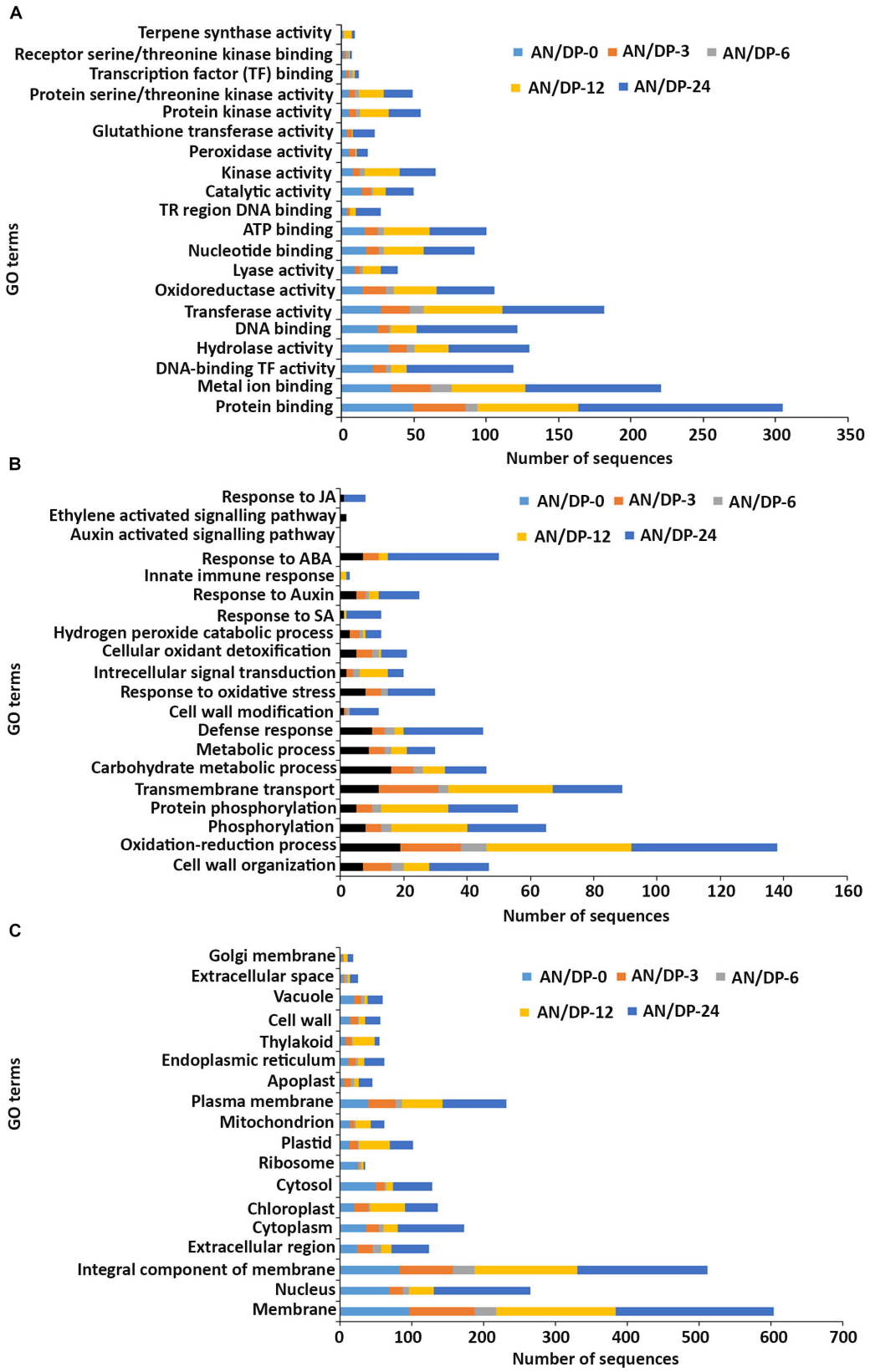


FIGURE 5 | Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) from the AN/DP treatment. The DEGs were categorized into panel **(A)** Molecular function, **(B)** Biological process, and **(C)** Cellular component.

acquired resistance was found only in the DP treatment at different time points after inoculation (**Figures 3–5**). However, the induction of expression of three major SAR genes (*PR1*, *PR5* and *NPR1*) was found in the qPCR validation of the RNA-Seq results (**Figures 6–8**).

In the **cellular component** GO category, up-regulated DEGs in all three of the seaweed extract-treated and inoculated plants were principally assigned to the categories membrane, nucleus, integral component of membrane, plasma membrane and extracellular region (**Figures 3–5**). A similar predominance of these GO categories was also found in the resistance of plants to incompatible pathogens (Song et al., 2019). For example, cell surface receptors are *trans*-membrane proteins that bind signal molecules in the extracellular space and generate different intracellular signals on the opposite side of the plasma membrane (Alberts et al., 2014).

KEGG pathway analysis

Kyoto Encyclopedia of Genes and Genomes analysis showed that different biosynthetic and metabolic pathways were up-regulated in response to *P. cinnamomi* infection of *A. thaliana* grown with the seaweed extracts. The most highly represented top five pathways that contained the largest numbers of up-regulated genes were purine metabolism, biosynthesis of antibiotics, thiamine metabolism, starch and sucrose metabolism and phenylpropanoid biosynthesis. The up-regulation of gene expression related to antibiotic biosynthesis in all three treatments revealed that the plant may use "antibiotic compounds" to combat the pathogen. Moreover, several genes were up-regulated that are associated with phenylalanine metabolism and terpenoid backbone biosynthesis which lead to the synthesis of antimicrobial phytoalexins, phytoanticipins and phenolic compounds that are known to be involved in plant defense against pathogens (Cho and Lee, 2015; **Supplementary Figure 3**).

Clustering and heatmap visualization of DEGs

To visualize the expression pattern of DEGs we performed a hierarchical clustering of the DEGs that were extracted at each time point for each treatment and the respective control. The clustering heatmap (**Supplementary Figure 4**) showed a complex pattern of gene expression at each time point for each treatment compared to the water control. The heatmap showed that the expression pattern of the following groups were similar: DP-12 and DP-24, AN/DP-3 and AN/DP -6, AN/DP -0 and DP-6, AN/DP -12 and AN-12, H-6 and AN-6 (**Supplementary Figure 4**). Further, we visualized the top 30 DEGs across the time points for each treatment. The result showed that most of the genes were up-regulated at 12 hpi followed by 24 hpi for the AN treatment. Moreover, *WRKY42* and *CML8* showed the most consistent up-regulation across the time series (**Supplementary Figure 5**). However, for those genes that were up-regulated most were only up-regulated at 12 and 24 hpi for the DP treatment (**Supplementary Figure 6**). In addition, the expression patterns of selected genes were slightly different for the AN/DP treatment where the genes were found to be more highly up-regulated at each time point except at 3 hpi (**Supplementary Figure 7**).

Gene set enrichment analysis (GSEA) using Reactome and MapMan databases

The list of DEGs were mapped using GSEA to Reactome and MapMan databases to reveal any pathways that contained a large proportion of genes. The GSEA categorized the DEGs at each time point for each treatment into a number of functional groups. Among those groups obtained from the Reactome database for the AN treatment batch, auxin signaling was dominant at all time points. Also, ABA- and ET-associated genes were enriched at various time points. In addition, a number of important categories related to plant defense reactions such as SA signaling, recognition of fungal and bacterial pathogen and immunity responses were dominant at 12 hpi for the AN treatment (**Supplementary Figure 8**). Similarly, auxin signaling was dominant at 0 hpi as well as at the early infection stages (6 and 24 hpi) for the DP treatment. The steroid phytohormone, i.e., brassinosteroids-group was enriched at 3, 6, and 24 hpi for DP-treated plants. Most importantly, the SA signaling was dominant at 12 hpi for the DP-treated plants. In addition, the ethylene signaling group was found to be represented at only 6 and 24 hpi (**Supplementary Figure 9**). The ABA and ET signaling and biosynthesis groups were dominant at 0 hpi and at the early infection time of 3 hpi for the AN/DP treatment whereas brassinosteroids, SA and secondary metabolism groups were found at 3 and up to 12 hpi. The auxin signaling group was found to be dominant at 12 hpi in the AN/DP-treated plants (**Supplementary Figure 10**).

The different GSEA categories defined using the MapMan databases of DEGs from different treatments are presented in **Supplementary Figures 11–13**. The biotic stress_PR proteins_Plant defensins group was dominant at 0 and 3 hpi in the AN treatment whereas the biotic stress- associated group was found at later time points (6—24 hpi). The calcium signaling, protein degrading serine proteases, signaling G-proteins and signaling MAP kinases dominated at different time points. Most importantly, secondary metabolism of phenylpropanoids and peroxidases were highly represented at 12 hpi for the AN treatment (**Supplementary Figure 11**). Similar categories were also found for the DP treatment. However, protein degrading aspartate proteases, BHLH transcription factor and transcription regulator categories were found at different hpi for the DP treatments. A BZIP transcription factor category was highly dominant at 12 hpi for the DP treatment (**Supplementary Figure 12**). Many of these categories were also similarly found in AN/DP treatments at different hpi, except for 24 hpi where biotic stress was dominated by metabolite transporter, leucine-rich repeat signaling receptor kinases and MYB transcription factor family proteins (**Supplementary Figure 13**).

Understanding the expression pattern of important stress-related genes at different time points following infection with the pathogen is necessary for pinpointing their specific contribution to plant defense. A closer look at the MapMan profile in regards to biotic stress pathways affected by each seaweed extract treatment clearly showed that R genes, proteolysis, cell wall, beta glucanase, phytohormones, respiratory burst, heat shock proteins, secondary metabolites and transcription factor- associated genes were up-regulated at all time points

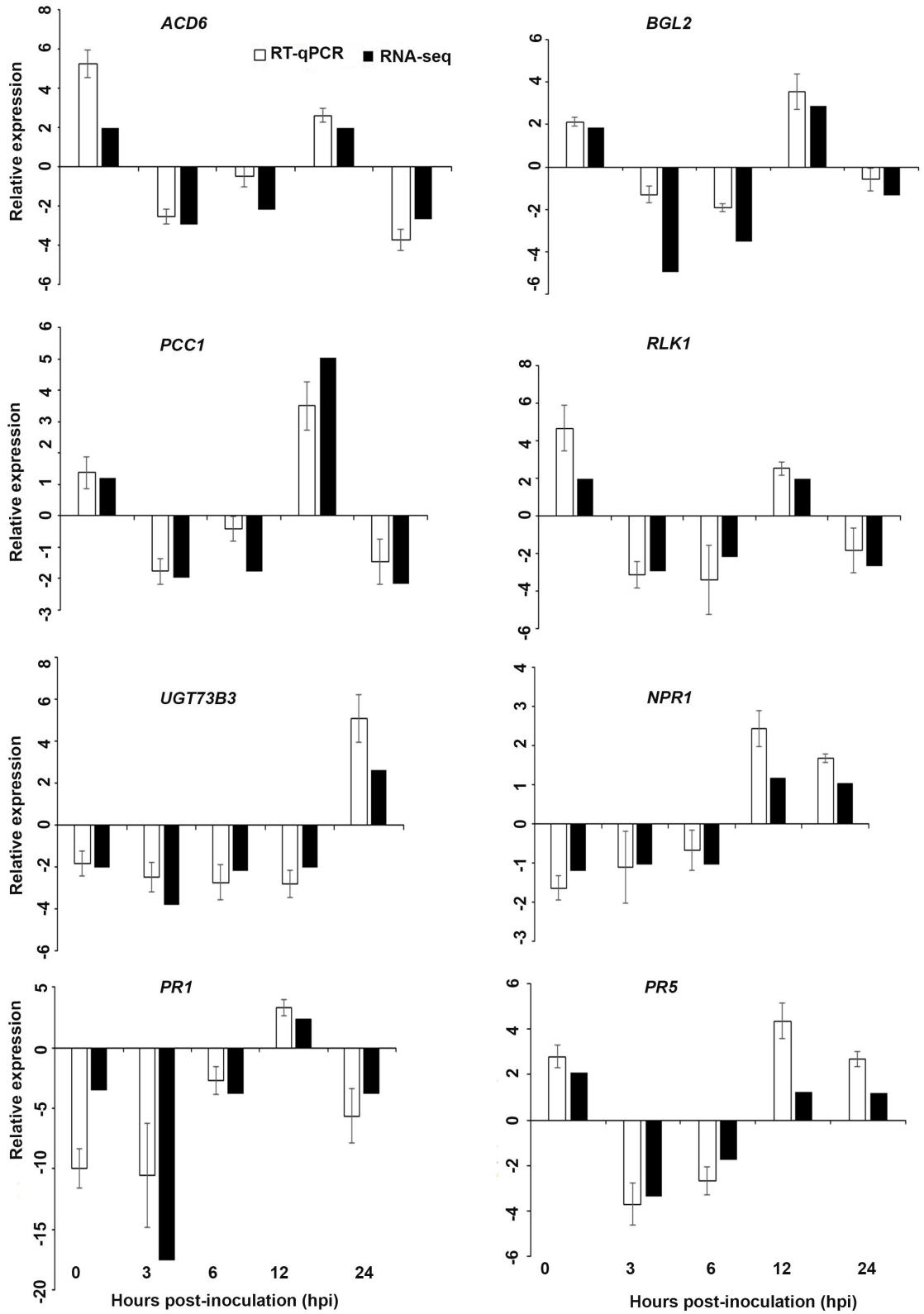


FIGURE 6 | Validation of the differentially expressed genes by RT-qPCR for *A. thaliana* plants treated with AN extract. Samples were collected from the plants grown with the seaweed extract and harvested at 0, 3, 6, 12, and 24 h after *P. cinnamomi* inoculation. All data were normalized to the expression level of *actin 2* (*ACT2*) and *actin 8* (*ACT8*). The data represent the fold change at each time point in the infected samples vs. the control sample. Bars show the standard error of the mean from three biological replicates.

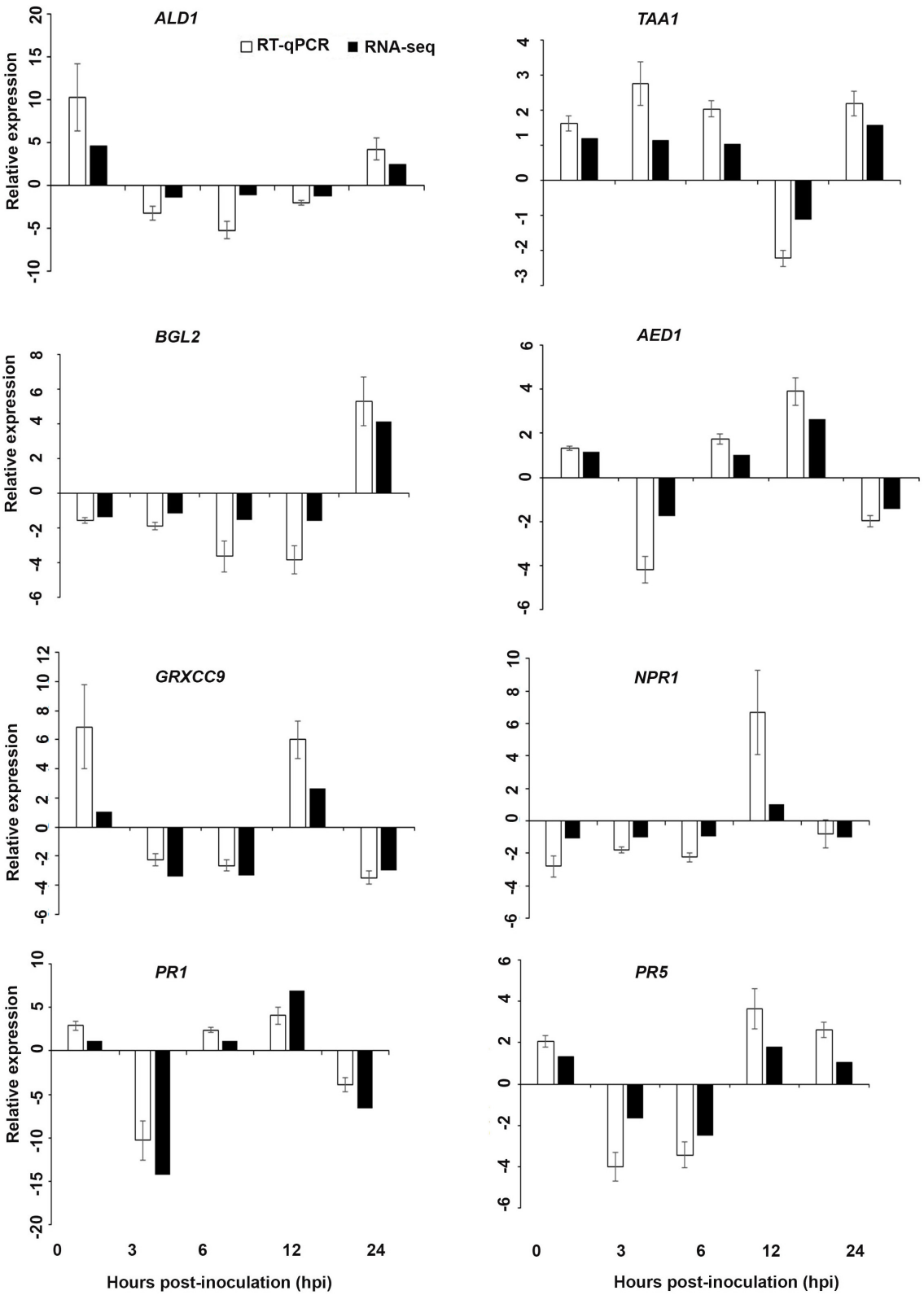


FIGURE 7 | Validation of the differentially expressed genes by RT-qPCR for *A. thaliana* plants treated with the DP extract. Samples were collected from the plants grown with the seaweed extract and harvested at 0, 3, 6, 12, and 24 h after *P. cinnamomi* inoculation. All data were normalized to the expression level of *actin 2* (*ACT2*) and *actin 8* (*ACT8*). The data represent the fold change at each time point in the infected samples vs. the control sample. Bars show the standard error of the mean from three biological replicates.

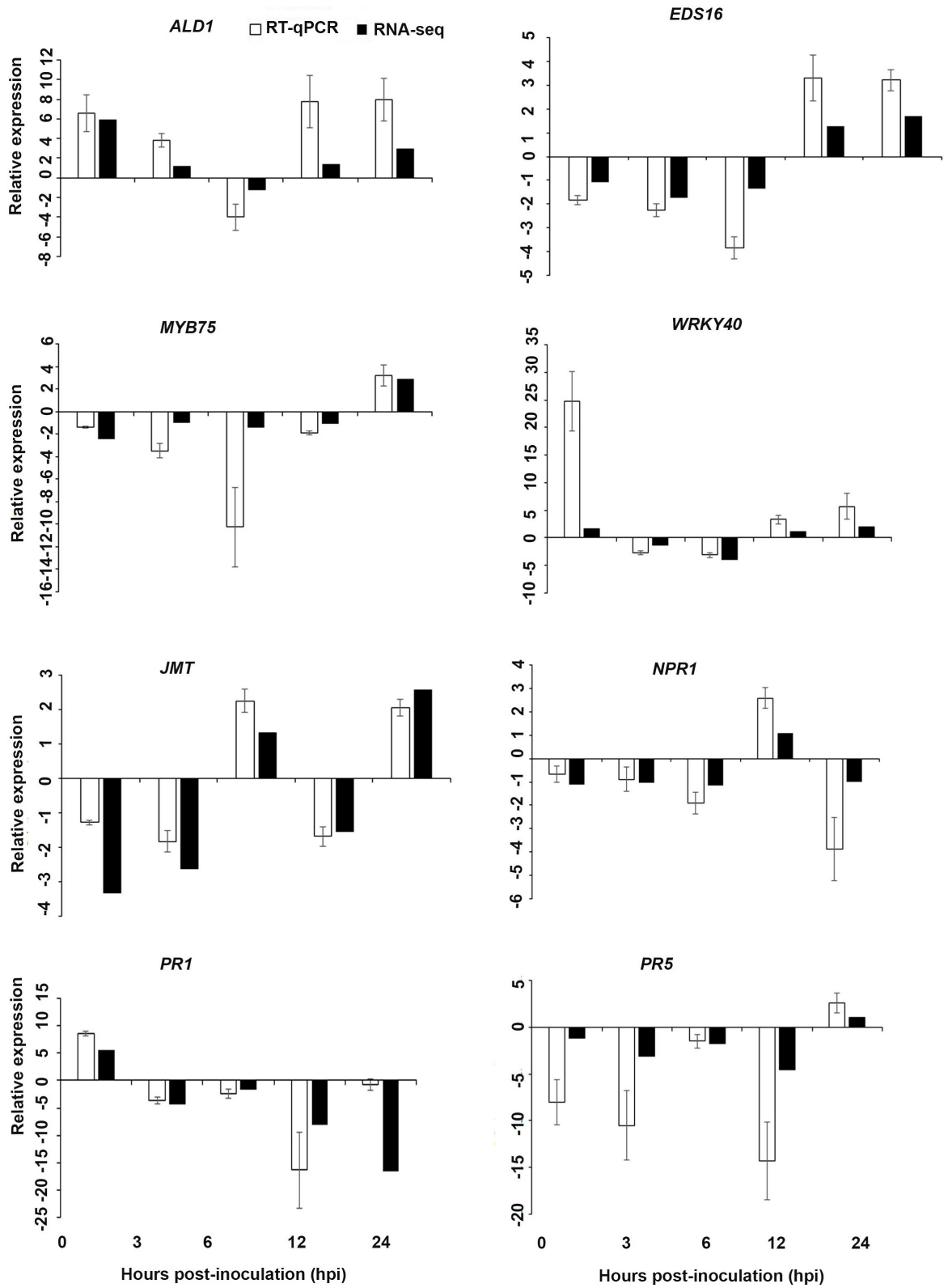


FIGURE 8 | Validations of the differentially expressed genes by RT-qPCR for *A. thaliana* plants treated with the AN/DP extract. Samples were collected from the plants grown with the seaweed extract and harvested at 0, 3, 6, 12, and 24 h after *P. cinnamomi* inoculation. All data were normalized to the expression level of *actin 2* (*ACT2*) and *actin 8* (*ACT8*). The data represent the fold change at each time point in the infected samples vs. the control sample. Bars show the standard error of the mean from three biological replicates.

for each treatment (**Figure 9** and **Supplementary Figure 14**). In terms of phytohormones, Auxin, BRs, SA, ABA and ET-associated genes were represented and up-regulated at most of the time points for each treatment. However, JA associated genes were only up-regulated in the AN treatments at 6 hpi. In terms of respiratory burst, redox state- and peroxidases-associated genes were induced in all treatments. However, glutathione-S-transferase (GST) was only up-regulated at 12 hpi for both the AN and DP treatments and at 24 hpi for treatment with AN/DP. Importantly, the greatest number of genes in each category were found to be up-regulated at 12 and 24 hpi for each extract treatment (**Figure 9** and **Supplementary Figure 14**).

Validation of RNA-Seq Expression

Based on their known involvement in plant defense pathways five genes from the DEG list for each treatment and three SAR-related genes were selected based on their initial gene expression analysis (see section "Analysis of SA and JA Related Gene Expression") for RT-qPCR using their specific primers to confirm the reliability of expression of DEGs obtained from RNA sequencing. The relative expression levels of the selected genes were determined at 0, 3, 6, 12, and 24 h after *P. cinnamomi* inoculation. All of the selected genes from the three seaweed extract treatments showed trends closely aligned to those found for the RNA-seq data (**Figures 6–8**). In most cases, the RT-qPCR relative expression was higher than that found for the RNA-seq data for both up-regulated and down-regulated genes across the various time points examined. These results confirmed and further indicated that genes in *A. thaliana* related to a plant defense response, phytohormone signaling and transduction and systemic acquired resistance (**Tables 2–4**) were induced by the seaweed extracts and that they may function together against infection by *P. cinnamomi.*

Key Plant Defense-Related Genes Significantly Up-Regulated in at Least One Time Point

The most important genes that are related to plant defense that were found to be significantly up-regulated in expression are shown in **Tables 2–4**. For the AN seaweed extract treatment the analysis of key defense-related genes revealed the presence of SA biosynthetic process and signaling associated genes, JA biosynthetic process and signaling-associated genes, pattern recognition receptors, plant defensin family gene and resistance-related gene active against oomycetes (**Tables 2**, **3**). From an analysis of the DP seaweed extract treatment key defense-related genes revealed were those for SA and JA signaling, R protein encoded, auxin biosynthetic process-associated, phytoalexin production regulating, SAR regulating, basal resistance-related and hydrogen peroxide production associated genes (**Tables 2**,**3**). For the AN/DP seaweed extract treatment key defense-related genes found were for SA and JA biosynthetic process-associated genes, ABA-signaling genes, receptor-like protein kinases, an innate immune response inducer gene, SAR-inducible gene, a transcriptional regulator gene and ethylene signaling gene (**Tables 2**,**3**). In addition to those sets of genes specific to individual extract treatments there were those that were commonly found across the treatments. Noticeably the three key SAR-associated genes (*PR1*, *PR5*, and *NPR1*) were all up-regulated at 12 hpi following the AN and DP treatments showing a clear involvement of a SA-stimulated pathway. However, for the combined extract treatment at 12 hpi only *NPR1* was up-regulated. In addition, the expression of auxin transporter, hydrogen peroxide responsive, receptor-like protein kinase and secondary metabolite biosynthetic genes were commonly found to be up-regulated across all three seaweed extract treatments (**Table 4**).

Detection of Hydrogen Peroxide in *A. thaliana* Roots

The production of H_2O_2 was identified as a reddish-brown precipitate that resulted from DAB staining of the *A. thaliana* roots. At 12 hpi, H_2O_2 was detected in the roots grown with all three seaweed extracts and inoculated with the pathogen (**Figures 10D,F,H**). No H_2O_2 was detected in control roots grown with water or mock-inoculated with water (**Figure 10A**). Moreover, a minimal level of H_2O_2 was found in all three extract-treated and mock inoculated roots (**Figures 10C,E,G**). In addition, image analysis of DAB stained roots showed significantly higher stain in each seaweed extract-treated and inoculated root compared to either non-inoculated of each seaweed extract-treated root or water control (**Supplementary Figure 15**).

DISCUSSION

Verification of the *Arabidopsis thaliana-Phytophthora cinnamomi* Plant-Pathogen System

This study used the model plant *A. thaliana* and the generalist, globally devastating pathogen *P. cinnamomi*, to examine the impacts of treatment of plants with two selected brown algal extract-based biostimulants or their combination, on pathogen growth and development in roots. Several previous studies have shown that applications of various brown algal extracts, either to soil or to foliage, enhanced root growth and plant development (Arioli et al., 2015; Mattner et al., 2018). Here we have used a sand culture system to grow *A. thaliana* with extracts from *A. nodosum* ("AN"), or *D. potatorum* ("DP"), or their combination ("AN/DP"). Studies on biostimulants and their impacts on plant disease establishment and progress have been reported (see for example, Gunupuru et al., 2019) although a comprehensive time course study of a root pathogen, and in this case an oomycete root pathogen, in the model plant *A. thaliana* has not been undertaken. The advantage of using *A. thaliana*, apart from its incredibly well detailed and characterized genome, is that there is a growing body of information around the interaction of this host with a range of oomycete pathogens including *P. cinnamomi* (Robinson and Cahill, 2003; Rookes et al., 2008), *Phytophthora porri* (Roetschi et al., 2001) and *P. parasitica* (Le Berre et al., 2017), *Hyaloperonospora arabidopsidis* (Kunz et al., 2008; Ried et al., 2019), and *Albugo candida* (Cooper et al., 2008), but none on their interactions with biostimulants.

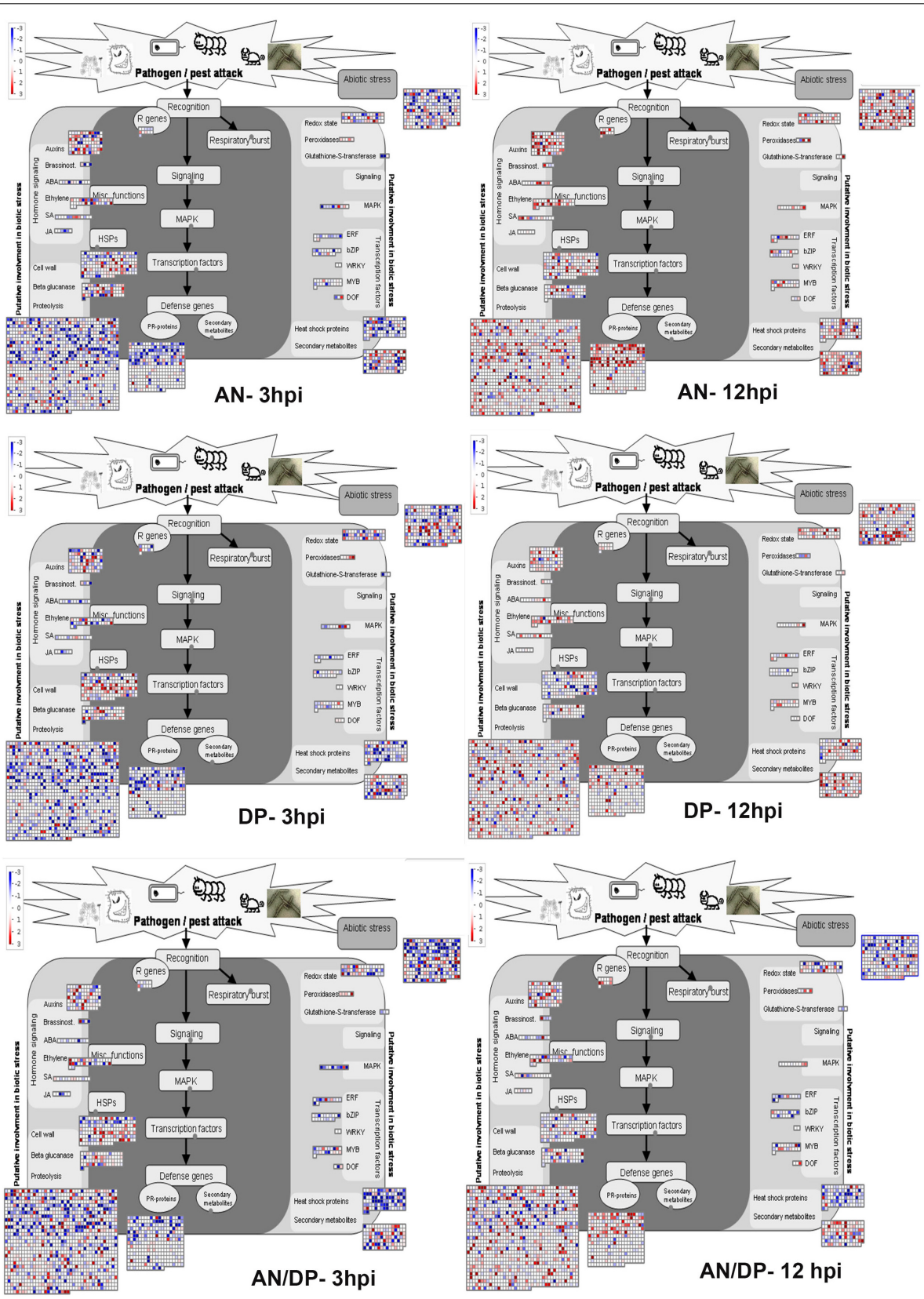


FIGURE 9 | Mapman overview of DEGs related to hormone, stress and metabolic responses in plants of *A. thaliana* following seaweed treatment and after inoculation with *P. cinnamomi* (at 3 and 12 hpi). The average fold change of genes are indicated by the color scale (red represents up-regulated genes and blue represents down-regulated genes).

TABLE 2 | Possible function in defense of individual DEGs significantly induced at least at one time point for each seaweed extract treatment.

Gene	Reported function	References
AN treatment		
PCC1	Salicylic acid (SA) and Jasmonic acid (JA) triggered pathogen-related response	Mir et al., 2013
ICS2	SA biosynthetic process	Shine et al., 2016
ACD6	Accelerated cell death 6, activator of the defense response against virulent bacteria and can activate SA-dependent cell death	Lu et al., 2003
UGT73B3	Glycosyltransferases, SA induced gene participates in regulation of redox status and general detoxification of ROS−reactive secondary metabolites	Simon et al., 2014
RLK1	Receptor like kinase1, pattern recognition receptor which induces innate immune defense	Chaliha et al., 2018
PDF2.3	Predicted to encode a PR (pathogenesis-related) protein, belongs to the plant defensin (PDF) family protein, defense response	Sels et al., 2008
KAT2	Peroxisomal 3-Ketoacyl-CoA Thiolase 3 (Pkt3), Kat 2, involved in JA biosynthetic process	Pye et al., 2010
VQ25	Involved in resistance to necrotrophic pathogen	Cheng et al., 2012
BAD1	Ankyrin repeat-containing protein BDA1, involved in plant defense, contribute upstream of NPR1 and WRKY70 to regulate plant defense	Yang et al., 2012
LURP1	LURP-one-like protein, required for full basal resistance through R protein to the oomycete pathogen *Hyaloperonospora parasitica*	Knoth and Eulgem, 2008
DP treatment		
GRXC9	CC-type glutaredoxin protein, involved in SA-dependent disease resistance pathway	Herrera-Vásquez et al., 2015
TAA1	Involved in auxin biosynthetic pathway	Stepanova et al., 2008
CRK5	Cysteine-rich receptor like protein kinase 5, pathogen-induced *Arabidopsis* gene, involved in multiple distinct defense responses. May function as a disease resistance (R) protein	Chen et al., 2003
ERF15	Ethylene responsive factor 15, Transcriptional activator, positively regulates immunity against bacteria and fungi	Zhang et al., 2015
MYB113	*MYB113* is critical in the production of anthocyanins which comprise specific stages of phenylpropanoid metabolism	Gonzalez et al., 2009
IOS1	Impaired Oomycete Susceptibility 1 (IOS1) has been implicated in defense-related signaling and is important for the resistance against bacteria	Yeh et al., 2016
MKK9	Map Kinase Kinase 9, Autophosphorylates and also phosphorylates MPK3 and MPK6. Independently involved in ETH and camalexin biosynthesis. Induces transcription of ACS2, ACS6, ERF1, ERF2, ERF5, ERF6, CYP79B2, CYP79B3, CYP71A13, and PAD3	Xu et al., 2008
SIB1	Sigma factor binding protein 1, plays a vital role in JA and SA mediated signaling pathway	Xie et al., 2010
HR2	RPW8-like protein 2, contributes to basal resistance to powdery mildew pathogen	Berkey et al., 2017
PEP1	Elicitor peptide 1, activates the transcription of plant defense genes and activates the synthesis of hydrogen peroxide	Huffaker et al., 2006
AN/DP treatment		
FMO1	Flavin-dependent monooxygenase1, involved in critical metabolic SAR signal	Návarová et al., 2012
EDS16	Enhanced disease susceptibility 16, involved in SA biosynthesis, Encodes a protein with isochorismate synthase activity. Mutants fail to accumulate salicylic acid. Its function may be redundant with that of ICS2	Wildermuth et al., 2001
WRKY40	Pathogen inducible transcription factor involved in ABA signaling pathway	Chen et al., 2010
JMT	JA carboxyl methyltransferase, JA biosynthetic pathway	Seo et al., 2001
MYB75	Transcriptional regulator of anthocyanin biosynthesis	Borevitz et al., 2000
CML24	CaM (Calmodulin)-like protein, acts to induce downstream NO synthesis as intermediary steps in a pathogen perception signaling cascade, leading to innate immune responses	Ma et al., 2008
ABR1	Abscisic acid-responsive 1, involved in cell death and defense signaling	Choi and Hwang, 2011
WAK1	Wall-associated kinase 1, Induced by SAR conditions, pathogen and defense related signaling molecules including methyl jasmonate (MeJA) and ethylene (Eth)	Meier et al., 2010
NHL13	Non-race-specific disease resistance1/harpin-induced1-like13, required for plant immunity to bacteria	Xin et al., 2015
PBL20	Probable serine/threonine-protein kinase PBL20, cytoplasmic receptor-like protein kinases, may be involved in plant defense signaling	Zhang et al., 2010

In other host−pathogen systems treatment with seaweed extract-based biostimulants have indicated that disease incidence and severity may be reduced following infection. For example, commercial seaweed extracts from *A. nodosum* and *D. potatorum* were found to suppress disease caused by *Plasmodiophora brassicae* in broccoli (Wite et al., 2015) and an extract from *A. nodosum* reduced the severity of *Fusarium* head blight caused by *F. graminearum* in wheat (Gunupuru et al., 2019). It is worth noting the diversity in seaweed extracts. Liquid seaweed extracts are processed from seaweed biomass using different chemical approaches (such as acid and alkaline extraction) and cellular disruption under pressure (Arioli et al., 2015). The extracts comprise diverse molecules that are heterogenous in nature and representative of the extraction process, which emphasizes the

TABLE 3 | Possible function in defense of individual DEGs significantly induced at least at one time point of at least at two treatments.

Gene	Reported function	References
AN and DP treatment		
BGL2	beta 1,3-glucanase, involved in systemic acquired resistance	Durrant and Dong, 2004
AED1	Apoplastic enhanced disease susceptibility-dependent 1, predicted aspartyl proteases, induced locally and systemically by infection and locally by SA	Breitenbach et al., 2014
SNC4	Suppressor of *NPR1-1*, constitutive4 (SNC4) encodes an atypical RLK, involved in plant innate immunity	Bi et al., 2010
DP and AN/DP treatment		
ALD1	Lys aminotransferase AGD2-like defense response protein 1, required for SAR activation	Song et al., 2004
BON1	BONZAI1, is a regulator of defense responses apparently through repressing activity of an R gene	Yang et al., 2006
AN and AN/DP treatment		
AOC3	Allene oxidase cyclase 3, Key gene in JA biosynthesis	Najafi et al., 2020
OPR3	12-oxophytodienoate reductase 2- an isoenzyme involved in JA biosynthesis	Schaller et al., 2000

TABLE 4 | Commonly up-regulated (at least at one time point) candidate genes following treatment with the three seaweed extracts.

Gene	Reported function
NPR1	SA mediated SAR signaling pathway (Eshraghi et al., 2011).
PR1	SAR marker gene (Eshraghi et al., 2011).
PR5	SAR marker gene (Eshraghi et al., 2011).
PIN2	PIN formed protein, Auxin transporter, plays a critical role in auxin gradient−mediated developmental processes, including lateral root formation and gravitropic growth (Sun et al., 2011).
GR1	Glutathione reductase 1, plays a crucial role in responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways (Mhamdi et al., 2010).
UGT73B4	UDP-glycosyltransferase 73B4, UGT plays an essential role in the biosynthesis of secondary metabolites in plants (Guo et al., 2016).
CRK15	Cysteine-rich receptor like protein kinase 15, involved in pathogen induced plant cell death in *bak1/serk4* mutant through regulation of ER quality control (ERQC) (de Oliveira et al., 2016).
MLO-8	Mildew resistance locus O-8, May be involved in modulation of pathogen defense and leaf cell death. Activity seems to be regulated by Ca^{2+}-dependent calmodulin binding and seems not to require heterotrimeric G proteins (Devoto et al., 2003).

need to characterize their properties. The results of our study show that the extent of colonization by *P. cinnamomi* of roots of *A. thaliana* was suppressed by pre-treatment of roots with the alkaline-based extracts from both *A. nodosum* and *D. potatorum* and a mixture of both. The generalist pathogen, *P. cinnamomi*, is an aggressive pathogen that is able to infect close to 5000 plant species (Hardham and Blackman, 2018). Therefore, the suppression of this pathogen by seaweed extracts is a significant finding that demanded further investigation of the details of the potential resistance mechanisms stimulated by different seaweed extracts against *P. cinnamomi* infection.

The availability of genetic and genomic tools for the model plant *A. thaliana* makes it a very good system in which to investigate the *in planta* action of seaweed extracts. Infections by *Phytophthora* spp. in *A. thaliana* have not been found under natural conditions, but have been achieved for several *Phytophthora* species under laboratory conditions (Herlihy et al., 2019). Ecotypic variation to infection by *P. cinnamomi* was described in an earlier study where ecotype Ler was found to be moderately susceptible (Robinson and Cahill, 2003). The microscopic analysis performed in the present study has confirmed that the system that was established and optimized was one in which the plants were successfully inoculated and that the pathogen grew both on the root surface and within roots.

Effect of Seaweed Extracts on Key Regulatory Resistance-Related Genes

Various seaweeds are a rich source of unique bioactive compounds, for example fucans, carrageenans, ulvans, and laminarins that have been shown to induce plant defense against a variety of pathogens (Cluzet et al., 2004; Shukla et al., 2016, 2019). These elicitor-like molecules may act as priming molecules or pathogen-associated molecular patterns (PAMPs) and thereby activate induced systemic resistance (ISR) and SAR responses. To first test this hypothesis in our system, we examined the expression of three SA- and two JA/ET-responsive marker genes (*PR1*, *NPR1, PR5* and *PDF1.2, THI2.1*, respectively) that are related to SAR (Eshraghi et al., 2011). In our study, each seaweed extract was found to enhance the expression of the key SA-marker genes from the earliest time point tested after inoculation following treatment with seaweed extracts. The JA-marker genes showed variation in expression depending on extract type and time after inoculation. Moreover, the genes were not induced when the plants were treated with the seaweed extracts alone. Other studies have shown similar upregulation of these genes at a single time point. For example, it had been shown earlier that the expression of the *PR1* gene in *A. thaliana* was up-regulated at 24 h post-treatment with an *A. nodosum*-based extract (Cook et al., 2018; Shukla et al., 2019). Furthermore, carrot plants primed by *A. nodosum*-derived extracts induced the accumulation of transcripts of the same or similar genes (Jayaraj et al., 2008). In contrast to these limited studies, the current study has identified the induction of key regulatory genes across a range of time points after pathogen infection therefore providing a post-infection, spatio-temporal analysis of induction following various seaweed extract treatments.

Transcriptome Analysis Revealed the Complexity of Resistance Induced by Seaweed Extracts

Summary of Transcriptional Changes Induced by Each Seaweed Extract With or Without Root Infection by *P. cinnamomi*

Transcriptome analysis using RNA-seq was performed to explore the whole plant transcriptome to reveal correlations between

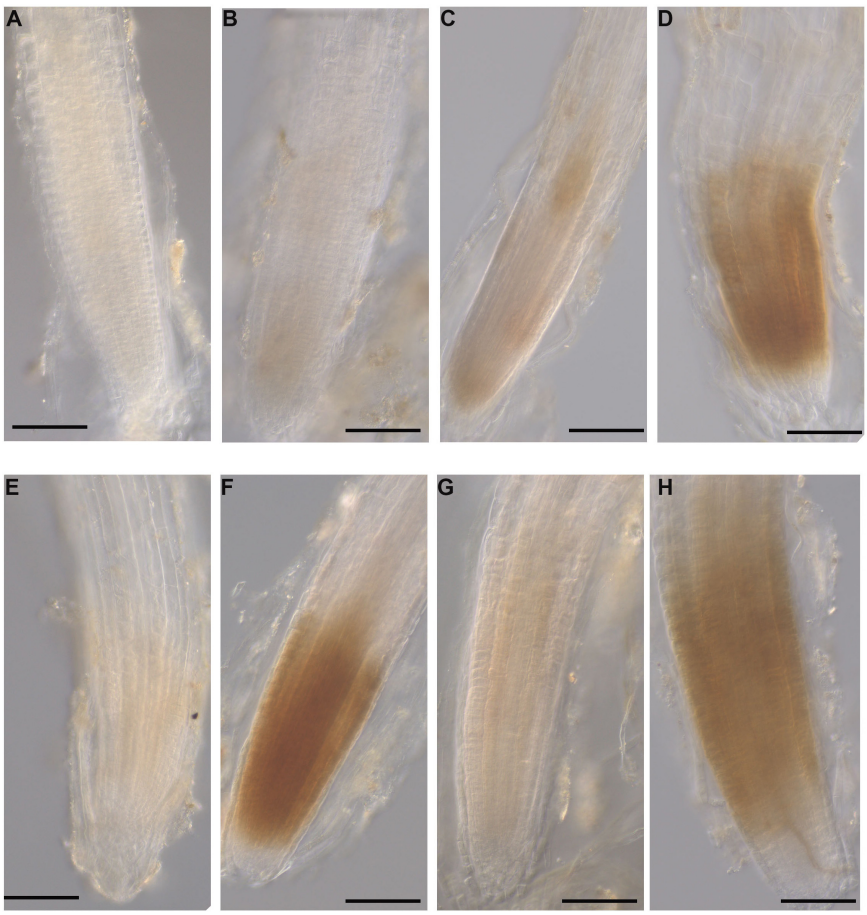


FIGURE 10 | Hydrogen peroxide detection in *A. thaliana* roots grown with seaweed extracts or water as the control and inoculated with *P. cinnamomi* or mock-inoculated with water. Hydrogen peroxide was detected using the 3,3 O-diaminobenzidine tetrachloride (DAB) stain, which resulted in a reddish-brown precipitate in the root tissue. **(A)** Control root grown with water and mock inoculated with water showing no H_2O_2 production. **(B)** Control root grown with water and inoculated with the pathogen showing no H_2O_2 production. **(C)** Infected root grown with seaweed extract AN and mock inoculated with water showing a low level of H_2O_2 production. **(D)** Infected root grown with seaweed extract AN and then inoculated with the pathogen showing H_2O_2 production. **(E)** Control root grown with seaweed extract DP and mock inoculated with water showing low level H_2O_2 production. **(F)** Infected root grown with seaweed extract DP and inoculated with the pathogen showing H_2O_2 production. **(G)** Control root grown with seaweed extract AN/DP and mock inoculated with water showing low level of H_2O_2 production. **(H)** Infected root grown with seaweed extract AN/DP and inoculated with the pathogen showing H_2O_2 production. Scale bar = 20 μM. Each image is representative of three biological replicates.

seaweed extract treatment and pathogen suppression that was found following quantitative analysis of the amount of pathogen within roots. Overall the results show that there was a large number of genes that were either up-regulated or down-regulated following exposure of plant roots to each of the extracts. In this study we have specifically concentrated on genes that were up-regulated in these interactions. Three major SAR-related genes were found to be up-regulated in common between extracts and were confirmed by both RNA-Seq and qRT-PCR validation. Equally importantly, each seaweed extract was found to exert its effect through different subsets of genes. A number of studies explored plant transcriptomes following abiotic and biotic stress (see for example: Tommasini et al., 2008; Allardyce et al., 2013; GonñI et al., 2016; Islam et al., 2018; Jithesh et al., 2019), however, the present study is the first report of a comprehensive transcriptome analysis, following treatment of plants with seaweed extracts, root pathogen infection and analysis over multiple time points.

The up-regulated genes were broadly identified as being involved in phytohormone signaling, defense responses, hydrolase activity and the biosynthesis of antibiotics, and also transcription factors and transcription regulators that were involved in metabolite biosynthesis. The GSEA using both Reactome and MapMan databases indicated that the DEGs were involved in a diverse range of activities during seaweed extract-induced plant defenses. For example, brassinosteroid (BR) signaling was commonly found across all three treatments. BRs are plant steroidal hormones that play vital roles in not only plant growth and development but also in plant defense through coordination with other phytohormones (Saini et al., 2015). Another example, proteases (serine or aspartic proteases), were commonly enriched in both AN and DP treatments. Plant

genomes encode a large number of proteases which play a regulatory role in a number of processes that are essential for immune responses, more specifically, programmed cell death (PCD) (Balakireva and Zamyatnin, 2018). Most importantly, an array of proteolysis-related genes and their increased expression was commonly found at all time points for each seaweed extract treatment. Proteolysis machinery acts mainly in a housekeeping role to remove non-functional proteins, however, proteolysis has also been shown to play a key role in the recognition of pathogens and the subsequently induced effective defense responses (van der Hoorn and Jones, 2004). Therefore, the results of our study indicated the deployment of multiple phytohormones and proteolytic machinery in seaweed extract-induced defense against *P. cinnamomi*. However, there is considerable scope to further investigate the role of individual proteases in seaweed extract-induced defenses.

The plant cell wall is a dynamic and highly controlled structure that is essential for growth and development. It is considered to be a passive defense barrier against a variety of attackers. Plants have mechanisms that maintain cell wall integrity which comprise a set of so-called "plasma membrane-resident sensors" and "pattern recognition receptors" (Bacete et al., 2018; De Lorenzo et al., 2019). When a pathogen alters the cell wall integrity during epidermal penetration or through deeper colonization of sub-epidermal cells, plants activate suites of genes for cell wall biosynthesis and remodeling as repair and defense responses. This activation of genes and the production of their downstream products is very effective at stopping, or slowing down, pathogen ingress. Several studies, including those that have used overexpressor mutants, have demonstrated the central importance of cell wall-related genes in enabling increased disease resistance (Miedes et al., 2014; Bacete et al., 2018). In our study, cell-wall associated genes were dominant at all-time points following infection for each seaweed extract treatment.

Cell walls are the first line of defense and their modification a very early response to pathogen attack. Each seaweed extract stimulated cell wall-related gene activity following pathogen attack that was well above that for the water-treated control. The induction of these genes at early stages of infection for each seaweed extract treatment was strongly indicative of their contribution toward strengthening the cell wall against pathogen penetration. The induction of similar genes at later stages suggested their contribution to cell wall repair and the fortification of new cell walls. For example, the *MYB46* transcription factor that was up-regulated at 12 hpi in the AN treatment, is directly involved in regulation of the expression of genes responsible for secondary cell wall formation including lignin and cellulose biosynthesis (Miedes et al., 2014). Another example, *CALS5* (Callose synthase 5) that was up-regulated at 6 hpi in the AN/DP treatment, is involved in callose synthesis and was also a pathogen-induced gene in *A. thaliana* infected with the downy mildew pathogen *Hyaloperonospora arabidopsis* (Dong et al., 2008). Callose is a well-known plant defense component and is considered an effective barrier against pathogen invasion including in various *A. thaliana* ecotypes infected by *P. cinnamomi* (Robinson and Cahill, 2003).

The baseline of plant defense is the activation of PRRs localized in the plasma membrane upon recognition of PAMPs/MAMPs (Bigeard et al., 2015). Indeed the induction of *RLK1* in AD-treated plants indicated the activation of PAMP-triggered immunity in these plants against the pathogen. The plant hormones SA, JA and ET have a significant role in plant defense against pathogens. The SA signaling pathway that activates programmed cell death is effective against biotrophic pathogens whereas JA and ET signaling pathways are effective against necrotrophic pathogens (Glazebrook, 2005). The upregulation of both JA and SA biosynthetic or signaling genes suggested the activation by seaweed extracts of both pathways in response to *P. cinnamomi*. The phytohormone, auxin, is well known to be a regulator of plant growth and development. However, auxin is also being recognized as a key regulator of plant defense (Wang and Fu, 2011; Wang et al., 2011). In our study, for example, the upregulation of *TAA1*, a gene involved in auxin biosynthesis, in DP-treated plants indicated the involvement of auxin signaling pathways in response to the pathogen. Similarly, ABA is mainly involved in abiotic stress tolerance as well as in biotic stress but it also may promote plant defense in a complicated network of synergistic and antagonistic interactions (Ton et al., 2009). The induction of an ABA biosynthesis-related transcription factor gene (*WRKY40*) and ABA responsive gene (*ABR1*) in plants treated with AN/DP, along with other key phytohormone-related genes suggested the activation of multiple phytohormone signaling pathways following seaweed extract treatment.

The second layer of plant defense is based on plant disease resistance, (R) gene, mediated resistance by recognition of the products of pathogen avirulence genes and subsequent effector-triggered immunity (ETI) (Andersen et al., 2018). For example, in the current study the induction of *CRK5*, which likely functions as a receptor-like kinase (Chen et al., 2003), in DP-treated plants indicated that ETI may have been triggered. WRKY transcription factors are encoded by a large gene superfamily with a broad range of roles in plants and several groups have reported that proteins containing a short VQ motif interact with WRKY motifs. One of these, VQ25, was reported by Cheng et al. (2012), to be involved in resistance to the necrotrophic pathogen *Botrytis cinerea*. The induction of the candidate resistance-related gene *VQ25* in plants treated with AN thus indicated a contribution of this gene to AN-induced plant defense.

The recent review published by Shukla et al. (2019) presented additional information on some of the plant defense components activated by different extracts from *A. nodosum*. The bioactive compounds present in the prepared *A. nodosum* alkali extract (ANE) were proposed to elicit defense responses to pathogens. The application of ANE enhanced the activation of various enzymes including peroxidases and phenylalanine ammonia-lyase. In addition, ANE also induced ISR against *P. capsici*, another oomycete pathogen, that caused disease in tomato. Further, ANE induced SA-related genes and several JA-related genes such as *PDF1.2* and plant immune response genes such as *WRKY30* and *CYP71A12*, that we also highlight in our study. The review emphasized the information gap around the role of phytohormones in activating defense-related genes that we have

now gone some way to fill. For example, the up-regulation of candidate genes, such as *PCC1*, *ACD6*, *GR1*, *ERF014*, *AOC3*, *ACS9*, and *ACS11* all hormone-related by the different extracts derived from both *A. nodosum* and *D. potatorum* and their combination used in our study.

The array of plant defense responses that are activated during pathogen invasion requires an abundant supply of energy which is predominantly derived from primary metabolic processes. These primary metabolic pathways are used by plants not only as a source of energy to drive diverse defense responses, but also as a source of signaling molecules to directly or indirectly, trigger defense responses (Rojas et al., 2014). In the current study primary metabolic pathway activation following pathogen infection was a key outcome of seaweed extract treatment and presumably acted as an energy provider and regulator of *Arabidopsis* defense responses. For example, purine metabolites provide an ongoing source of nitrogen for *A. thaliana* growth. One of the purine metabolites, allantoin, plays a role in a JA-signaling pathway, suggesting that the role of purine metabolism not only underpins normal plant growth but, as others have found, is also a player in stress hormone homeostasis and signaling (Takagi et al., 2016). In our study, a large number of DEGs from each treatment were classified into purine metabolism through KEGG analysis. Therefore, this result indicated that purine metabolites acted to maintain plant growth during pathogen infection as well as contributing to defense-related hormone signaling pathways. In addition, at 12hpi the highest number of up-regulated purine metabolism genes was found for both AN and DP treatments whereas it was only at 24 hpi for the AN/DP treatment. This difference may have indicated a more sustained defense activation and supply of energy in the AN/DP-treated plants during pathogen infection.

Thiamine metabolism has an important function in many metabolic reactions including in glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. In addition, thiamine is also related to the induction of SAR and is involved in plant adaptation toward biotic and abiotic stresses (Kamarudin et al., 2017). For example, several studies reported that thiamine treatment of plants, including *A. thaliana,* activated plant defense and enhanced resistance to disease (Ahn et al., 2007; Boubakri et al., 2012; Kamarudin et al., 2017). Therefore, up-regulation of thiamine metabolism which was demonstrated in our study has strong implications for its involvement in the induction of defense, as well as adaptation, during infection by *P. cinnamomi*.

Common Plant Defense-Related DEGs That Were Up-Regulated Following Inoculation With *P. cinnamomi* in Extract-Treated Plants

Two hundred and sixty three genes (1.3% of the genome) were commonly found to be up-regulated for at least one time point following inoculation with *P. cinnamomi* of extract-treated plants. A number of candidate resistance-related genes were found to be up-regulated across all treatments including *PIN2*, *GRI*, *UGT73B4*, *CRK15*, and *MLO-8* which have been implicated in diverse resistance-related roles in different host and pathogen combinations. In addition to these genes, even though not above the cutoff by our RNASeq analysis, the pathogenesis-related genes *PR1*, *NPR1*, and *PR5* were confirmed to be commonly up-regulated following treatment with the extracts through our preliminary semi-quantitative PCR analysis as well as in the RNA-seq validation that used quantitative PCR. The PR proteins are a group of proteins that are induced by phytopathogens through activation of specific defense-signaling pathways and are fundamental components of resistance regulation (Backer et al., 2019). After pathogen infection, activation of defense-signaling pathways, such as those regulated by SA and JA take place which further leads to the accumulation of PR proteins that stops pathogen growth and development within host tissues. The SA pathway is especially active following infection by biotrophic pathogens and which stimulates the transcription of *NPR1* which in turn leads to activation, as well as accumulation, of SA-induced *PR* signature gene (*PR1*, *PR2*, and *PR5*) products locally and systemically that leads to SAR (Ali et al., 2018; Backer et al., 2019). PIN proteins are responsible for polar localization in the plasma membrane that determines the direction and rate of intercellular auxin flow (Sun et al., 2011). Moreover, *GR1* plays a crucial role in coordinating gene expression through both SA- and JA-signaling pathways (Mhamdi et al., 2010). The induction of these genes and other phytohormone-related genes in our study suggested that all three extracts induced defense against *P. cinnamomi* that was dependent on the activation of multiple phytohormone signaling pathways. In addition to all the above defense interactors, receptor-like kinases such as *CRK15* found to be up-regulated across treatments in our study, are fundamental signaling components that regulate a variety of cellular processes (Lee et al., 2017).

Plant secondary metabolites have numerous functions in plant−pathogen interactions and experimental evidence has demonstrated their important contributions in plant innate immunity (Piasecka et al., 2015). Plant-produced antibiotics are antimicrobial secondary metabolites and can be broadly classified as phytoalexins and phytoanticipins (Morrissey and Osbourn, 1999). UGT for example, plays an essential role in the biosynthesis of secondary metabolites in plants (Guo et al., 2016) and the induction of *UGT73B4* and other genes associated with the biosynthesis of antibiotics in all seaweed extract treatments indicated the synthesis of potentially novel antimicrobial compounds as a reaction to infection by *P. cinnamomi.*

The diverse patterns of differential gene expression found in our study were consistent with seaweed extracts having complex and pleiotropic modes of action that involved a cascade of gene activation for different plant responses. The commonality in the transcriptome profiles suggested that, at least for the seaweed extracts derived from the brown seaweeds used in the current study, behaved in a similar, but not identical, way.

Novel Genes That Were Up-Regulated That Provide Insight Into the Mechanisms of Action of Seaweed Extracts Against *P. cinnamomi*

WRKY transcription factors play important roles in plant responses to various biotic and abiotic stresses. WRKYs act as substrates of calcium-dependent protein kinases and calmodulin (CaM) is a Ca^{2+} -binding protein that is involved in various

cellular functions (Bakshi and Oelmüller, 2014). The function of calmodulin-like (CML) proteins is largely unknown. However, one of these, CML8 was found to be up-regulated in our study and has been shown to be involved in *Arabidopsis* immunity against *Pseudomonas syringae* (Zhu et al., 2017). The strong and consistent upregulation of *WRKY42* and *CML8* in the AN treatment indicated a correlation with, and the increased involvement of, calcium signaling in defense activation.

The *A. thaliana* genome has four jasmonate-induced oxygenases (JOXs) and one of them hydroxylates jasmonic acid to 12-OH-JA (Caarls et al., 2017). In our study the higher expression of *JOX1* in the DP treatment at 6 hpi indicated the involvement of other phytohormone signaling pathways at this stage of the interaction with the pathogen. The expression of the *POLARIS* (*PLS*) gene that encodes a 36-amino acid peptide that regulates plant root growth and vascular development, is related to auxin transport and coordinates the ethylene signaling pathway (Chilley et al., 2006). The strong up-regulation of *PLS* expression in AN/DP treatments suggested that *PLS* also contributed to *A. thaliana* root growth, as well as functioning in regulation of phytohormone-induced signaling pathways, that resulted in suppression of *P. cinnamomi*. In addition, the strong up-regulation of other uncharacterized genes, in all three treatments, suggested the contribution of unknown novel mechanisms in AN and DP extract-induced defense.

Other publications have compared the transcriptional profiles of plants treated with seaweed extracts derived from the brown seaweed *A. nodosum* (Nair et al., 2012; GonñI et al., 2016; Santaniello et al., 2017; Jithesh et al., 2019; Omidbakhshfard et al., 2020). The extracts used in these studies varied in their chemical nature (including alkaline, neutral and acid extracts) and extraction approaches. Despite the differences observed among the transcriptional profiles following extract-treatment of plants, the overall results demonstrate the highly dynamic and responsive nature of plants to different types of seaweed extracts, and the inherent capacity for the seaweed extracts to simultaneously enhance plant growth and tolerance to biotic and abiotic stresses.

Role of ROS in Seaweed Induced Plant Defense

To examine the production of defense-related components prior to and following the upregulation of defense-related transcripts, the production of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), was examined in *A. thaliana* roots grown with each seaweed extract and infected, or not, with *P. cinnamomi*. The induction of hydrogen peroxide was found only in those roots treated with the three seaweed extracts individually and infected with *P. cinnamomi*. This result is somewhat different to that of a previous study (Cook et al., 2018) that showed the induction of reactive oxygen species in seedlings treated only with an *A. nodosum* extract. This variation in results between the two studies may reflect differences in preparation of the extract and the treatment and analysis methods. The method used in our study gave a direct visualization of the location and intensity of ROS in the roots, something that is not possible using alternative assays.

The *PEP1* gene, which was found to be up-regulated in our study in DP-treated plants, is involved in activation of the synthesis of enzymes associated with hydrogen peroxide formation (Huffaker et al., 2006). Also, plant peroxidases participate in various physiological processes, such as lignification, suberisation, auxin catabolism and defense mechanisms that are activated during pathogen infection. They are considered to catalyze the generation of aromatic oxyl radicals from several aromatic compounds and the peroxidase-dependent production of such organic radicals often results in the generation of reactive oxygen species (Kagan et al., 1990; Kawano, 2003). The induction of peroxidases in all extract treatments suggested their involvement in ROS generation and potentially other aspects of extract-induced defense mechanisms.

The Trade-Off Between Growth and Defense and Priming for Defense

New insights into how plants balance growth while responding to stress has implications for advanced agriculture. The compromise between growth and stress response is based on plants having limited resources which need to be prioritized for growth, or toward responses to the abiotic and/or biotic stresses they encounter (Huot et al., 2014; Karasov et al., 2017). The trade-off concept is supported by research that has demonstrated that plant-fitness costs are associated with the induction of defense genes (Huot et al., 2014; Karasov et al., 2017), and that maintaining activated plant response systems is metabolically costly (Karasov et al., 2017; Buswell et al., 2018). Nevertheless, emerging research has uncovered chemical priming of immunity that provides defense without costs to plant growth (Buswell et al., 2018).

In our research we observed a balanced trade-off between root growth and the activation of specific defense pathways. Our transcriptomic analysis identified the up-regulation of specific defense-associated pathways (such as those regulated by SA, JA, ET, and PPP) and genes associated with plant resistance (for example *PR1*, *MLO*, and others), while root length growth continued, despite the interior of the roots being actively infected by *P. cinnamomi*. In our experimental design pretreating the plants with seaweed extract was an important pre-requisite. This approach may have contributed to a favorable trade-off that utilized a natural plant priming system. More generally, extracts of different seaweeds have been shown to activate broad spectrum defense systems in plants (Kerchev et al., 2019; Shukla et al., 2019). Collectively the research supports the notion that seaweed extracts may act as a plant priming stimulant, particularly if pre-applied.

Plant-priming is an adaptive and low-cost defensive mechanism that, upon activation by a priming stimulus, results in a faster and/or stronger induction of inducible defenses. Plant-priming occurs in a wide range of plant species and is often associated with enhanced abiotic and biotic stress tolerance (Martinez-Medina et al., 2016). The idea that priming is the result of treatment with a specific seaweed extract is supported by our transcriptomics analysis particularly based on the molecular and

cellular GO categories: for example, genes up-regulated for (i) DNA Binding Transcription Factor Activity (ii) Transcription Regulatory Region DNA Binding, (iii) the increased number of transcripts found in the nucleus, (iv) and up regulation of genes associated with redox signaling and sensing. Also ROS are key molecules involved in the priming process (Borges et al., 2014). Our data showing the accumulation of hydrogen peroxide in root cells at 12 hpi, in plants pretreated with seaweed extract, was consistent with ROS acting as a latent signal involved in priming plant resistance (Gonzalez et al., 2009). The priming of plant resistance can also be achieved by exogenous application of synthetic and natural compounds (Aranega-Bou et al., 2014). Hexanoic acid, for example, is a natural primer (Aranega-Bou et al., 2014) which is a component of one of the seaweed extracts used in this study (AN/DP, data not published).

CONCLUSION

This study showed that *A. thaliana* was a very useful model plant for studies on the impact that a seaweed extract-based biostimulant had on interactions at the molecular level with a root pathogen. We also demonstrated the up-regulation of key SAR-related genes and phytohormone- associated genes at various critical time points post-inoculation following treatment with extracts of the selected brown seaweeds. Importantly, each seaweed extract induced multiple defense-related pathways prior to penetration and infection by the pathogen. These observations were characteristic of a primed response, and closely associated with ROS production. Transcriptomic analysis has proven to be a powerful approach to elucidate the timing of activation of defense-related mechanisms and the subsequent suppression of pathogen growth. Our results can now be used in future studies that use specific plant mutants that are impaired in various resistance-related pathways or, for example, gene edited hosts to investigate the role of individual defense components in seaweed extract-induced defense. Further, we propose that the approach used in the current study could be applied to agriculturally important crop species to investigate the impact of seaweed extract-treatment on their reaction to a pathogen.

DATA AVAILABILITY STATEMENT

The datasets presented in this study have been deposited in online repositories. The names of the repositories and their accession number(s) can be found either in this article or in the/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MI, DC, and TA conceptualized and designed the project. MI performed the laboratory work, analyzed the RNA-seq transcriptome data, and wrote and revised the manuscript. HG contributed to RNA-seq data processing. MZ performed the functional analysis of DEGs. HH contributed to the critical analysis of the RNA-seq data and manuscript draft. DC and TA contributed to the manuscript draft and final version. All authors revised and approved the final version to be published and agreed with all aspects of the work.

FUNDING

This project was funded through the Faculty of Science, Engineering and Built Environment (SEBE), Deakin University, Australia post-doctoral support for industry projects scheme and jointly with Seasol International Pty Ltd., Australia.

ACKNOWLEDGMENTS

We would like to thank Drs. Pavani Nadiminti and Xinhua Lu, Deakin University, for assistance with growing plants and other aspects of the laboratory experiments for the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00852/full#supplementary-material

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Conflict of Interest: Seasol® was manufactured by the company Seasol International (SI). TA and HH are researchers in the SI R&D Department and employed by SI. SI provided in-kind support as salary for TA and HH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 17 July 2020
doi: 10.3389/fpls.2020.00999

Physiological, Ecological, and Biochemical Implications in Tomato Plants of Two Plant Biostimulants: Arbuscular Mycorrhizal Fungi and Seaweed Extract

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The worldwide use of plant biostimulants (PBs) represents an environmentally friendly tool to increase crop yield and productivity. PBs include different substances, compounds, and growth-promoting microorganism formulations, such as those derived from arbuscular mycorrhizal fungi (AMF) or seaweed extracts (SEs), which are used to regulate or enhance physiological processes in plants. This study analyzed the physiological, ecological, and biochemical implications of the addition of two PBs, AMF or SE (both alone and in combination), on tomato plants (*Solanum lycopersicum* L. cv. "Rio Fuego"). The physiological responses evaluated were related to plant growth and photosynthetic performance. The ecological benefits were assessed based on the success of AMF colonization, flowering, resistance capacity, nonphotochemical quenching (NPQ), and polyphenol content. Biochemical effects were evaluated *via* protein, lipid, carbohydrate, nitrogen, and phosphorous content. Each PB was found to benefit tomato plants in a different but complementary manner. AMF resulted in an energetically expensive (high ETR_{MAX} but low growth) but protective (high NPQ and polyphenol content) response. AMF + nutritive solution (NS) induced early floration but resulted in low protein, carbohydrate, and lipid content. Both AMF and AMF + NS favored foliar instead of root development. In contrast, SE and SE + NS favored protein content and root development and did not promote flowering. However, the combination of both PBs (AMF + SE) resulted in an additive effect, reflected in an increase in both foliar and root growth as well as protein and carbohydrate content. Moreover, a synergistic effect was also found, which was expressed in accelerated flowering and AMF

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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 01 February 2020
Accepted: 17 June 2020
Published: 17 July 2020

Citation:
González-González MF, Ocampo-Alvarez H, Santacruz-Ruvalcaba F, Sánchez-Hernández CV, Casarrubias-Castillo K, Becerril-Espinosa A, Castañeda-Nava JJ and Hernández-Herrera RM (2020) Physiological, Ecological, and Biochemical Implications in Tomato Plants of Two Plant Biostimulants: Arbuscular Mycorrhizal Fungi and Seaweed Extract. Front. Plant Sci. 11:999. doi: 10.3389/fpls.2020.00999

colonization. We present evidence of benefits to plant performance (additive and synergistic) due to the interactive effects between microbial (AMF) and nonmicrobial (SEs) PBs and propose that the complementary modes of action of both PBs may be responsible for the observed positive effects due to the new and emerging properties of their components instead of exclusively being the result of known constituents. These results will be an important contribution to biostimulant research and to the development of a second generation of PBs in which combined and complementary mechanisms may be functionally designed.

Keywords: plant biostimulant, *Rhizophagus intraradices*, *Padina gymnospora*, additive property, synergistic property, emergent properties

INTRODUCTION

The current challenges associated with horticultural production are growing due to the ever-increasing worldwide demand for efficient, environmentally friendly, and sustainable food production. Previously proposed variants of the term "Plant biostimulant" (PB) included biogenic stimulator (Filatov, 1951a; Filatov, 1951b), organic biostimulant (Russo and Berlyn, 1991), biostimulator (Goatley and Schmidt, 1991), biostimulant (Schmidt, 1992), and others as reviewed and cited in Yakhin et al. (2017). An *ad hoc* revision of PBs, which was published as "The Science of PBs - A bibliographic Analysis" (Du Jardin, 2012), was carried out by the European Commission. PBs were defined therein as highly heterogeneous materials that could be classified into eight categories: humic substances, complex organic materials, beneficial chemical elements, inorganic salts, seaweed extracts (SEs), chitin and chitosan derivates, antitranspirants, and free amino acids and N-containing substances. However, this PB classification did not include any microbial biostimulants. Three years later, Colla and Rouphael (2015), in their special issue article titled "Biostimulants in horticulture," proposed six nonmicrobial (i.e., chitosan, humic and fulvic acids, protein hydrolysates, phosphites, SEs, and silicon) and three microbial [i.e., arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria, and *Trichoderma* spp.] PBs. Thus, PBs are any applied substance or microorganism, including those of commercial products, which stimulate natural processes and improve nutrient uptake and use efficiency, abiotic stress tolerance, and crop quality (Du Jardin, 2015). However, PB definitions and classifications have recently been the focus of controversy. The new proposed definition of a PB is "a formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds" (Yakhin et al., 2017). This particular conceptualization of PBs, in addition to allowing for a better understanding of their physiological and biochemical modes of action, has contributed to the development of PB science, industry, and legislation. Despite the notable progress in PB science in recent years, there are still many questions that remain open as well as many challenges and opportunities to identify patterns in complex data and elucidate the inherent activity and potential synergistic effects of the combination of microbial and nonmicrobial PBs for agricultural purposes (Rouphael and Colla, 2018).

The application of microbial and nonmicrobial PBs may efficiently improve yields without increasing the quantity of applied nutrients. Furthermore, the resultant effect of the combined application of microbial and nonmicrobial PBs may be antagonistic, additive, or synergistic. In antagonistic interactions, the combined effect of the PBs is lower than the sum of the PB effects when they are applied independently. In additive interactions, the combined effect of the PBs is equal to the sum of the PB effects when they are applied independently. Synergistic interactions occur when the combined effect of the PBs exceeds the sum of the PB effects when they are applied independently (Rouphael and Colla, 2018; Rouphael and Colla, 2020). However, knowledge of potential synergistic effects among PBs is scarce, and limited published data is available with respect to nutrient uptake efficiency or plant performance (Rouphael et al., 2017; Rouphael et al., 2018; Rouphael and Colla, 2018). Therefore, this study aimed to analyze the physiological, ecological, and biochemical implications of the addition of two PBs, AMF and SE, both independently and in combination, on the development of tomato plants (*Solanum lycopersicum* L. cv. "Rio Fuego") to better elucidate the causal/functional mechanism of action.

AMF comprise an important microbial PB category composed of a soil biota functional group that has been found to positively affect crop production and support ecosystem sustainability (Rouphael et al., 2015a). The advantages plants derive from symbiosis go far beyond the nutritional benefits they obtain. Mycorrhizal plants have shown improved tolerance and resistance to a broad range of environmental stressors caused by both abiotic (e.g., drought or salinity) and biotic (e.g., pests and pathogens) factors due to the protection they gain from changes in various physiological parameters (Song et al., 2015; Begum et al., 2019 and literature cited therein). The mechanisms that mediate benefits from AMF are diverse and depend on the characteristics of the stress, and in most cases, these have been reported to be finely regulated by phytohormones (Pozo et al., 2015). Considering this complexity, further efforts are needed to unravel the mechanisms underlying the enhanced ability of host

plants to overcome adverse conditions. This knowledge contributes to the promotion of the use of AMF as biostimulants and bioprotectors in agricultural practices as environmentally-friendly alternatives to traditional crop management strategies, which have generally depended on chemical fertilizer and pesticide application. AMF and plants live in symbiosis, and AMF hyphae grow into plant roots (Harrison, 2005; Gutjahr et al., 2009; Gutjahr and Paszkowski, 2013). Further, mycorrhizal ontogenesis has been linked to both host and symbiont growth and development (Chaudhary et al., 2019). Photosynthetic products are obtained from the host plant and utilized by fungi, who in turn supply the plant root system with soil nutrients, such as phosphorus, nitrogen, copper, and zinc (Ferrol et al., 2019). This form of symbiosis has been found to promote secondary metabolite synthesis, such as that of phenolic acids or flavonoids, which are essential for elevating abiotic stress tolerance in plants (Morandi, 1996; Strack and Fester, 2006; Schliemann et al., 2008; Tavarini et al., 2018). The economic and ecological value of AMF comes from approximately 80% of all land plants interacting with AMF, including agronomically important crops (Azcón-Aguilar and Barea, 1997). Recent studies using omics technologies have allowed researchers to further elucidate the important protective mechanisms resulting from AMF interactions that protect plants from abiotic stress, [i.e., Transcriptomics (Salvioli et al., 2012), Proteomics (Bernardo et al., 2017), and Metabolomics (Bernardo et al., 2019)].

The mechanism that has been proposed to explain the biostimulant activity of AMF with regard to plant performance is root biomass regulation that may enhance nutrient uptake and translocation, resulting in an increase in total carbohydrate, protein content, and phenolic levels while promoting growth, biomass production, stress tolerance, and disease resistance (Abbas, 2013; Colla et al., 2015a; Colla et al., 2015b; Rouphael et al., 2015a; Rouphael et al., 2017; Fiorentino et al., 2018; Rouphael and Colla, 2018; Lucini et al., 2019). In addition, AMF hyphal networks can enhance the quality of the soil by improving soil particle aggregation and reducing soil erosion by either wind or water. Further, AMF limit the amount of nutrients that are leached from the soil and thus promote nutrient retention while decreasing the risk of ground water contamination (Rouphael et al., 2015a; Chen et al., 2018; Tavarini et al., 2018).

Another important nonmicrobial PB category is based on SEs from brown, green, and red macroalgae, which have been proposed as sustainable amendments to improve crop yields without adverse environmental impacts (Khan et al., 2009). SEs are inexpensive and easy to prepare and use (Hernández-Herrera et al., 2014a). In addition, beneficial effects may be achieved with small SE doses (diluted to 1:1,000 or more; Crouch and van Staden, 1993). The active SE components have been identified as macroelements and microelements, such as nutrients, amino acids, vitamins, sugars (e.g., carbohydrates and oligo- and polysaccharides), growth hormones [e.g., cytokinins, auxins, gibberellins, and abscisic acid (ABA)-like growth substances], or low-weight molecular components (e.g., polyamines and brassinosteroids), all of which have been found to affect cellular metabolism and enhance crop growth and yield (Khan et al., 2009; Battacharyya et al., 2015; Shukla et al., 2019). Furthermore, SE components that are present in moderate or large quantities, such as polyphenols (e.g., phloroglucinol and eckol) or polysaccharides (e.g., alginate, fucoidan, laminarian, carrageenan, and their derived oligosaccharides), have also been found to promote plant growth (Hong et al., 2007; Khan et al., 2009; Craigie, 2011; González et al., 2013; Battacharyya et al., 2015; Rengasamy et al., 2015a; Rengasamy et al., 2015b; Hernández-Herrera et al., 2016; Mzibra et al., 2020).

SEs also provide an alternative means to manage pests and prevent plant disease (Baloch et al., 2013), increase plant tolerance to abiotic stress (EL Boukhari et al., 2020), and improve the number of fruits or other quality traits (Hamed et al., 2018). These are a result of the combined actions of various complex pools of bioactive molecules found within SEs (Khan et al., 2009; Ertani et al., 2018). According to Shukla et al. (2019), a specific mode of action for SEs is the role they play in plant growth by regulating genes involved in nutrient acquisition and thus enhancing nutrient uptake.

We hypothesized that the independent application of AMF and SE would be favorable but would differ with regard to plant development. However, we assumed that when microbial and nonmicrobial PBs were used in combination, their combined effect on the plants would be far superior (synergistic) to that of either PB when applied independently due to the new and emerging properties of the constituent complex.

MATERIALS AND METHODS

Experimental Design and Plant Material

The greenhouse experiment was performed in a complete randomized block design with a total of six treatments that contained 12 experimental units (replicas) each (n = 72 plants). The experiment was carried out from February to May in 2019. The treatments consisted of (1) plants grown without any PBs and only irrigated with Rorison nutritive solution (NS); (2) plants treated with a microbial-based biostimulant containing *Rhizophagus intraradices*, a type of AMF; (3) plants treated with a microbial-based biostimulant containing AMF and irrigated with the NS (AMF + NS); (4) plants treated with a nonmicrobial-based biostimulant from a *Padina gymnospora* extract (SE); (5) plants treated with a nonmicrobial-based biostimulant from SE and irrigated with the NS (SE + NS); and (6) plants grown with both PBs and irrigated with the NS (AMF + SE + NS). A control group of plants irrigated with only water was also included, although the plants did not survive until the end of the experiment, and thus the data are not shown.

Solanum lycopersicum L. cv. "Rio Fuego" seeds (Kristen Seed, San Diego, USA) were surface-sterilized in 3% sodium hypochlorite for 10 min, triple-rinsed in sterile distilled water, and planted individually in 1-L pots with a sterile soil mixture composed of vermiculite:sand (1:2, v/v) that had been autoclaved thrice. The plants were cultivated under natural light conditions. Daily temperature in the greenhouse was always maintained below 27°C ± 2°C, and the night temperature

was always higher than 15°C ± 2°C. The average day/night relative humidity was ~ 85%.

PB Application

The AMF microbial-based biostimulant containing *Rhizophagus intraradices* (N.C. Schenck and G.S. Smith) C. Walker and A. Schüßler, previously referred to as *Glomus intraradices*, was produced by Experimental Field Bajio at the National Research Institute of Forestry, Agriculture, and Livestock (INIFAP). The *R. intraradices* AMF was comprised of a mixture of mycelia, root segments, spores, and soil-sand and is commercialized as Mycorrhiza-INIFAP® (INIFAP®, Celaya, Guanajuato, Mexico).

The SE was obtained from the Biotechnology Research Laboratory of the Universidad de Guadalajara (Guadalajara, Mexico). The methodology for its preparation followed that of Hernández-Herrera et al. (2014a; 2014b). Briefly, 8 g of dry powder from the brown seaweed *Padina gymnospora* (Kützing) Sonder was added to 1 L of distilled water, constantly stirred for 15 min, and autoclaved at 121°C for 1 h at 1.21 kg cm^{-2}. The hot extract was filtered through Whatman No. 40 filter paper from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and stored at −4°C until further use. The chemical composition of the *P. gymnospora* SE at a 0.8% concentration was analyzed, and the results are shown in **Supplementary Table S1**.

For the experiment, the tomato seeds were sown in sand and divided into six groups. Two groups of seeds were first treated by covering them with a microbial-based biostimulant solution of AMF at a concentration of 3 g L^{-1} (~ 100 spores g^{-1}) and then watered (one group with NS and the other with only water). Similarly, two other groups of tomato seeds were treated with the nonmicrobial biostimulant, SE. For the nonmicrobial PBs treatment, the SE was added directly to the substrate (50 mL of *P. gymnospora* at a concentration of 0.8%) on planting day (one SE group was watered with NS and the other with only water). Furthermore, a group of tomato seeds was treated with a combination of both biostimulants (AMF and SE) and then watered with NS. In all treatments, the initial application of the biostimulant took place on planting day with an additional application on day 15 when the tomato seedlings had already emerged. After which, watering took place every two weeks for a total of five applications of the PBs during the experiment. The control treatment was comprised of seeds that were planted in sand without PB and only watered with the NS. All of the plants of the treatments that were watered with NS were irrigated weekly with the NS known as Rorison nutrient solution (Hewitt, 1966) with modifications. The phosphate concentration of the solution was diluted to 0.05 mM to decrease the KH_2PO_4 content to favor AMF colonization. The composition of the Rorison NS used in this work is shown in **Supplementary Table S2**. Furthermore, all plants in all treatments were watered with additional distilled water according to their needs.

Physiological Responses of Tomato Plants

After 96 d, the effects of AMF and SE on plants were analyzed by measuring physiological descriptors. Initially, photosynthetic performance was measured in six randomly selected plants per treatment *via* nonintrusive pulse amplitude modulated chlorophyll fluorometry using a Junior PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The leaves were dark-acclimated with leaf clips for 20 min before the start of a rapid light curve (RLC) routine. Actinic illumination in the RLC trial was increased in 12-step increments from 5-1500 μmol photon m^{-2} s^{-1} with a total of 30 s for each light level. The maximum electron transport rate (ETR_{MAX}) was calculated from the RLC according to the methodology of Murchie and Lawson (2013) and used as a proxy for plant photosynthetic performance. In the same fluorescence trial, the maximum photochemical quantum yield efficiency of PSII in a dark-adapted state (F_V/F_M) and nonphotochemical quenching (NPQ) were also obtained to evaluate the photoinhibition state and photoprotection capacity of the plants due to PB addition.

Once the chlorophyll fluorescence parameters were measured in the living plants, the plants were harvested to evaluate their growth characteristics. The plants were carefully removed from their plastic pots and submerged immediately in bowls filled with water at ~20°C for 20 min. Then, the 12 plants per treatment were photographed and growth characteristics were measured using ImageJ v. 1.52a software (https://imagej.nih.gov/ij/download.html). Data were used to obtain the ratio of the projected shoot, root, and total length; leaf and root area; and the number of leaves and flowers. After growth was measured, the root and foliar system were also carefully washed to eliminate sand particles and subsequently dried with blotting paper for further analysis.

Biochemical Characteristics of Tomato Plants

The effects of AMF and SE on plants were evaluated by measuring the biochemical composition of the 12 replicas per treatment (n = 12 tomato plants). The samples were oven dried at 65°C for 72 h. A 100-g sample of dry material was used for quantifying protein, lipid, total carbohydrate, nitrogen, phosphorous, and total polyphenol content. The methods followed those of the Association of Official Analytical Chemists (A.O.A.C., 1990) for lipids (954.04), nitrogen (955.04), and proteins using a factor of 6.25 (954.04). Phosphorous determination was performed according to Mengel and Kirkby (1987), and total carbohydrate content was evaluated with the DuBois method (DuBois et al., 1956), which included a standard glucose calibration curve (Merck KGaA). Finally, the Folin-Ciocalteu colorimetric method, based on the procedure of Singleton and Rossi (1965), was used to estimate total polyphenol content using gallic acid as the standard. All chemical measurements were performed in triplicate (each replicate consisted of a mix of four plants). A separate sub-sample of the roots per plot was frozen immediately with liquid nitrogen and kept at −80°C for subsequent gene expression analyses.

Ecological Benefits and AMF Molecular Colonization in Tomato Plants

The success of the mutual association between AMF and tomato plants was determined at the end of the experiment based on root measurements. Root systems were washed in cold water and

their fresh weights were recorded. Small root samples were stored in liquid nitrogen for RNA extraction, and a fresh root fraction was fixed in formalin/acetic acid/ethanol (FAE, 13:5:200 [v/v/v]) for 24 h to determine the degree of AMF colonization. The roots were cut into 1-cm pieces and then placed in 10% KOH at 99°C for 1.5 h. The samples were then stained with 0.05% (w/v) trypan blue in lactophenol following the methods described by Phillips and Hayman (1970). The AMF were examined under a Primo Star compound light microscope (Carl Zeiss, Gottingen, Germany). Fungal colonies were estimated as described by Trouvelot et al. (1986) using the MYCOCALC program[1] by means of the AMF colonization in the root system (M%) and arbuscule abundance (A%) quantified from six randomly selected roots per treatment (each one with 30 replicates). A total of 180 fragments from each treatment group were evaluated.

In addition, another sub-sample of the same six roots per treatment was immediately frozen with liquid nitrogen and kept at −80°C for subsequent gene expression analyses. The expression of symbiosis marker genes (i.e., *RiEF* and *LePT4)* was analyzed to confirm mycorrhizal colonization. Total RNA was extracted from the roots obtained from a pool of six plants using Trizol reagent (Invitrogen, Carlsbad, USA) according to the protocol of the manufacturer. cDNA was obtained from 2 µg of RNA using M-MLV reverse transcriptase (Promega, Madison, USA) and oligo-dT (12–18) primers. To determine gene expression, real-time quantitative PCR (RT-qPCR) amplifications were performed in 96-well plates using SYBR Green (Thermo Fisher Scientific, Germantown, USA) detection chemistry in a StepOnePlus™ RT-qPCR System (Applied Biosystems, Foster City, USA). Reactions were prepared in a total volume of 15 µl, with 2 µl of cDNA template (1:10), 7.5-µl SYBR® Select Master Mix (Applied Biosystems), and forward and reverse primers (300 nM). The gene-specific primers were designed from GenBank sequences (**Supplementary Table S3**). The cycling conditions were set as follows: initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing at 60°C for 30 s. A melting curve analysis was used to evaluate reaction specificity. The baseline and cycle threshold (Ct) were automatically determined using the RT-qPCR system software. Relative expression was calculated using a comparative cycle threshold method (Livak and Schmittgen, 2001). Transcript abundance was normalized using the housekeeping gene of the SAND family proteins as an endogenous reference (Expósito-Rodríguez et al., 2007).

Statistical Analysis

Twelve plants per treatment were used for each analysis (mean ± SD). An analysis of variance (ANOVA) was performed to evaluate significant differences among treatments for all physiological, ecological, and biochemical descriptors with Statgraphics Centurion XV (Statpoint Technologies, Inc., The Plains, USA). Significance ($P \leq 0.05$) was identified with the general linear model (GLM) procedure and the least significant difference (LSD) mean comparison test. A joint principal coordinates analysis (PCoA) was performed on the physiological variables to evaluate their interdependence, including photosynthetic performance (ETR_{MAX}) and growth parameters (shoot, root, and total length and leaf and root area). A second PCoA analysis was performed for biochemical and ecological benefits (i.e., the success of the mutual association *via* AMF colonization), flowering, resistance capacity (NPQ), and polyphenol content. In addition, a Cluster-Simprof analysis was performed on the Euclidian distance matrixes constructed from square root-transformed descriptor data to identify similar patterns among the PB treatments. The cluster groups, PCoA figures, and correlation values were generated using Primer 7 + Permanova (PRIMER-E Ltd., Plymouth, UK) according to the methodology of Anderson et al. (2008).

[1] https://www2.dijon.inrae.fr/mychintec/Mycocalc-prg/download.html

RESULTS

Physiological Characteristics of Tomato Plants in Response to PB Addition

The physiological parameters of photosynthesis and growth performance were significantly ($P \leq 0.05$) affected by AMF and SE PBs as well as the combination of AMF and SE (**Tables 1** and **2**). **Figure 1** shows an example of a growth image pertaining to a representative plant of each PB treatment. A biplot analysis was used to confirm the relationship that was expressed in the ANOVA among the PBs and the physiological parameters of the tomato plants. The PCoA graphs in **Figures 2A–E** show a comparison among the physiological parameters of the PB-treated plants. Two factors explained 92.5% of the total variance. Factor 1 (PCO1) explained 82.3% of the variance and was negatively correlated with root area and fresh weight, while also being positively correlated with ETR_{MAX} (**Supplementary Table S4**). Factor 2 (PCO2) explained 10.2% of the variance and was positively correlated with shoot length. By plotting data according to PCO1 and PCO2, three clusters were identified that showed a clear separation among tomato plants from the different PB treatments.

The first group was composed of plants treated with AMF and plants irrigated with NS. The AMF-treated plants presented the lowest root length growth and fresh weight, although their shoot length was significantly larger than that of the NS plants ($p \leq 0.05$), highlighting the biostimulant activity of AMF. Moreover, AMF conferred an extremely high resistance to environmental stress in their host plants, as determined from the high polyphenol content as well as the high NPQ and F_V/F_M values (**Tables 2** and **3**, **Figure 3E**) that reflected an augmentation of antioxidant and photoprotective mechanisms. In contrast to the AMF plants, the NS plants that grew without PB addition but that were irrigated with the NS presented low leaf growth yet large and heavy roots (**Table 1**) as well as significantly lower NPQ values (**Table 2**). This result indicates that the NS plants presented lower antioxidant and photoprotective capacity than that of the AMF plants.

As no differences in the degree of photoinhibition were present between the AMF and NS plants, which was evident in their similar F_V/F_M values, it appears that the high ETR_{MAX} energy in

TABLE 1 | Growth of tomato plants treated with plant biostimulants.

Treatments	Length (cm)			Area (cm^2)		Weight (g)		Total Flowers
	Shoot	Root	Total	Leaf	Root	Fresh	Dry	
NS	16.9 ± 1.2^{b}	24.4 ± 8.2^{b}	39.1 ± 9.1^{a}	11.3 ± 2.3^{a}	43.4 ± 13.8^{b}	1.65 ± 0.51^{a}	0.71 ± 0.32^{b}	0
AMF	21.1 ± 4.1^{c}	18.0 ± 1.3^{a}	39.1 ± 4.6^{a}	23.8 ± 8.1^{b}	11.5 ± 4.9^{a}	1.67 ± 0.49^{a}	0.18 ± 0.08^{a}	0
AMF + NS	21.9 ± 1.9^{c}	31.5 ± 5.1^{c}	53.6 ± 8.6^{d}	104.3 ± 4.3^{d}	143.1 ± 28.4^{d}	12.65 ± 1.86^{c}	1.30 ± 0.80^{cd}	5
SE	12.5 ± 3.6^{a}	30.8 ± 5.3^{c}	45.4 ± 6.1^{c}	31.8 ± 9.8^{c}	50.9 ± 8.4^{b}	4.13 ± 0.84^{ab}	1.23 ± 0.23^{c}	0
SE + NS	12.4 ± 2.3^{ab}	33.8 ± 5.2^{c}	44.7 ± 5.4^{bc}	34.8 ± 6.2^{c}	58.6 ± 11.2^{b}	4.32 ± 0.93^{b}	1.31 ± 0.26^{cd}	0
AMF + SE + NS	24.8 ± 2.0^{d}	32.5 ± 2.4^{c}	58.4 ± 2.9^{e}	120.5 ± 20.3^{e}	117.8 ± 5.03^{c}	14.46 ± 1.65^{d}	1.56 ± 0.29^{d}	13

Tomato plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing Rhizophagus intraradices (AMF), treated with a AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from Padina gymnospora extract (SE), treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants and irrigated with nutritive solution (AMF + SE + NS). Values are mean ± standard deviation (n = 6. Different letter (a–d) within the columns indicate significant differences according to the least significant differences (LSD) mean comparison test (p = 0.05).

TABLE 2 | Maximum electron transporter rate (ETR_{MAX}), photochemical quantum yield of PSII (F_V / F_M), and nonphotochemical quenching (NPQ) measured in tomato plants treated with plant biostimulants.

Treatments	ETR_{MAX}	F_V/F_M	NPQ
NS	262.00 ± 15.87^{d}	0.802 ± 0.00^{b}	0.84 ± 0.10^{a}
AMF	268.84 ± 32.9^{d}	0.801 ± 0.01^{b}	1.30 ± 0.10^{b}
AMF + NS	104.98 ± 9.07^{a}	0.757 ± 0.00^{a}	0.99 ± 0.11^{a}
SE	112.64 ± 14.93^{a}	0.778 ± 0.02^{ab}	0.81 ± 0.15^{a}
SE + NS	197.63 ± 31.14^{c}	0.786 ± 0.01^{a}	0.99 ± 0.14^{b}
AMF + SE + NS	151.92 ± 13.14^{b}	0.783 ± 0.01^{a}	0.96 ± 0.25^{b}

Tomato plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing Rhizophagus intraradices (AMF), treated with a AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from Padina gymnospora extract (SE), treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants and irrigated with nutritive solution (AM F+ SE + NS). Values are mean ± standard deviation (n = 6). Different letters (a–d) within the columns indicate significant differences according to the least significant difference (LSD) mean comparison test (p = 0.05).

NS plants may have been spent repairing photosynthetic machinery and developing larger root systems to obtain missing nutrients. The second group was composed of SE-treated plants (SE) and SE-treated plants irrigated with the NS (SE + NS). This group exhibited greater shoot and root length, root surface area, and fresh weight than those of the AMF and NS plants. These results highlight the PB power of SE. Interestingly, a significant beneficial effect was observed in the third group that included AMF-treated plants irrigated with the NS (AMF + NS) as well as AMF- and SE-treated plants irrigated with the NS (AMF + SE + NS). This third plant group displayed the highest growth values and a down-regulation of the electron transport rate at the PSII level (ETR_{MAX}), which suggests an optimization of energetic resources (**Figures 2A–E**).

Biochemical and Ecological Effects in Response to PB Addition

By relating plant parameters with AMF and SE PBs through a PCoA, two factors were found to explain 99.9% of the total variance (**Figures 3A–E**). Factor 1 (PCO1) explained 95% of the variance and was negatively correlated with AMF colonization in the root system (M%) and the number of flowers. Factor 2 (PCO2) explained only 4.9% of the variance and was positively correlated with polyphenol content and NPQ values (**Supplementary Table S5**).

Plotting data according to PC1 and PC2 resulted in three clusters that were clearly separated by the PB properties of the different treatments. Group 1 was composed of NS plants, SE-treated plants, and SE + NS plants. These plants showed the lowest polyphenol content, low NPQ induction, and no flower development. Logically, no mycorrhiza colonization was observed. Cluster 2 was composed of AMF-treated plants. These plants showed the lowest AMF colonization in the root system but presented the most effective NPQ induction and the highest polyphenol content. Increasing NPQ may play a key role in dissipating excess energy to prevent the photosynthetic machinery from being destroyed by excess light energy. Polyphenols may also help to destroy ROS molecules that affect the PS II photosynthetic core protein D1. Therefore, AMF treatment alone conferred ecological advantages to the tomato plants under low nutrient conditions (**Figures 3A–E**). Finally, the third cluster included AMF + NS plants and AMF + SE + NS plants. This final group of plants displayed high AMF root colonization as well as the greatest biomass yield and number of flowers (**Figures 3A–E**).

With respect to the biochemical composition of the tomato plants treated with the combination of both PBs and irrigated with the NS (AMF + SE + NS), these plants showed the greatest yield with regard to both fresh and dry biomass as well as high protein, carbohydrate, and phosphorous content in leaf tissues (**Tables 1** and **3**).

Root Mycorrhizal Colonization

Plants were collected 96 d after AMF treatment. Fungal structures were stained within the roots and showed well-established mycorrhizal symbiosis in the AMF-treated and AMF + SE + NS plants, with ample fungal colonization and well-formed arbuscules at the root cortex. The absence of fungal structures was confirmed in the roots of the plants from the nonmycorrhizal treatments (**Figure 4**). The AMF colonization in the root system (91.5%), arbuscule abundance (84%), and vesicle number (125) were more abundant in the roots of plants treated with the combination of both PBs and irrigated with the NS (AMF + SE + NS). The AMF colonization levels agreed with the expression level of the *RiEF1-α* gene marker of *Rhizophagus intraradices*. Gene expression was elevated in AMF + NS plants and slightly more so in AMF + SE + NS plants (**Table 4**).

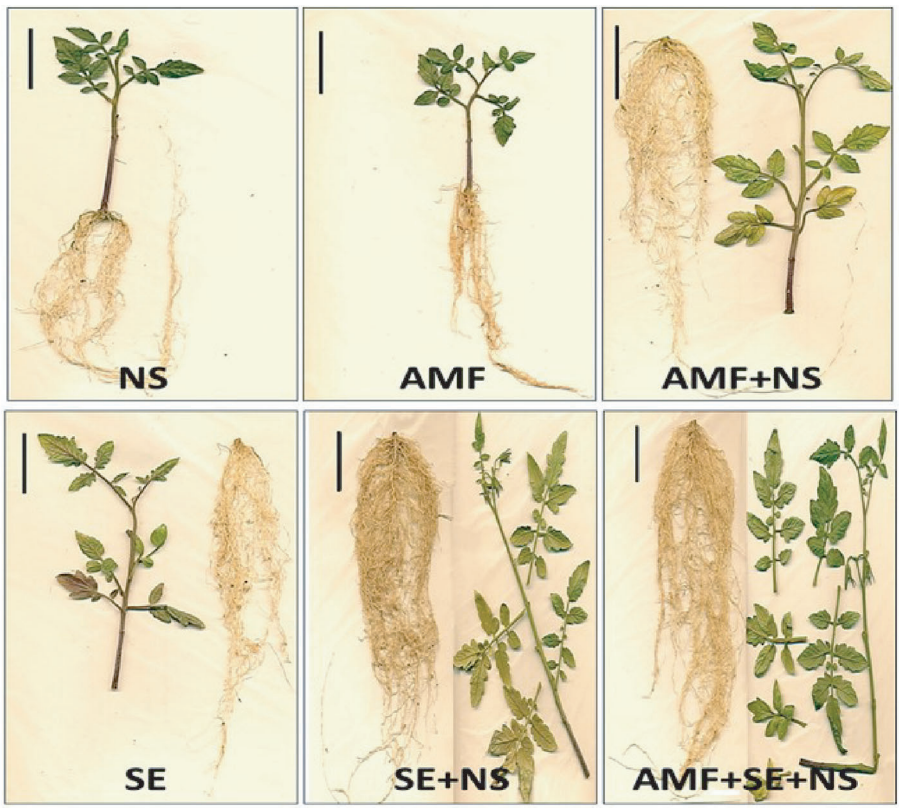


FIGURE 1 | Tomato plant growth after the 96-day experiment. Treatments included plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing *Rhizophagus intraradices* [arbuscular mycorrhizal fungi (AMF)], treated with an AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from *Padina gymnospora* extract [seaweed extract (SE)], treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants plus irrigation with nutritive solution (AMF + SE + NS). Bar, 5 cm.

Functional symbiosis was evaluated by analyzing *LePT4* expression. In arbuscule-containing cells in which a notable proportion of nutrient exchange occurs, the *LePT4* phosphate transporter is induced, and thus *LePT4* expression may be used to evaluate functional symbiosis. No difference was observed in AMF-treated or AMF + NS plants, and *LePT4* expression was similar among treatments. However, when plants were treated with a combination of AMF and SE and irrigated with NS (AMF + SE + NS), a strong induction of *LePT4* expression was detected (1.8-fold greater than that of AMF + NS plants), indicating that adding SE promoted AMF development, mycorrhizal establishment, and symbiosis (**Table 4**).

DISCUSSION

In recent years, a lot of agricultural research has focused on the discovery of environmentally friendly management strategies that ensure food safety. The application of PBs has been one of the best strategies to fulfill these terms, and consequently the use of biostimulants has evolved over time to produce increasingly better results with regard to plant performance. The first generation of biostimulants (1.0) was composed of products created from bioactive substances and/or microorganisms found in organic materials. These products improved nutrient uptake and use efficiency by stimulating physiological and molecular processes, which enhanced tolerance to abiotic stress and increased product yield and quality (Du Jardin, 2015). Then, efforts were directed to the discovery, development, characterization, and production of new PBs that were very different from those of generation 1.0. The new obtained PBs allowed researchers to identify the components of a successful PB formulation by elucidating complex biomolecular modes of action and identifying new opportunities for the creation of novel or improved PBs (Povero et al., 2016). With this approach, natural substance functions and the manner in which they modulate plant physiology may be predicted, resulting in the development of PBs that perform well under diverse conditions, including under stress (De Pascale et al., 2017; Parađiković et al., 2019). This approach relies on a powerful combination of chemistry, biology, and omics to identify patterns in complex data and elucidate the inherent activity and synergy of potential microbial and nonmicrobial PBs for commercial agricultural applications (Rouphael and Colla, 2018). The synergistic effects among microbial and nonmicrobial biostimulants, including those of different origins, have enabled researchers to design

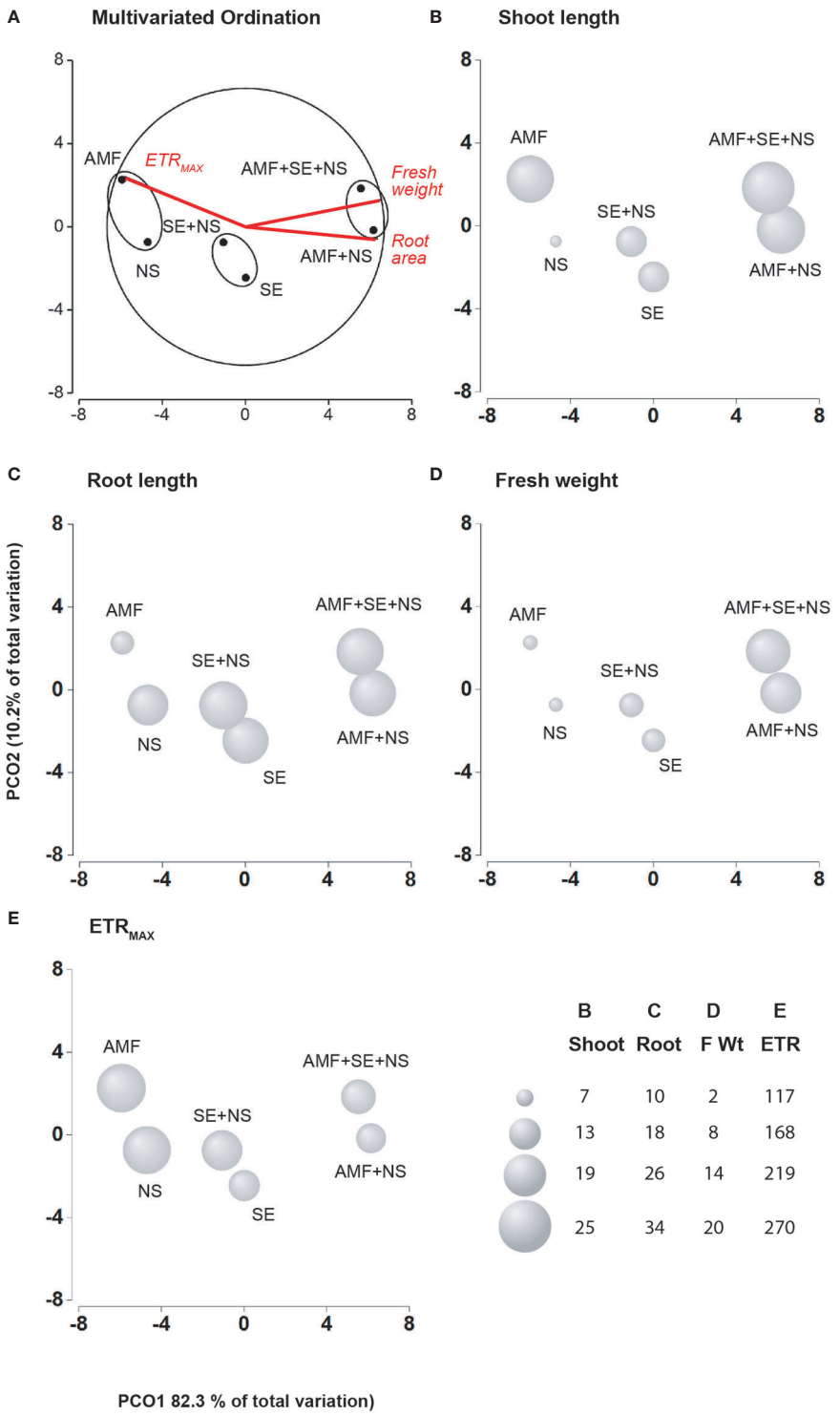


FIGURE 2 | Principal coordinates analysis (PCoA) of the physiological characteristics of the tomato plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing *Rhizophagus intraradices* [arbuscular mycorrhizal fungi (AMF)], treated with an AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from *Padina gymnospora* extract [seaweed extract (SE)], treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants and irrigated with nutritive solution (AMF + SE + NS). **(A)** Multivariate Ordination. **(B)** Shoot length. **(C)** Root length. **(D)** Fresh weight. **(E)** Maximum electron transport rate (ETR_{MAX}; n = 6 plants).

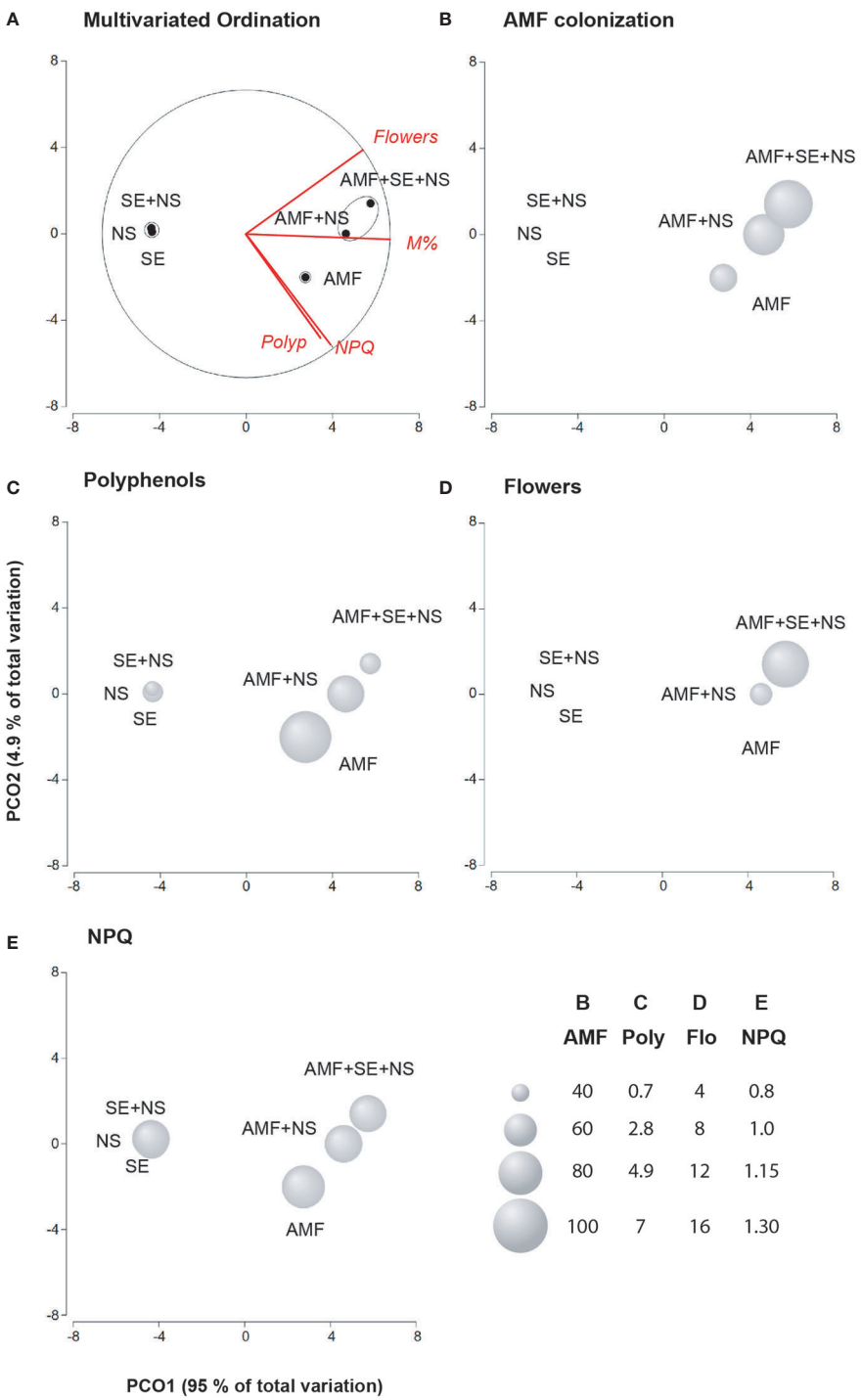


FIGURE 3 | Principal coordinates analysis (PCoA) of the ecological benefits of tomato plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing *Rhizophagus intraradices* [arbuscular mycorrhizal fungi (AMF)], treated with a AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from *Padina gymnospora* extract [seaweed extract (SE)], treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants and irrigated with nutritive solution (AMF + SE + NS). **(A)** Multivariate ordination; **(B)** AMF colonization of the root system; **(C)** Polyphenols; **(D)** Flowers, **(E)** Nonphotochemical quenching (NPQ; n = 6 plants).

TABLE 3 | Biochemical content of tomato plants treated with plan biostimulants.

Treatments	Protein (%)	Lipids (%)	Carbohydrates ($mg \cdot g^{-1}$)	Polyphenols ($mg \cdot g^{-1}$)	Nitrogen (%)	Phosphorous ($mg \cdot Kg^{-1}$ P)
NS	10.81 ± 0.41^{b}	4.51 ± 0.27^{c}	4.672 ± 0.35^{e}	0.050 ± 0.01^{a}	1.2 ± 0.01^{a}	0.119 ± 0.05^{b}
AMF	10.58 ± 0.19^{b}	2.44 ± 0.13^{b}	2.134 ± 0.39^{b}	0.687 ± 0.01^{d}	2.3 ± 0.01^{b}	0.014 ± 0.05^{a}
AMF + NS	9.29 ± 0.16^{a}	2.73 ± 0.23^{b}	3.750 ± 0.16^{d}	0.352 ± 0.01^{c}	2.1 ± 0.01^{b}	0.223 ± 0.05^{c}
SE	12.20 ± 0.21^{c}	2.46 ± 0.11^{b}	0.952 ± 0.01^{a}	0.108 ± 0.02^{b}	1.9 ± 0.01^{a}	0.080 ± 0.05^{b}
SE + NS	10.44 ± 0.24^{b}	5.49 ± 0.12^{d}	2.944 ± 0.14^{c}	0.057 ± 0.01^{a}	2.1 ± 0.01^{b}	0.100 ± 0.05^{b}
AMF + SE + NS	13.22 ± 0.19^{d}	1.42 ± 0.36^{a}	5.164 ± 0.22^{f}	0.114 ± 0.00^{b}	1.8 ± 0.01^{a}	0.201 ± 0.05^{c}

Tomato plants irrigated with nutritive solution (NS), treated with a microbial-based biostimulant containing Rhizophagus intraradices (AMF), treated with a AMF and irrigated with the nutritive solution (AMF + NS), treated with not microbial-based biostimulant from Padina gymnospora extract (SE), treated with SE and irrigated with nutritive solution (SE + NS), and a combination of both plant biostimulants and irrigated with nutritive solution (AMF + SE + NS). Values are mean ± standard deviation (n = 3, each replicate is a mix of four plants). Different letters (a–d) within the columns indicate significant differences according to the least significant difference (LSD) mean comparison test (p = 0.05).

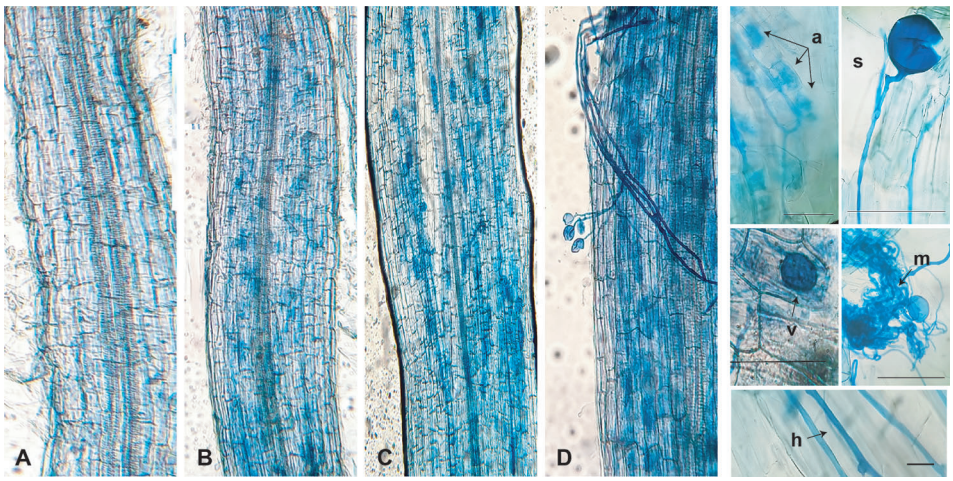


FIGURE 4 | Optical micrographs of the tomato roots and morphological structures of *Rizophagus intraradices* during mycorrhizal symbiosis **(A)** Non-arbuscular mycorrhizal fungi (AMF) in root, **(B)** AMF in root, **(C)** AMF in root and irrigated with nutritive solutions (AMF + SN), and **(D)** AMF in root with seaweed extract and irrigated with nutritive solution (AMF + SE + NS). Spore (s), hyphae (h), mycelium (m), arbuscule (a), vesicle (v). Scale bars represent 200 µm 40×. (n = 6 plants).

TABLE 4 | *Rizophagus intraradices* colonization measured on the tomato root system. AMF colonization of the root system (M%) and arbuscule abundance (A%) and relative expression values of symbiosis marker genes.

Treatment	Spores	Hyphae	Mycelium	Vesicles	M%	A%	*Ri EF-1α*	*LePT4*
AMF	2	128	27	70	52.2	44.6	1.09 ± 0.6	1.00 ± 0.0
AMF + NS	6	144	40	112	74.0	67.9	15.09 ± 2.8	0.99 ± 0.8
AMF + SE + NS	0	163	120	125	91.5	84.0	21.14 ± 2.5	1.85 ± 0.76

Plants treated with arbuscular mycorrhiza fungi (AMF), treated with AMF and irrigated with nutritive solution (NS) and combination of both microbial (AMF) and nonmicrobial seaweed extract (SE) and watered with nutritive solution (AMF + SE + NS). Data shown n = 6. Expression values of AMF symbiosis marker genes (mean ± standard deviation).

and formulate efficient PB products (2.0) with specific yield characteristics, particularly with regard to nutritional and functional qualities (Rouphael and Colla, 2018).

In this work, we found that each PB positively affected the physiological, ecological, and biochemical composition of the tomato plants in different but complementary ways (**Figure 5**). Initially, we analyzed the physiological responses in the plants in each PB treatment. For example, AMF conferred specific benefits related to plant physiology, including enhanced plant growth (preferential growth in the aerial portion instead of the plant root) and development (accelerated floration). Numerous studies have reported root system changes in response to AMF that have promoted root branching and increased the volume of the root system (reviewed in Gutjahr and Paszkowski, 2013; Pozo et al., 2015). Root-associated AMF trigger root biomass increases that have been attributed to increased auxin levels. A mechanism has been proposed to explain this in which mycorrhizal symbiosis modifies the development of the plant structure below the ground, including affecting the timing, extent (e.g., number and volume of lateral roots and root hairs), and degree (e.g.,

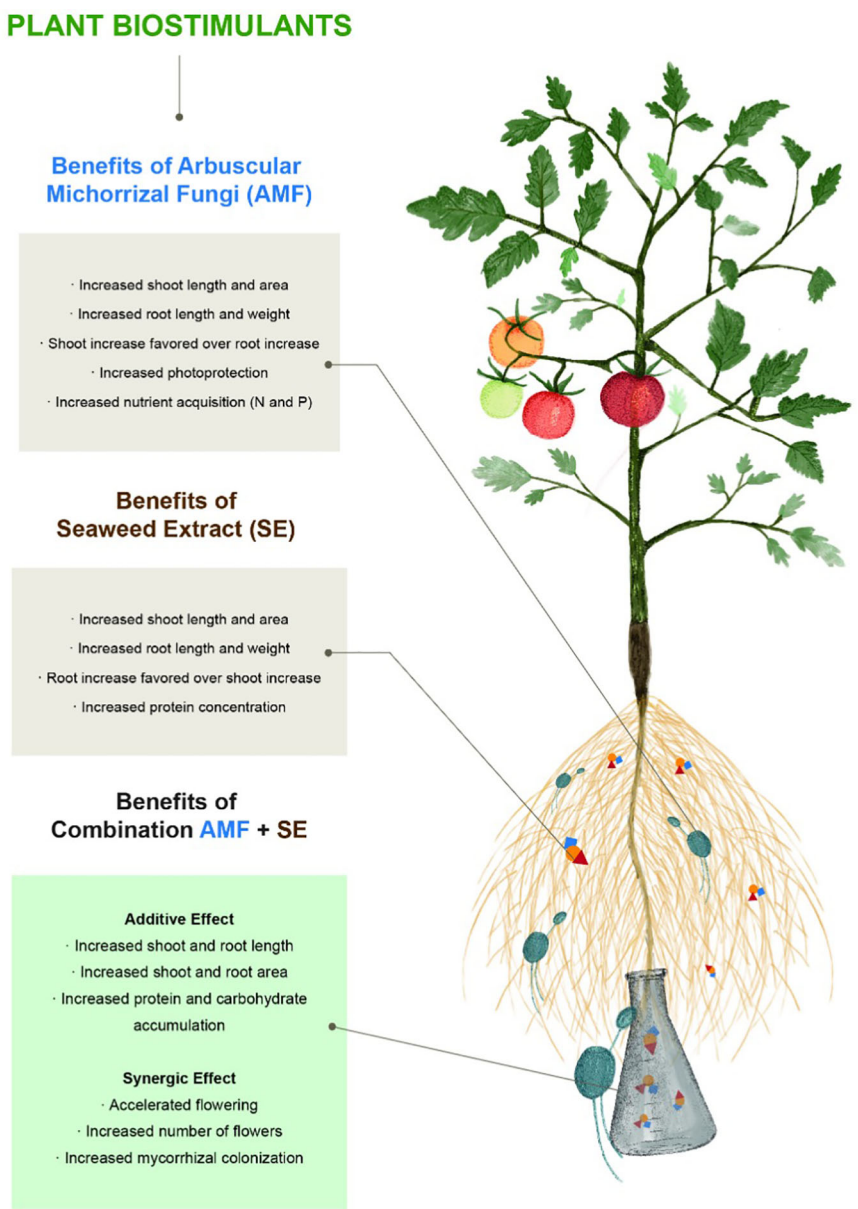


FIGURE 5 | Plant biostimulants contributions of arbuscular mycorrhizal fungi (AMF) and seaweed extract (SE), both alone and in combination on tomato plants (*Solanum lycopersicum* L. cv. "Rio Fuego").

secondary and tertiary roots) of root branching during primary root growth. This can be simply summarized by auxin inhibiting root elongation while lateral root development is strongly promoted (Sukumar et al., 2013). All of these effects are believed to change the hormonal state of the plant and consequently increase nutrient assimilation (Yakhin et al., 2017). Rapid plant growth depends on the ability of roots to provide sufficient water and nutrients to meet the requirements of acquisitive leaves with high photosynthetic rates and evaporative demands (Reich, 2014). However, our results show that the complete mechanisms are much more complex since root and foliar biomass varied according to nutrient conditions, in what seemed to be mycorrhiza-plant coordination.

Other important beneficial effects of microbial biostimulants have been frequently associated with improved photosynthetic apparatus functioning and pigment biosynthesis (Colla et al., 2015b and references cited therein; Rouphael et al., 2015b). However, as Demmig-Adams et al. (2017) recently discussed, photosynthesis and photosynthetic parameters change in many ways according to the response of the organism as a whole to environmental or even ecological conditions. Therefore, we consider that the photosynthetic responses of our treatments

should not only be taken as either high or low with regard to the PBs added but as the response of the whole organism and the PB to the environmental conditions of the experiment, such as the presence of NS. In our work, the plants grown under low-nutrient conditions and treated with AMF showed high ETR_{MAX} values that were not reflected in plant growth. This implies that arbuscular mycorrhization promoted PSII photochemistry in low nutrient soil. Nevertheless, the fixed carbon in the plant was mainly used to feed the AMF instead of for plant growth. It is also possible that part of the energy produced was directed to the plant-microorganism mutualistic induction of photoprotective and antioxidant mechanisms that preserved the PSII machinery under the detrimental conditions of nutrient limitation.

It is already known that plants share their photosynthetically fixed carbon with their associated microbial community, while microorganisms confer the ability to use previously nonavailable nutrients to their host plants, creating an important mechanism with which to resist abiotic stress (Smith et al., 2015; Bernardo et al., 2017; Lata et al., 2018). Therefore, our AMF-plant interactions under low nutrient conditions required energy to synthetize and share these biochemical compounds to resist abiotic stress and show a strong photosynthetic response (high ETR_{MAX}, high NPQ values, and high polyphenol content). In this sense, although our mutualistic interaction experiment with tomato plants and AMF in a low-nutrient medium was found to be energetically expensive, it conferred to the plants the capacity to survive under harsh conditions through the assimilation of normally nonavailable nutrients from the soil and increased plant resistance to stress. This is supported by what was observed in the control plants given that control plants without any PBs or NS died in the early stages. Clearly, AMF mutualistic interactions confer an important ecological advantage to tomato plants under low-nutrient conditions.

SEs are comprised of a multifaceted mixture of bioactive compounds, including polysaccharides, fatty acids, vitamins, phytohormones, and mineral nutrients and may induce various positive physiological responses that are reflected in improved root length, biomass production of the aerial portion of the plant, and plant weight (Craigie, 2011; Battacharyya et al., 2015). In this study, we also observed physiological and ecological benefits in plants treated with the algal extract. The addition of SE enabled the plants to survive in low nutrient soil (i.e. sand). The most characteristic benefit of SE in this work was related to the promotion of root length. These results agree with those of Hernandez-Herrera et al. (2014a), who attributed their similar findings to the minerals present in the SEs. Also, Hernández-Herrera et al. (2014a; 2014b; 2016) reported that SE and polysaccharide-enriched extracts from *P. gymnospora* conferred strong root growth-promoting activity in tomato plants and resulted in mung bean hypocotyl cuttings with longer roots when compared with those of the control plants and plants treated with Indole-3-butyric acid, a synthetic rooting hormone. These results suggest that oligosaccharides may behave as signaling molecules that trigger changes in the endogenous phytohormone metabolism of treated plants by a selective regulation of associated genes (De Pascale et al., 2017).

It has also been shown that different SE treatments promote biotic and abiotic responses in tomato plants, including the activation of defense-related enzymes, such as SOD, POD, PPO, and PAL (Kumar and Pandey, 2013; Hernandez-Herrera et al., 2014b; Drobek et al., 2019) and phenolic compounds (El Modafar et al., 2012) as well as the accumulation of proline and soluble sugars (Goñi et al., 2018). According to Mzibra et al. (2020), the tomato plant cell wall may recognize the hydrolysis of SE polysaccharides that can behave as signaling molecules and induce phytochemical biosynthesis, including that of polyphenols. In this study, surprisingly, polyphenol content was high in the SE-treated plants when no nutrients were added, but low when nutrients were supplied *via* irrigation, and no accelerated flowering was detected with regard to what was observed with AMF addition. Therefore, results showing a 2-fold increase in polyphenol content in the SE-treated plants vs NS-treated plants in this study could be attributed to tomato plant growth under stressful conditions. In addition, SE + NS-treated plants showed significantly higher NPQ than that of the untreated plants with NS. These results agree with those of Santaniello et al. (2017) who reported similar NPQ results in SE-treated plants under dehydration conditions, supporting the idea that SE confers photoprotection under stressful environmental conditions.

The information available with regard to the influence of SEs on fungal growth, root colonization, and infection rates in tomato plants indicates that the potential mechanisms for stimulating AMF hyphal growth acted *via* various low-weight isolated compounds, such as 5-deoxy-5-methylamino-adenosine as well as alginic acid, mannitol, and some polysaccharides from various seaweed species (Kuwada et al., 1999; Ishii et al., 2000; Kuwada et al., 2005; Kuwada et al., 2006a; Kuwada et al., 2006b; Khan et al., 2009; Paszt et al., 2015), which led to increased phosphorus acquisition. In this study, the application of *Padina gymnospora* SE enhanced the growth of the lateral roots of un-mycorrhized plants and improved root colonization by AMF; however, no improvement was observed in phosphorus acquisition or accumulation. Thus, the enhanced fungal growth and root colonization may have been due to the alginic acid, alginate oligosaccharides, or mannitol contained in the SE, which has been observed in the brown seaweed *P. gymnospora* (Chennubhotla et al., 1977). Another possible explanation for the improvement in lateral root growth by the SE may have been due to a change in the hormonal balance as a consequence of the auxins contained in the SEs, which are root hair growth regulators that promote elongation through the up-regulation of associated root epidermis genes, and thus mycorrhization was consequently improved as a result of greater root development (Zhang et al., 2019). In the same way, the polyphenol and carbohydrate content in the *P. gymnospora* SE at 0.8% (**Supplementary Table S1**) may have been used by the AMF, which may have been reflected in the observed improvements in fungal growth and root colonization.

In particular, the combined effect of both PBs resulted in high values of the physiological parameters evaluated, although PB efficacy was only equal to the sum of the independent effects. Therefore, a clear additive interaction on the physiological parameters was observed when AMF and SE were applied simultaneously to the tomato plants. The plants grown under optimal conditions (irrigated with the NS) that were treated with AMF and SE showed high growth, high fresh biomass, and low ETR_{MAX}. Clearly, the photoprotective and photorepair mechanisms as well as the costly alternative metabolic routes for nutrient assimilation were not necessary under less-stressful conditions when compared to those of the AMF-plants grown in sand and water. Therefore, under less-stressful environmental conditions, most of the fixed carbon could be redirected to maximize growth and development, which suggests an optimization of resources that is beneficial to both organisms of the mutual interaction (the plant and the mycorrhizal fungi). According to Rouphael et al. (2017), the application of combined PBs resulted in beneficial effects that could be associated with increased chlorophyll biosynthesis, a greater ability to maintain the photochemical activity of PSII, and a favorable nutritional status in leaf tissues compared with those of plants grown in the absence of combined PB addition.

In this experiment, the values of F_V/F_M found in all treatments can be considered slightly suboptimal. However, they may be associated with a natural response of self-reduction and dynamic photoinhibition due to the natural variation of environmental variables instead of to the chronic photoinhibition associated with PSII damage (Demmig-Adams et al., 2017). However, no detailed photoinhibition experiment was conducted. Further investigation is needed to assess the high photoprotection offered by AMF alone in low nutrient soils. The activation of the Xanthophyll cycle and acceleration of D1 PSII-core protein expression as well as the ability to detoxify ROS may constitute important mechanisms that help to improve physiological performance and ecological fitness, which require further study.

In this work, we analyzed some of the biochemical and ecological characteristics of the plants in each PB treatment. We identified that each PB (AMF or SE) affected the plants in a different manner. Based on both morphological and transcriptomic data, mycorrhization has been found to accelerate flowering and fruit development in tomato plants (Salvioli et al., 2012). Perhaps the most important benefit of the microbial AMF PB was related to early floriation, which is a promising finding that reflects the economic potential of the use of this PB to increase production. Also, AMF has been found to significantly increase tomato dry biomass and citric acid concentrations (Bona et al., 2017). In addition, AMF was found to increase the polyphenol concentration 6-fold. Both abiotic stress and fungal infections have been found to induce phenol and secondary metabolite accumulation in plant tissues (Zhi-Lin et al., 2007).

In contrast, SE increased protein accumulation, which agrees with what was found by Pise and Sabale (2010), who reported that the increases in protein content of SE-treated plants may have been the result of seedlings being able to absorb most required elements. Also, enhanced N uptake and translocation have been frequently associated with the highest protein content observed in PB-treated plants (Calvo et al., 2014). However, the total N content in the tomato plants in this study did not support this observation.

The combination of both PBs, enriched by the NS, resulted in a synergistic interaction of the PBs and was reflected in a favorable and significant enhancement of the biochemical (protein and carbohydrate levels) and ecological (AMF colonization and flowering) characteristics of the tomato plants. The elevated protein content may have been the result of amino acids that were directly used for protein biosynthesis after being incorporated by the plant. Nonetheless, increased protein content has been found to be associated with increased carbohydrate concentrations in plant leaves (Abbas, 2013). High sugar content in the leaves generally accelerates the nitrate assimilation pathway. Carbohydrates are required for the incorporation of ammonia in amino acids and accelerate the biosynthesis of proteins (Bulgari et al., 2015). Although chemical composition was measured in vegetative tissue in this experiment, it is highly probable that stable responses could be present in tomato fruits, in which PB application has been shown to shorten the ripening time (Yao et al., 2020). The results of this study are also consistent with those obtained by Faris (2013); Bettoni et al. (2014), and Rouphael and Colla (2018) who demonstrated that different PB combinations provided reproducible plant growth and production benefits, either in the form of additive or synergistic interactions.

When the ecological parameters were analyzed, such as mutual association success (AMF colonization) and flowering, a greater number of flowers were present on AMF-treated tomato plants compared to that of the SE-treated tomato plants. Flowering is driven by the requirements for carbon and nutrients and phytohormones, which may be affected by AMF (Torelli et al., 2000; Boldt et al., 2011). The AMF accelerated flowering and fruit production but also increased the amino acid concentration in the fruits, especially that of glutamine and asparagine (Salvioli et al., 2012). Poulton et al. (2002) demonstrated that AMF colonization can enhance host plant fitness by positively affecting its reproductive functions. In addition, SEs may improve flower yield and fruit production by behaving as chelators (Battacharyya et al., 2015; Yuvaraj and Gayathri, 2017; Pohl et al., 2019). Also, hormonal responses are mainly attributed to the cytokinins present in the SE or to their production, transport, and mobilization into developing tissues, either vegetative or floral (Ganesan et al., 2015). Similarly, Bona et al. (2017) reported that the farmed tomato plants treated with either AMF or PBs or their combination showed improved flower number and tomato fruit size and weight as well as enhanced industrial fruit (i.e., dry biomass, pH, and nitrate and citrate concentrations) and nutritional (i.e., sugar, ascorbate, and lycopene content) characteristics.

Additionally, applying PBs to plants has been shown to produce healthy plants with a significantly higher number of fruits and weights compared to those of plants grown in the absence of PBs (Hamed et al., 2018; Rouphael and Colla, 2018). In this study, we have shown that the addition of SE to AMF-treated plants accelerated flowering and resulted in a larger

flower number compared to that of plants only supplied with AMF. Interestingly, the combined effect of both PBs resulted in high values of the physiological parameters evaluated, although the efficacy of the PBs was only equal to the sum of their independent effects. Therefore, a clear additive interaction on physiological parameters was observed when AMF and SE were applied simultaneously to the tomato plants. However, superior crop performance was related to enhanced nutrient availability, which was driven by the combined application of SE and AMF that resulted in a synergistic interaction. Understanding the effects of the different PBs, either alone or in combination, on the tomato plants in our study implies that we could select the appropriate PB to obtain preferred product characteristics, which constitutes an important biotechnological application.

Currently, most biostimulants are complex chemical mixtures that are either derived from or extracted from biological processes and materials. In fact, AMF and SE mixture complexity has been thought to be a critical determinant of how well the biostimulant performs. Moreover, PB properties may be the result of the collective sum of all of their components and may not be fully understood by evaluating either component characteristics independently or particular component combinations. These properties are known as "emergent properties" and cannot be fully understood from a functional decomposition analysis (Yakhin et al., 2017). These novel emergent properties arise when an elevated degree of structural complexity is attained with lower-complexity components.

Based on an analysis of all data *via* the PCoA, we have shown how scale-specific observations may not be reflective of responses at the whole-plant level. We have highlighted the limitations of classical individual analysis based on the empirical evidence generated in this study. Nevertheless, modularity (AMF or SE) or approaches that consider the whole as the sum of its parts (AMF + SE) are unable to produce a complete understanding of physiological, biochemical, and ecological implications of SE and AMF PBs in tomato plants. Indeed, the integration of modules allows for the emergent properties of biological systems to become known resulting in the creation of unique individual entities. Therefore, we can adopt the term "emergent properties" in this work to describe the appearance of novel properties due to a particular degree of structural complexity forming from multiple components. The combined application of AMF and SE resulted in responses that could not be identified through functional decomposition. By identifying additive and synergistic results as well as additional effects from the combined use of both PBs, we suggest that beneficial, novel, and emergent properties arise from the constituent complex that forms following the application of both PBs and not as the result of the independent effects of known essential plant nutrients, growth regulators, or protective compounds.

CONCLUSION

When AMF and SE PBs were used individually, each was found to positively stimulate plant growth and yield in a different but complementary manner, since AMF promoted foliar growth while SE promoted root enhancement. However, the benefits of the combined application of both AMF and SE on crop growth and yield should be emphasized. SE was found to boost the AMF population; therefore, the benefits of the combined application of the PBs were additive with regard to the physiological variables but synergistic with regard to the ecological and productivity related parameters. The main advantages of the AMF and SE PBs were reflected in positive impacts on plant quality and performance. The findings of this study have identified patterns in complex data and elucidated the inherent activity and synergy of potential microbial and nonmicrobial PBs that may be used in commercial agriculture.

According to the data provided by FAOSTAT, 182,301,395 tons of tomatoes were produced globally in 2017, the cultivation of which is highly dependent on mineral nutrition (FAOSTAT, 2019). Mexico produces 4,243,058 tons of tomatoes annually, and utilizes 92,993 ha of land for tomato plantations. In fact, Mexico is the top tomato exporting country in the world, accounting for approximately 24.5% of the total tomato exports worldwide. Unfortunately, data of tomatoes produced with the use of biostimulants does not exist. In Mexico, the *Norma Oficial Mexicana* (NOM-077-FITO-2000) enables the Secretary of Agriculture and Rural Development to regulate phytosanitary and plant nutritional aspects of agricultural production, establishing the specifications, criteria, and procedures to regulate studies of the biological effectiveness of plant nutrition inputs. "The plant nutrition inputs provide essential elements to stimulate the growth and development of plants, correct or prevent any nutritional deficiency, or temporarily improve the properties of the soil, in order to increase the yield and quality of agricultural products" (NOM-077-FITO-2000, 2000). These inputs are highly diverse (e.g., organic and organic-mineral fertilizers, organic soil improvers, inoculants, soil humectants, and types 1, 2, and 3 growth regulators) and many have been developed with the intention to be registered and commercialized in Mexico, making it necessary to demonstrate their effectiveness in the field. Specifically, biological effectiveness is measured when applying a plant nutritional input, which may be said to be a biostimulant if it improves nutritional uptake efficiency, tolerance to abiotic stress, and/or crop quality. In Mexico, the production and use of biostimulants is still limited, and PBs have rarely been incorporated into the established cultivation practices, which is in part due to a lack of understanding on behalf of farmers regarding biostimulant functions and application. This gap in understanding has resulted in a hesitancy to use biostimulants based on a fear of additional cultivation costs and a reduction in the quality and quantity of plants and overall crop profitability. Therefore, it is important to introduce a truthful understanding of PBs to replace the use of organic fertilizers and agrochemicals. Biostimulants have negligible nutrient concentrations and act on the metabolism of a plant, unlike fertilizers. PBs may facilitate nutrient acquisition and translocation by enhancing metabolic processes that take place in the soil and in plants. For example, AMF development or the addition of highly diluted SE solutions induce the mineral exchange of P and N found in the soil,

making these nutrients available to plants (De Pascale et al., 2017; Lucini et al., 2019; Shukla et al., 2019).

An elucidation of the functions of AMF and SE as first-generation PBs, when they are applied either alone or in combination, would allow for the development of a second generation of biostimulants. Specifically, understanding improved nutrient use efficiency, plant quality, and tolerance to abiotic stress will allow for the development of second-generation PBs with specific synergistic and complementary biostimulant actions, the mechanisms of which could be functionally designed. In agriculture, the application of both microbial and nonmicrobial PBs could substantially promote sustainability efforts. Consequently, greater collaboration between farmers, industrial sectors, researchers, and governmental entities is required to improve production systems and PB quality to create and implement improved and environmentally friendly agricultural practices.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in the TRY Plant Trail Database: https://www.try-db.org/TryWeb/Data.php#56.

AUTHOR CONTRIBUTIONS

Conceptualization: RH-H. Data curation: MG-G and RH-H. Formal analysis: HO. Funding acquisition: RH-H. Investigation: MG-G. Methodology: HO-A, CS-H, and FS-R. Project administration: RH-H. Resources: FS-R, CS-H, and KC-C. Software: AB-E, HO-A, and JC-N. Supervision: RH-H and HO-A. Validation: FS-R. Visualization: RH-H and HO-A. Writing—original draft: RH-H and HO-A. Writing—review and editing: RH-H and HO-A.

FUNDING

This work was supported by the project number UDG-PTC-1329 of PRODEP to RH-H; UDG-PTC-1460 of PRODEP to HO-A; Universidad de Guadalajara by PROSNI-2017 and P3E to HO-A and PROSNI-2019 and P3E to RH-H.

ACKNOWLEDGMENTS

The authors thank the journal reviewers for their valuable comments, suggestions, and guidance. The authors thank Andrea Liévana MacTavish for her valuable suggestions and English language review and Carolina Mateos-Salmón for the artistic illustration in **Figure 5**. AB-E thanks Catedras-Conacyt.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00999/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 August 2020
doi: 10.3389/fpls.2020.01281

Biochar Administration to San Marzano Tomato Plants Cultivated Under Low-Input Farming Increases Growth, Fruit Yield, and Affects Gene Expression

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Biochar is a rich-carbon charcoal obtained by pyrolysis of biomasses, which was used since antiquity as soil amendant. Its storage in soils was demonstrated contributing to abate the effects of climate changes by sequestering carbon, also providing bioenergy, and improving soil characteristics and crop yields. Despite interest in this amendant, there is still poor information on its effects on soil fertility and plant growth. Considerable variation in the plant response has been reported, depending on biomass source, pyrolysis conditions, crop species, and cultivation practices. Due to these conflicting evidences, this work was aimed at studying the effects of biochar from pyrolyzed wood at 550°C, containing 81.1% carbon and 0.91% nitrogen, on growth and yield of tomato plants experiencing low-input farming conditions. San Marzano ecotype from Southern Italy was investigated, due to its renowned quality and adaptability to sustainable farming practices. Biochar administration improved vegetative growth and berry yield, while affecting gene expression and protein repertoire in berries. Different enzymes of carbon metabolism and photosynthesis were over-represented, whereas various stress-responsive and defense proteins were down-represented. Molecular results are here discussed in relation to estimated agronomic parameters to provide a rationale justifying the growth-promoting effect of this soil amendant.

Keywords: biochar, tomato, proteomics, climate change, low-input farming

OPEN ACCESS

Edited by:
Andrew Merchant,
The University of Sydney, Australia

Reviewed by:
Agnieszka Klimek-Kopyra,
University of Agriculture in Krakow, Poland
Giovanni Avola,
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *03 February 2020*
Accepted: *06 August 2020*
Published: *27 August 2020*

Citation:
Tartaglia M, Arena S, Scaloni A, Marra M and Rocco M (2020) Biochar Administration to San Marzano Tomato Plants Cultivated Under Low-Input Farming Increases Growth, Fruit Yield, and Affects Gene Expression. Front. Plant Sci. 11:1281. doi: 10.3389/fpls.2020.01281

INTRODUCTION

While World's food demand is raising as a result of the rapidly growing of population, increasing atmospheric CO_2 concentration and global climate changes, due to anthropogenic activities, greatly impact on agriculture, reducing crop yields, and decreasing the availability and the quality of soils in terms of water and nutrient content or heavy metal contamination (Lafferty, 2009; Auffhammer et al., 2012). In agricultural soils, the addition of amendants rich in organic carbon has been proposed as a sustainable remediation to improve soil fertility and increase crop productivity (Marris, 2006; Lehmann, 2007a). This practice also provides a mean to permanently sequester carbon, thereby reducing CO_2

release in the atmosphere and mitigating global climate changes. Biochar is a carbon-rich product produced by pyrolysis of biomasses of different origin, under high-temperature and low-oxygen conditions (Lehmann, 2007b; Laird et al., 2009). Due to its peculiar structural features, like porosity, high surface area of particles and affinity for charged compounds (Keech et al., 2005), biochar has been proposed for different purposes, such as soil management, feedstuff for livestock, and water purification. Recently, the renewed attention to sustainable practices in agriculture prompted extensive use of biochar to increase the fertility of soils and to improve the productivity of crop plants (Biederman and Stanley Harpole, 2013; Laghari et al., 2016). In fact, the addition of biochar to the soil improves its water and nutrient retention capacity (Laird et al., 2010), increases bioavailability of phosphate and potassium (Laghari et al., 2016), and stimulates soil microbial activity (Steinbeiss et al., 2009); conversely, it decreases that of heavy metals (Park et al., 2011) and N_2O emission. Although negligible to adverse effects have also been reported (Spokas and Reicosky, 2009; Jeffery et al., 2011; Spokas et al., 2011), recently, comprehensive meta-analyses of published studies demonstrated that biochar, on the overall, has a positive effect on ecosystems and cultures in terms of plant productivity, nutrient uptake, and soil properties (Jeffery et al., 2011; Biederman and Stanley Harpole, 2013). Contradictory results have also been obtained in studies aimed at testing the effectiveness of biochar as a primer of plant defense responses to pathogens. In fact, a reduction of the severity of infection has been reported for some fungine foliar diseases, such as powdery mildew, anthracnose, or gray mold (Elad et al., 2011; Harel et al., 2012; Mehari et al., 2015), and for nematode root infections (Huang et al., 2015), whereas inconsistent or negative effects have been observed in other root-pathogen interactions (Elmer and Pignatello, 2011; Akhter et al., 2015; Shoaf et al., 2016) or foliar diseases (Copley et al., 2017).

The effects of biochar seem greatly influenced by the feedstock used to produce it and by conditions of pyrolysis (Méndez et al., 2013), which can impact on structure, nutrient and phenolic content, and pH value of the final product (Novak et al., 2009). Moreover, the effects of biochar on plant cultivations also vary in dependence of the nature (type, mineral, and nutrient content) and conditions (fertilization and humidity) of the soil to which it is added (Van Zwieten et al., 2010a; Van Zwieten et al., 2010b; Biederman and Stanley Harpole, 2013). Tomato (*Lycopersicon esculentum*, Mill.) is one of the most economically important vegetable crop, especially in the Mediterranean area, and has been used as a model crop species for genomics and proteomics studies (Rocco et al., 2006; Sant'Ana and Lefsrud, 2018). Nowadays, information on the effect of biochar administration to tomato cultivation in terms of growth, yield and quality is very poor. In a field experiment, Vaccari and coworkers reported that biochar addition to a fertile soil improved tomato (Pietrarossa cultivar) growth but not yield (Vaccari et al., 2015). Polzella et al. reported that biochar administration to tomato plants (San Marzano ecotype) in a neutral and low in nutrients soil did not significantly improved growth and yield performances; results by proteomic and qRT-PCR analysis pointed to a limited impact of biochar on photosynthesis and defense genes (Polzella et al., 2019).

Considering the above-reported variability in plant productivity and the scarce information concerning tomato growth and yield performances as a result of biochar administration, this work was aimed at investigating the impact of biochar produced from wood under controlled conditions (550°C pyrolysis temperature, 81.1% carbon, 0.91% nitrogen) on the aboveground growth and productivity of tomato plants of the S. Marzano ecotype cultivated in an acidic soil, under low-input conditions. The San Marzano ecotype was chosen for this study because it is a traditional cultivar from Campania, South Italy, which has become a top quality variety owing to its peculiar organoleptic traits (Ercolano et al., 2008; Ercolano et al., 2014); furthermore, as local accession, it is also well suited to low-input cultivation or organic farming (Negri, 2003). Agronomic parameters such as height and number of flower buds, and number and weight of berries were evaluated and related to molecular data obtained from qRT-PCR and proteomic analyses.

MATERIALS AND METHODS

Plant Material

Verfofood biochar was purchased from Green Biochar, Torino, Italy. This fine grain coal is produced from wood at a maximum pyrolysis temperature of 550°C. It contains 81.1% of carbon and 0.91% nitrogen; it also has a pH value of 8.21 and an ash content of 7.74%. The soil matrix used for the growth of tomato plants was a typical Mediterranean agriculturally managed soil, classified as Eutric Cambisol collected in Campania, Italy. After sampling, the soil was dried at 40°C for 24 h, and sieved (<2 mm). The soil pH in water was 5.6 and its Water Holding Capacity (WHC) and ash content was 35.85% and 88%, respectively. Seeds of the 204-San Marzano 2 accession (Consorzio Agrario, Parma, Italy) were sowed in plastic pots (10 l) containing soil or soil with 5% of biochar. Ten seeds each pot were sowed and five pots containing soil plus five pots containing soil and 5% biochar were prepared; all pots were irrigated with the same volume of water two times a week. Fifteen days after sowing, a single plant for each pot was selected to continue the experiment, and pots were placed in greenhouse, under controlled conditions, with 14 h of light/day. After 50 days of growth, agricultural parameters, like height and number of flowers buds, were estimated every 15 days. At the end of the experiment (177 days after sowing), tomato fruits from each pot were collected and their number and weight registered. Immediately after collection, fruits were cut longitudinally into four parts and frozen in liquid N_2 following seeds removal. Fruits were stored at −80°C until their use.

Protein Extraction and 2-D Electrophoresis

Fruit samples (2.5 g of frozen tomato fruits) were powdered in a mortar using liquid N_2, and suspended in 7.5 ml of extraction buffer (700 mM sucrose, 500 mM Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol, and 1 mM PMSF) for 15 min, on ice. After the addition of an equal volume of Tris-saturated phenol (500 mM Tris-HCl, pH 7.5), the mixture

was vortexed for 10 min and then centrifuged at 10,000 × *g*, for 15 min, at 4°C. The upper phenol phase was removed and extracted twice with the extraction buffer. Proteins were precipitated from the phenol phase by the addition of five volumes of saturated ammonium acetate in methanol, overnight at −20°C. Precipitated proteins were centrifuged at 10,000 × *g*, for 30 min (Rocco et al., 2006).

Protein pellets were washed once with ice-cold methanol and three times with ice-cold acetone, dried and solved in IEF buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT, and 1% w/v carrier ampholytes pH 3–10) (BioRad, Hercules, CA, USA). Protein concentration was quantified using the BioRad protein assay, using BSA as a standard. IPG strips (17 cm, pH 4–7, BioRad ReadyStrip, BioRad) were rehydrated with 300 µl of IEF buffer containing 400 µg of total proteins, overnight. Proteins were focused using a Protean IEF Cell (BioRad, Segrate MI, Italy) at 12°C, applying 250 V (90 min), 500 V (90 min), 1,000 V (180 min), and 8,000 V, for a total of 53 KVh. After focusing, proteins were reduced by incubating the IPG strips with 1% w/v DTT in 10 ml of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS, and a dash of bromophenol blue, for 15 min, and alkylated with 2.5% w/v iodoacetamide in 10 ml of the same buffer, for 15 min. Electrophoresis in the second dimension was carried out using a Protean apparatus (BioRad, Segrate MI, Italy) and 12% polyacrylamide gels in 25 mM Tris (pH 8.3), 1.92 M glycine and 1% w/v SDS, with 120 V applied for 12 h. Gels were stained with colloidal Coomassie G-250. Analyzes were done on two technical replicates for three biological samples.

Image Acquisition and Analysis

2-DE gel images were acquired using a GS-800 calibrated densitometer (BioRad, Segrate MI, Italy). Image analysis was performed using the PD Quest software (BioRad, Segrate MI, Italy). Spot detection and matching between gels were performed automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole gel densities, the percentage volume of each spot was averaged for six different (two replicates of three samples) gels; Student's t-test analysis ($p < 0.05$) was performed to find out statistically significant spot volume fold changes (>1.5 or <0.66) associated with biochar presence in soil.

Spot Digestion, Mass Spectrometric Analysis, and Protein Identification

Spots from 2-DE were excised from the gel and shattered. Proteins were *in-gel* reduced with dithiothreitol, S-alkylated with iodoacetamide, and then digested with trypsin. Resulting peptide mixtures were subjected to a desalting/concentration step on µZip-TipC_{18} devices (Millipore, Bedford, MA, USA) before MS analysis. Recovered peptides were then analyzed for protein identification by nanoLC-ESI-LIT-MS/MS, using an LTQ XL mass spectrometer (Thermo Fisher Scientific, USA) equipped with a Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Denmark). Peptides were resolved on an Easy C_{18} column (100 mm × 0.075 mm, 3 µm) (Proxeon) (Paiardini et al., 2014). Mobile phases were 0.1% v/v formic acid (FA) (solvent A) and 0.1% v/v FA in ACN (solvent B), running at a total flow rate of 300 nl/min. Linear gradient was initiated 20 min after sample loading; solvent B ramped from 5 to 35% over 45 min, from 35% to 60% over 10 min, and from 60% to 95% over 20 min. Spectra were acquired in the range m/z 400–2000. Each peptide mixture was analyzed under CID-MS/MS data-dependent product ion scanning procedure, enabling dynamic exclusion (repeat count 1 and exclusion duration 60 s), over the three most abundant ions. Mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

Raw nanoLC-ESI-LIT-MS/MS data were searched with v.2.2.06 MASCOT software (Matrix Science, UK) against an updated (07/2017), non-redundant UniProtKB database (taxonomy *Viridiplantae*) to identify protein(s) present within each gel spot. Database searching was performed by using Cys carbamidomethylation and Met oxidation as fixed and variable protein modifications, respectively, a mass tolerance value of 1.8 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, and a missed cleavage maximum value of 2. Other MASCOT parameters were kept as default. Protein candidates assigned on the basis of at least two sequenced peptides with an individual peptide expectation value <0.05 (corresponding to a confidence level for peptide identification >95%) were considered confidently identified. Definitive peptide assignment was always associated with manual spectra visualization and verification.

RNA Extraction and cDNAs Synthesis

The MirPremier microRNA isolation kit (Sigma-Aldrich, Milan, Italy) was used to extract RNA from tomato berry samples. The samples (0.1-g fresh weight) were homogenized in liquid N_2, with mortar and pestle, and aliquoted (0.07 g) into a pre-cooled tube containing 750 µl of kit lysis solution adding 1% of v/v β-mercaptoethanol. After 5-min incubation at 55°C, samples were centrifuged at 14,000 × *g*, and the supernatant loaded onto a filtration column included in the kit. The filtrate was recovered by centrifugation at 14,000 × *g*, for 1 min, which was added with 1.5 vol of binding solution from the same kit. The mixture was loaded onto the binding column, and the corresponding filtrate removed by centrifugation at 14,000 × *g*, for 1 min. To remove any traces of phospholipids, proteins, and carbohydrates, the column was washed with 700 µl of pure ethanol, 500 µl of binding solution and 500 µl of pure ethanol. In order to elute nucleic acids, 30 µl of nuclease-free H_2O were added to the column and the eluate was recovered by centrifugation at 14,000 × *g*, for 1 min. To degrade genomic DNA and obtain pure RNA, we used the RNeasy/QIamp columns and RNase-free DNase set (Quiagen, Milan, Italy), following the manufacturer's instructions. Extracted RNA was retrotranscribed to cDNA by using the ImProm-II Reverse Transcription System kit (Promega, Milan, Italy) and the mini thermal cycler BioRad (Segrate MI, Italy). To 10 µl of purified RNA, 1 µl of Primer Oligo (dt) s and 1 µl of dNTP were added; the mixture was incubated in the thermal cycler at 70°C, for 5 min. After the addition of 8 µl of master mix (20 µl Improm-II 5X reaction buffer, 10 µl $MgCl_2$, 5 µl Recombination RNasin ribonuclease inhibitor and 5 µl Improm-II Reverse Trascriptase), the mixture was incubated again in the thermal

cycler at 25°C, for 5 min, at 50°C for 60 min, and at 70°C, for 15 min. After retrotranscription, the samples were stored at −20°C.

RT-qPCR

Gene primers were selected according to (Viger et al., 2015) and designed using the NCBI Primer Blast tool; their nature is reported in **Table 1**. For RT-qPCR, the EvaGreen 2X qPCR MasterMix-R kit (Applied Biological Materials Vancouver, Canada) was used. A 7300 Real-time PCR system (Applied Biosystems) was set to perform an initial denaturation at 95°C for 1 min, an annealing phase at 95°C, for 5 min, and 40 subsequent cycles of denaturation at 95°C, for 30 s, annealing at 60°C, for 30 s, and extension at 72°C, for 30 s. Relative gene expression quantification was carried out using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Experiments were carried out in triplicate for each biological sample.

Data Analysis

Agronomic parameter data are reported as mean ± SD of three independent experiments. Statistical analysis was performed by ANOVA followed by the Student-Newman-Keulus test, with the minimum level of significance being $p < 0.05$. Statistical significance of differences in protein spot densities from densitometric analysis of 2D-electrophoretic gels was assessed automatically by PD Quest software (Bio-Rad), using the Student's t test ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Biochar Addition on Soil Characteristics

Analysis of soil with and without the addition of biochar for corresponding pH, and carbon (C), phosphorus (P), potassium (K), magnesium (Mg), and total nitrogen (N) content values is reported in **Table 2**. These results demonstrated that biochar addition highly modified the pH value as well as C and N content of soil, while it poorly affected the other constitutive soil parameters.

TABLE 1 | Forward and reverse primer sequences designed and used for RT-qPCR experiments.

Primer	Sequence
Nb BETA ACTIN F	TGGACTCTGGTGATGGTGTC
Nb BETA ACTIN R	CCTCCAATCCAAACACTGTA
PDF1.2a F	GCTGCTTTCGGTGAGTAATAATG
PDF1.2a R	CCATGTCCCACTTGGCTTCT
PDF1.2b F	GCAGCTTTTGGTTAGTAATGCTCT
PDF1.2b R	AGTACCACTTGGCTTCTCGC
JAZ F	AGCCAACAAACAGAACCCCA
JAZ R	AATTCCGTCTCGCGATTGGT
LOX F	GCCTCAATTGTCGATGGTGC
LOX R	TCGTTGCGATCCCAGTCAAA

Effect of Biochar on Growth and Productivity of Tomato Plants

In order to evaluate the effect of a 5% biochar administration on the aboveground growth and productivity of San Marzano tomato plants, the corresponding height and the number of flower buds were measured every 14 days, starting from the 50th day after sowing (**Figure 1**). Similarly, the average number and weight of ripe berries was estimated at the end of the growth period (177 days after sowing) (**Figure 2**). Results demonstrated that biochar administration accelerated the growth of treated plants, particularly in the early phases of growth, whereas the effect was reduced at the end of the growing period (**Figure 1**). On the other hand, treated plants showed a marked increase in the number of floral buds at every stage of the cultivation, a parameter that is strictly related to their productivity (**Figure 2**). In fact, the average number (**Figure 3A**) and weight (**Figure 3B**) of ripe berries at the end of cultivation period resulted markedly increased in San Marzano plants treated with 5% biochar, when compared to untreated plants. These results demonstrated that biochar has a marked effect on tomato plant growth and productivity, when this specific biochar type and plants experiencing low-input farming conditions are considered.

Proteomic Analysis of Tomato Berries From Plants Grown in the Presence of Biochar

Differential proteomic characterization of tomato fruits at final maturation stage from San Marzano plants grown in soil amended with 5% biochar and control soil was achieved by 2-DE analysis of corresponding proteins resolved in the pI range 4–7 (first dimension) and the mass range 10–150 kDa (second dimension), followed by gel staining with colloidal Coomassie blue (see experimental section for details). This analysis allowed a comparison of the protein repertoire of biochar-amended and control samples to determine statistically significant quantitative variations due to biochar addition to soil. In particular, average proteomic maps for berries from biochar-treated and control plants showed 875 and 805 reproducible spots, respectively, with a similarity degree of 68%. The master gel of this proteomic analysis is shown in **Figure 4**. To ascertain quantitative changes in corresponding proteomic maps, relative spot densities were evaluated by software-assisted analysis. Statistical analysis ($p > 0.05$) revealed 56 protein spots as differentially represented between biochar-amended and control samples. Differentially

TABLE 2 | Chemical parameters of soil samples with and without biochar addition.

Sample	pH	C (%)	N (%)	P (%)	K (%)	Mg (%)
Soil	5.62 ± 0.02	3.20 ± 0.01	0.16 ± 0.01	0.26 ± 0.01	1.62 ± 0.01	0.75 ± 0.01
Biochar	8.21 ± 0.03	81.10 ± 0.02	0.91 ± 0.02	0.08 ± 0.01	0.88 ± 0.01	0.41 ± 0.01
Soil + biochar	7.10 ± 0.02	17.81 ± 0.02	1.21 ± 0.02	0.27 ± 0.01	1.74 ± 0.01	0.89 ± 0.01

Data on corresponding pH value, and carbon (C), phosphorus (P), potassium (K), magnesium (Mg), and total nitrogen (N) percentage content are shown for comparison.

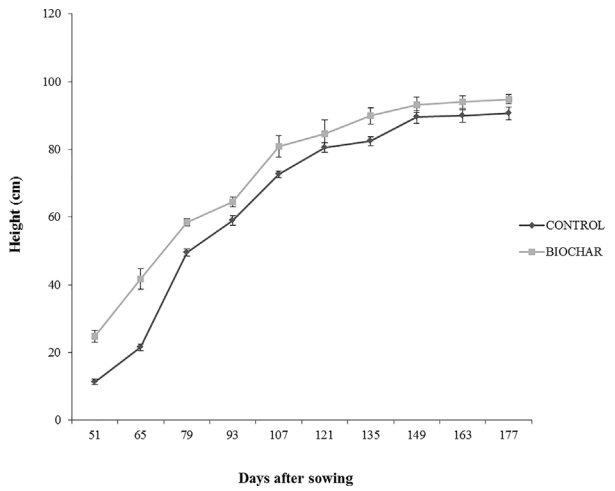


FIGURE 1 | Average height (in cm) of tomato plants experiencing low-input farming conditions in soils amended with or not with 5% Verfofood biochar. Measurements were performed the 50th day after sowing using one sample per five pots.

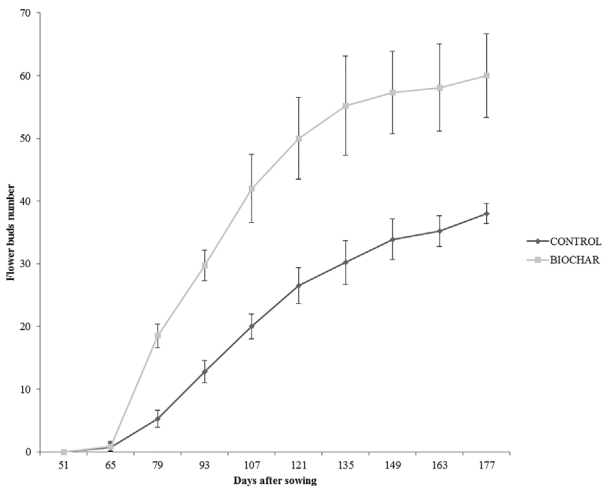


FIGURE 2 | Average number of flower buds in tomato plants experiencing low-input farming conditions in soils amended with or not with 5% Verfofood biochar. Measurements were performed the 50th day after sowing using one sample per five pots.

represented spots were excised from gels, trypsinolyzed, and subjected to nanoLC-ESI-LIT-MS/MS analysis for protein identification. The list of the identified proteins, together with their quantitative variations between Biochar amended and control plants, is reported in **Table 3**. Functional categorization according to Gene Ontology annotation and literature data allowed grouping identified proteins into two main functional categories, namely, energy/carbon metabolism and stress/defense. Different proteins with uncharacterized function or not grouping in the above categories were also identified. Above-mentioned groups are discussed in the dedicated sections reported below.

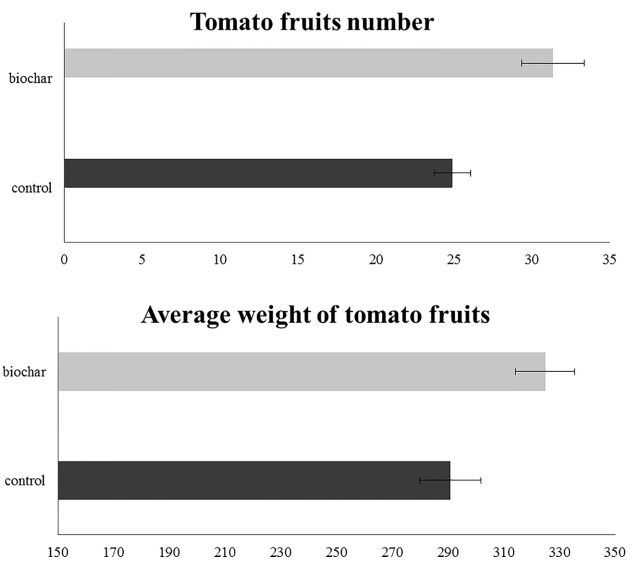


FIGURE 3 | Fruit characteristics in tomato plants experiencing low-input farming conditions in soils amended with or not with 5% Verfofood biochar. **(A)** Average number of fruits per plant at the end of the experiment. **(B)** Average weight of the fully-ripen fruits. Measurements were performed at the end of plant treatment using one sample per five pots.

Carbon and Energy Metabolism

Most of the differentially represented proteins in the fruits of from tomato plants grown in soil amended with 5% biochar belong to the carbon/energy metabolism functional group, which included 17

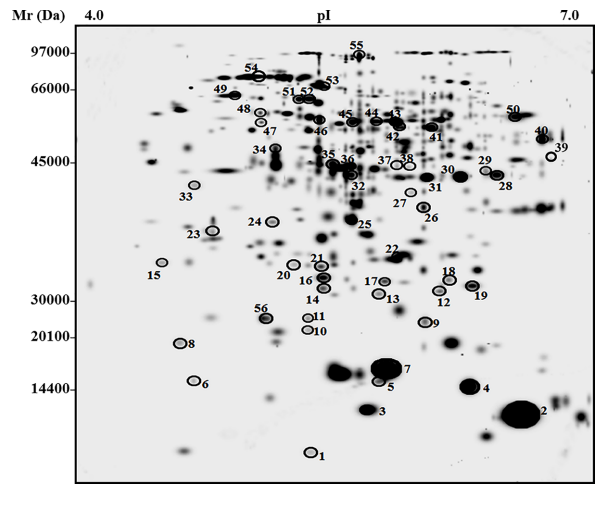


FIGURE 4 | Two-dimensional electrophoretic reference map of fruits from tomato plants experiencing low-input farming conditions in soils amended with or not with 5% Verfofood biochar. Proteins were resolved on IPGs (first dimension) and 12% SDS-PAGE (second dimension) and were visualized by colloidal Coomassie blue staining. Spot numbering coincides with that reported in **Table 3**; experiments were carried out in triplicate for each biological sample.

TABLE 3 | Differentially represented proteins in tomato fruits from plants treated with 5% Verfofood biochar as identified by combined 2-DE and nanoLC-ESI-LIT-MS/MS of corresponding spot digests.

Spot	Protein description	UniProtKB accession	Gene name	Organism	Unique peptides	Sequence coverage (%)	pI_{th}/Mw_{th}	Fold change (biochar vs. control)
Energy and carbon metabolism								
1	Ribulose bisphosphate carboxylase small chain 2A	P07179	RBCS-2A	SL	7	34	6.58/20278	4.6
8	Chloroplast inorganic pyrophosphatase	K4B2L1	PPA6	SL	3	11	6.01/32383	1.9
12	Probable adenylate kinase 2	Q9FIJ7	KAD2	AT	5	19	7.11/31452	3.5
16	Triose phosphate isomerase	K4B3X5	TIM	SL	31	48	6.45/34665	2.2
18	Triose phosphate isomerase cytosolic isoform	Q6T379	TPIP1	SC	12	60	5.73/27040	2.2
20	Triose phosphate isomerase	K4B3X5	TIM	SL	5	21	6.45/34665	2.3
21	Soluble inorganic pyrophosphatase	K4B2L1	PPA6	SL	3	11	6.01/32383	1.6
24	Oxygen-evolving enhancer protein 1-2	K4BCF4	PSBO2	SL	3	17	5.84/35271	2.3
28	Malate dehydrogenase cytosolic	K4CW40	MDH1	SL	6	15	5.91/35384	3.3
29	Glyceraldehyde 3-phosphate dehydrogenase	K4BYG6	GAPC2	SL	7	14	6.34/36651	7.8
30	Mitochondrial NAD-dependent malate dehydrogenase	Q8L5A6	mMDH	ST	15	33	8.87/35661	3.5
31	Malate dehydrogenase cytosolic	K4CW40	MDH1	SL	19	23	5.91/35384	3.9
32	Ribulose bisphosphate carboxylase/ oxygenase activase	O49074	RCA	SPE	5	12	8.61/50701	2.4
35	Ribulose bisphosphate carboxylase/ oxygenase activase	O49074	RCA	SPE	7	18	8.61/50701	5.1
36	Ribulose biphosphate carboxylase/ oxygenase activase	O49074	RCA	SPE	8	16	8.61/50701	5.4
37	Enolase	P26300	PGH1	SL	10	25	5.68/47798	2.3
38	Phosphoglycerate kinase	K4CHY3	T8K14.3	SL	13	40	5.78/42489	3.1
39	Fructose-bisphospate aldolase	Q9FUG7	SCA1	FA	2	23	6.93/38515	2.0
40	Ribulose bisphosphate carboxylase large chain	P48698	rbcL	DS	18	36	6.54/51948	3.0
41	UTP-glucose-1-phosphate uridylyltransferase	K4D601	UGP2	SL	41	48	5.84/51819	2.2
42	Enolase	P26300	PGH1	SL	53	59	5.68/47798	2.2
43	Enolase	P26300	PGH1	SL	46	49	5.68/47798	2.0
44	ATP synthase β-subunit	K4BX20	ATPB	SL	38	21	5.74/59607	2.5
45	ATP synthase β-subunit	K4BX20	ATPB	SL	64	19	5.74/59607	2.3
46	ATP synthase CF1 beta subunit	A0A0C5CEC7	atpB	SL	21	44	5.28/53467	5.8
47	ATP synthase CF1 beta subunit	A0A0C5CEC7	atpB	SL	17	39	5.28/53467	7.5
Oxidative stress								
3	Superoxide dismutase [Cu-Zn]	K4AX22	SODC	SL	21	54	5.47/15285	0.6
10	Thioredoxin peroxidase 1	Q7Y240	TPx1	SL	11	64	5.18/17437	0.5
15	Glutathione S-transferase L3	K4D3M6	GSTL3	SL	7	26	5.06/27183	0.4
17	L-ascorbate peroxidase 2	K4CQB7	APX2	SL	16	46	5.63/27635	0.4
19	Glutathione-S-transferase φ class	K4C3T2	GSTF8	SL	29	61	5.98/23723	0.4
22	Ascorbate peroxidase	Q52QQ4	APX	SL	17	60	5.86/27322	0.6
23	Ascorbate peroxidase	Q52QQ4	APX	SL	4	14	5.86/27322	0.6
27	Lactoylglutathione lyase	K4B9T4	GLX1	SL	31	39	6.62/38542	0.6
Stress and defense								
2	Fruit-ripening protein	O82575	Asr1	SL	6	58	6.48/12555	0.4
4	17.6 KDa Class I sHSP	Q96489	HSP17.6	SP	32	44	6.32/17613	0.3
5	TSI-1 protein	O49881	TSI-1	SL	13	74	5.61/20221	0.5
6	Glycine-rich RNA-binding protein	K4B3H9	GRP1	SL	6	43	5.59/17343	0.3
7	17.8 KDa Class I sHSP	G5DGD2	HSP17.8	SL	40	56	5.82/17639	0.3

(Continued)

TABLE 3 | Continued

Spot	Protein description	UniProtKB accession	Gene name	Organism	Unique peptides	Sequence coverage (%)	pI_{th}/Mw_{th}	Fold change (biochar vs. control)
9	Chloroplast sHSP	Q95661	HSP21	SL	9	42	7.84/26227	0.5
11	Metacaspase 1	Q8H272	MCA1	SL	2	11	4.78/44864	0.4
13	Chloroplast sHsp	Q95661	HSP21	SL	7	29	7.84/26227	0.6
14	Chaperonin 21	K4DC13	CPN21	SL	23	53	5.32/22416	0.6
25	Embryo-abundant EMB	Q8S271	P0415C01.10	OS	4	31	6.60/33104	0.4
26	Protein phosphatase 2C	Q6QLU0	PP2C	SL	7	52	5.67/30944	7.2
33	Dehydrin ERD10	K4BVU7	ERD10	SL	6	37	5.12/23111	0.2
34	Heat shock protein 70 (fragment)	Q40151	MED37C	SL	7	13	5.17/71515	0.4
48	HSP70-interacting protein 1	K4CNT4	HIP1	SL	6	18	4.92/45598	0.2
49	Chaperonin-60 α-sub	K4DAD5	CPN60A1	SL	21	25	5.21/61955	0.2
50	Stress-induced protein STI-1-like protein	A0A1V1H194	OB_Ba0011H08.28	OB	32	61	6.49/57217	0.1
51	Heat shock protein 60	K4CWE4	CPN60	SL	30	45	5.52/61560	0.5
52	Chaperonin 60 beta subunit	K4AV63	CPN60B2	SL	9	18	5.72/62992	0.2
53	Heat shock protein 70	K4D9L9	MED37C	SL	23	30	5.10/71414	0.4
54	Heat shock protein 70	K4D9L9	MED37C	SL	33	40	5.10/71474	0.6
55	Chaperone protein ClpB3	K4BC16	CLPB3	SL	13	14	6.17/110377	0.2
56	Mitochondrial HSO70 2	K4B2I9	HSP70	SL	26	35	5.75/72970	0.1

Spot number, protein name, UniProtKB accession, gene name, organism, unique detected peptides, sequence coverage (%), theoretical pI and molecular mass values, and fold change (biochar vs. control) are shown. Proteins are reported according to their belonging to functional classes (stress and defense, oxidative stress, and energy/carbon metabolism). SL, Solanum lycopersicum; SP, Solanum peruvianum; OS, Oryza sativa; OB, Oryza brachyantha; AT, Arabidopsis thaliana; SC, Solanum chacoense; ST, Solanum tuberosum; SPE, Solanum pennellii; FA, Fragaria ananassa; DS, Datura stramonium.

unique protein entries (present in 26 spots). All resulted over-represented in the berries of biochar-treated plants. In tomato berries, glycolysis and respiration represent the main carbon and energetic fluxes that fuel substrates to sustain the respiratory burst as well as biosynthesis of cofactors, pigments, and metabolites during the maturation process (Livak and Schmittgen, 2001; Sarry et al., 2004). Interestingly, seven proteins involved in the oxidative phase of glycolysis were over-represented in the berries of biochar-treated plants, namely, cytosolic fructose-bisphosphate aldolase 6 (spot 39), triose phosphate isomerase cytosolic and chloroplastic isoforms (spots 16, 18 and 20), glyceraldehyde 3-phosphate dehydrogenase (spot 29), phosphoglycerate kinase (spot 38) and enolase (spots 37, 42, and 43). These findings suggest that biochar addition brought a stimulation of carbon catabolism during ripening, which resulted in improved growth and yield of mature berries. In agreement with above-mentioned observation, levels of cytosolic (cMDH) (spot 28 and 31) and NAD-dependent mitochondrial (mMDH) (spot 30) malate dehydrogenase were also increased in biochar-treated berries. MDH is a pivotal enzyme for regulation of malate concentration; its over-representation in biochar-challenged plants fairly correlates with their increased sugar metabolism during ripening as well as the stimulatory effect of this amendant on tomato growth and ripening. In fact, massive malate oxidation takes place during the last phase of maturation, in order to sustain the respiratory burst and to provide through the Krebs cycle carbon intermediates for secondary metabolites and volatiles biosynthesis, which accumulates in the mature berries (Carrari and Fernie, 2006). In line with augmented carbon metabolism in biochar-challenged plants were also the observed increased levels of UTP-glucose-1-phosphate uridylyltransferase (spot 41), and cytoplasmic (spot 21) and plastidial (spot 8) inorganic pyrophosphatase, which are involved in sucrose synthesis and thus promoted sucrose or hexose accumulation in treated plants (Carrari and Fernie, 2006).

In biochar-challenged plants, above-mentioned proteomic changes corresponded to a parallel over-representation of enzymes involved in photosynthetic carbon assimilation, namely, ribulose bisphosphate carboxylase large (spot 40) and small (spot 1) chain, and ribulose biphosphate carboxylase/oxygenase activase (spots 32, 35, and 36), and in (photosynthetic) energy production, e.g., ATP synthase β-subunit (spots 44 and 45), ATP synthase CF1 beta subunit (spots 46 and 47), and oxygen-evolving enhancer protein 1-2 (spot 24), which directly contributed in stabilizing/synthesizing molecules essential to provide the fruit energetic supply and to maintain the berry endogenous O_2 balance. These data suggest that different energetic processes are activated in San Marzano as result of biochar addition to the soil, ultimately determining improved growth and yield of mature berries.

Stress and Defense

Additional differentially represented proteins categorized in the broad functional group of stress- and defense-related proteins, for a whole of 27 unique protein entries (present in 30 spots). In particular, seven components of the cellular antioxidant machinery showed down-represented levels in biochar-treated plants, namely, (Cu-Zn) superoxide dismutase (spot 3), two isoforms of ascorbate peroxidase (spot 17, 22, and 23), glutathione-S-transferase L3 and φ class (spot 15 and 19), thioredoxin peroxidase 1 (spot 10), and lactoylglutathione lyase (spot 27). In mature berries, antioxidant enzymes are abundant proteins since fruit ripening is generally paralleled by an increase of oxidative metabolism, with

accumulation of H_2O_2 and reactive oxigen species (ROS) as well as of membrane lipid peroxidation (Carrari and Fernie, 2006). This ROS accumulation is often balanced by the activity of different cellular antioxidant enzymes and scavenging systems, such as catalases, superoxide dismutases, and the ascorbate-gluthathione cycle. In this context, superoxide dismutases and ascorbate peroxidases are the major enzymes deputed to H_2O_2 removal from the plant cell (Jimenez et al., 2002). Besides detoxification of xenobiotics, plant glutathione-S-transferases also function as GSH-dependent peroxidases, which reduce organic hydroproxides to monohydroxyalcohols, thus limiting oxidative injury (Rocco et al., 2006). On the other hand, thioredoxin peroxidase is a key component for the control of mitochondrial H_2O_2 metabolism (Huang et al., 2007), while lactoylglutathione lyase participates into the glutathione-based detoxification of methylglyoxal that is produced by carbohydrate and lipid metabolism (Jimenez et al., 2002). On the overall, our result demonstrate that biochar treatment of San Marzano plants experiencing low-input farming conditions determined a significant reduction of antioxidant enzymes, which may suggest the occurrence of other compensative mechanisms to face the augmented levels of ROS generally observed during fruit ripening.

A number of other components involved in the plant response to stress, which belong to the heat shock protein (HSP) or chaperonin protein families, were also detected as down-represented in tomato plants challenged with biochar. They were 17.6 (spot 4) and 17.8 (spot 7) kDa class I sHSPs, chloroplast sHSP (spot 9 and 13), two HSP70 isoforms (spot 34, 53, and 54), mitochondrial HSO70 2 (spot 56), HSP-interacting protein 1 (spot 48), HSP 60 (spot 51), chaperonin 21 (spot 14), chaperone protein ClpB3 (spot 55), and chaperonin 60 (spot 49) and β (spot 52) subunits. While functional characterization of chaperonins in plants is quite limited, more information is available on HSPs. The latter are a family of ubiquitous molecular chaperones whose main function is to prevent protein aggregation during stress, which group into five classes according to their molecular mass (Jacob et al., 2017). sHSPs are the most prevalent in plants, where they are expressed in distinct cellular compartments not only in response to a wide range of environmental stresses, including the oxidative one, but also during fruit development and maturation (Paull and Jung Chen, 2000). Biochar treatment of tomato plants brought about a consistent reduction in the abundance of HPSs and chaperonins in the corresponding berries, ranging from about 50% to 80%, as compared to untreated counterparts.

Finally, other proteins involved in the plant response to different stress conditions were also ascertained with decreased concentration levels in tomato plants treated with biochar, namely, fruit-ripening protein (spot 2), TSI-1 protein (spot 5), embrio-abundant EMB (spot 25), dehydrin ERD10 (spot 33), stress-induced protein sti1-like protein (spot 50), glycine-rich RNA-binding protein (spot 6), and metacaspase 1 (spot 11). Fruit-ripening protein, embrio-abundant EMB, dehydrin ERD10, and stress-induced protein STI-1-like protein are molecules generally related to plant response to various environmental stresses. In particular, fruit-ripening protein shares 90% sequence identity with abscisic acid stress ripening protein 1, a nuclear protein that interacts with chromatin and accumulates in leaves in response to water stress and in fruits during maturation (Rocco et al., 2006). Analogously, glycine-rich RNA-binding protein, TSI-1 protein and metacaspase 1 are generally involved in plant response to biotic stresses. In particular, metacaspase 1 belongs to the type I class of putative cysteine proteases, which are distantly related to caspases (Rocco et al., 2006). In plants, metacaspases have been shown to act as cell death regulators in the hypersensitive response (HR) to pathogen attack (Lema Asqui et al., 2018).

In the whole, above-reported data demonstrate that the addition of 5% biochar to San Marzano low-input cultivation brought a general and consistent down-representation of proteins involved in the response of plants to abiotic and (to a reduced extent) biotic stresses. Even though conflicting data exist as far as the biochar-mediated modulation of stress and defense response in plants, our proteomic results are in line with gene expression studies on *Arabidopsis* and lettuce treated with biochar (Viger et al., 2015), where a general up-regulation of growth-promoting genes and a down-regulation of stress and defense ones occurred following administration of this soil amendant.

RT-qPCR Analysis of Jasmonic Acid-Related Genes

Recently, microarray investigations have suggested that genes related to the signaling pathway of the defense response hormone jasmonic acid (JA) are down-regulated in biochar-treated plants (Viger et al., 2015). Since we did not recorded here by proteomics any evidence regarding the modulation of proteins related to above-mentioned genes, we extended our analysis to verify the accordance of the model of San Marzano plants experiencing low-input farming conditions together with administration of 5% biochar with that of the growth-defense trade-off model mentioned above (Viger et al., 2015). To this purpose, a RT-qPCR analysis of genes related to JA-modulated pathways was carried out. Results shown in **Figure 5** demonstrate that biochar treatment in our case determined a down-regulation of plant defensin PDF1.2A, defensin PDF1.2B, and lipoxygenase (LOX) genes in tomato berries, whereas a repressor gene of the JA response pathway, namely, iasmonate-zim domain protein (JAZ), was up-regulated. These results are in agreement with that already reported by Viger and coworkers for *Arabidopsis* and lettuce plants.

CONCLUSION

Nowadays, agronomic data on the effects of biochar administration on tomato growth, yield, and fruit quality are scarce and contrasting (Vaccari et al., 2015; Polzella et al., 2019), whereas those on related molecular information are practically absent. The effectiveness of biochar treatments very likely depend on the characteristics of the soil to which it is added, as well as on the farming conditions (use or not of fertilizers) generally adopted. Best effects are usually obtained with acid and low nutrients soils; in fact, biochar administration generally increases soil pH value and modifies soil characteristics to improve water retention and nutrient availability (N, P, and K). In

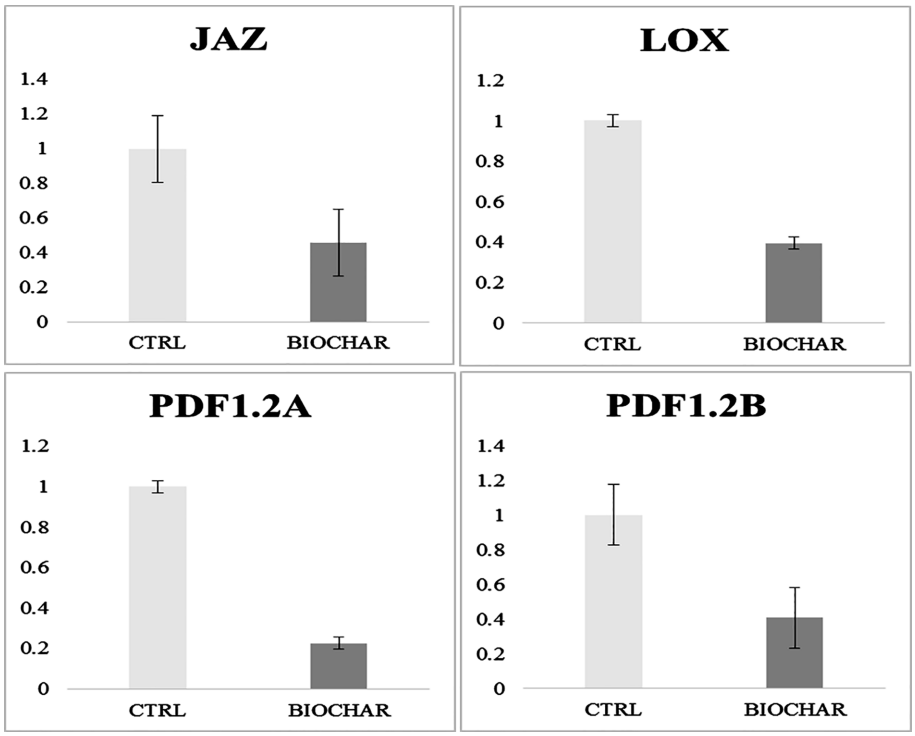


FIGURE 5 | Gene expression analysis in fruits from tomato plants experiencing low-input farming conditions in soils amended with or not with 5% Verfofood biochar. Reported are data on genes involved in JA pathway, namely, plant defensin factor 1.2A and 1.2B (PDF1.2A and PDF1.2B, respectively), lipoxygenase (LOX) and jasmonate-zim domain (JAZ). Data were analyzed with the 2-ΔΔCt method.

field agronomic studies on tomato cultivars, Vaccari and coworkers reported that biochar addition to a fertile soil improves tomato growth but not yield (Vaccari et al., 2015), whereas Polzella and colleagues showed that biochar administration to plants growing in a neutral and low-input (for nutrients) soil did not significantly ameliorate growth and yield performances (Polzella et al., 2019). Under our farming conditions (slightly acidic pH value of the soil and no use of fertilizers), addition of 5% biochar to San Marzano tomato plants modified soil characteristics, at the same time significantly affecting vegetative growth and fruit yield. This fact confirms that the outcome of the treatment with this amendant is more dependent on agronomic site features than on crop species and/or biochar type (Jay et al., 2015). At the molecular level, unique reference studies are the above-mentioned ones on *Arabidopsis* and lettuce, which were performed by a gene expression platform on plants grown in a soil treated or not with biochar (Viger et al., 2015). These investigations identified auxin and brassinosteroid signaling pathways (Camoni et al., 2018) as key determinants for the growth-promoting effect of biochar. They also proposed a growth-defense trade-off model, where up-regulation of growth-promoting genes is accompanied by down-regulation of a large set of plant defense genes. The proteomic and gene expression results presented in this study are in line with this model, since plant treatment with biochar determined an opposite quantitative trend of genes related to the last phase of reproductive growth (ripening) and of genes related to stress and defense responses. In conclusion, our data suggest that biochar administration under controlled farming conditions can be a useful sustainable practice to improve tomato growth and yield. At molecular level, besides amelioration of water and nutrient availability, this study suggests that the biochar growth-promoting effect is underwent by the activation of specific signaling pathways and molecular mechanisms that deserve a dedicated deeper analysis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MT and MR were responsible for design of and planning the experiments, analyzed data, and drafted manuscript. MT and SA performed experiments. MM, AS, and MR interpreted results of experiments, edited, and revised manuscript. MR approved final version of manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 February 2021
doi: 10.3389/fpls.2020.550169

Plant-Dependent Soil Bacterial Responses Following Amendment With a Multispecies Microbial Biostimulant Compared to Rock Mineral and Chemical Fertilizers

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 08 April 2020
Accepted: 17 December 2020
Published: 04 February 2021

Citation:
Mickan BS, Alsharmani AR, Solaiman ZM, Leopold M and Abbott LK (2021) Plant-Dependent Soil Bacterial Responses Following Amendment With a Multispecies Microbial Biostimulant Compared to Rock Mineral and Chemical Fertilizers. Front. Plant Sci. 11:550169. doi: 10.3389/fpls.2020.550169

Biostimulants are gaining momentum as potential soil amendments to increase plant health and productivity. Plant growth responses to some biostimulants and poorly soluble fertilizers could increase soil microbial diversity and provide greater plant access to less soluble nutrients. We assessed an agricultural soil amended with a multispecies microbial biostimulant in comparison with two fertilizers that differed in elemental solubilities to identify effects on soil bacterial communities associated with two annual pasture species (subterranean clover and Wimmera ryegrass). The treatments applied were: a multispecies microbial biostimulant, a poorly soluble rock mineral fertilizer at a rate of 5.6 kg P ha^{-1}, a chemical fertilizer at a rate of 5.6 kg P ha^{-1}, and a negative control with no fertilizer or microbial biostimulant. The two annual pasture species were grown separately for 10 weeks in a glasshouse with soil maintained at 70% of field capacity. Soil bacteria were studied using 16S rRNA with 27F and 519R bacterial primers on the Mi-seq platform. The microbial biostimulant had no effect on growth of either of the pasture species. However, it did influence soil biodiversity in a way that was dependent on the plant species. While application of the fertilizers increased plant growth, they were both associated with the lowest diversity of the soil bacterial community based on Fisher and Inverse Simpson indices. Additionally, these responses were plant-dependent; soil bacterial richness was highly correlated with soil pH for subterranean clover but not for Wimmera ryegrass. Soil bacterial richness was lowest following application of each fertilizer when subterranean clover was grown. In contrast, for Wimmera ryegrass, soil bacterial richness was lowest for the control and rock mineral fertilizer. Beta diversity at the bacterial OTU level of resolution by permanova demonstrated a significant impact of soil amendments, plant species and an interaction between plant type and soil amendments. This experiment highlights the complexity

of how soil amendments, including microbial biostimulants, may influence soil bacterial communities associated with different plant species, and shows that caution is required when linking soil biodiversity to plant growth. In this case, the microbial biostimulant influenced soil biodiversity without influencing plant growth.

Keywords: biostimulants, soil bacteria, microbial inoculant, pasture, soil biology

INTRODUCTION

In grassland systems where the aim is to promote plant biomass, application of fertilizers is the most common and important management practice (Ikoyi et al., 2018; Carswell et al., 2019). However, the use of biostimulants to compliment fertilizers is gaining interest (Caradonia et al., 2019). Plant growth responses to some biostimulants could influence the soil microbial community and provide greater plant access to less soluble nutrients (Calvo et al., 2014). Biostimulants include multispecies microbial inoculants and may be used alone (e.g., Assainar et al., 2018) or in combination with fertilizers (e.g., Assainar et al., 2020).

The success of microbial inoculants as biostimulants is varied and may not be predictable (Qiu et al., 2019). For example, in previous studies of the use of a multispecies microbial inoculant applied to wheat, there was a positive response in grain yield (Assainar et al., 2018). The microbial inoculant influenced the relative abundance of rhizosphere bacteria, especially Actinobacteria. However, in another study using the same multispecies microbial inoculant, there was no benefit from the introduction of the multispecies microbial inoculant in terms of fertilizer use efficiency for wheat (Assainar et al., 2020). Despite the rapid expansion of interest in commercial use of microbial inoculants (Qiu et al., 2019; Sammauria et al., 2020), further investigation is required to assist farmers discriminate among management practices that involve microbial products as biostimulants in terms of their efficacy (Abbott et al., 2018).

Conventional P fertilizers promote pasture growth but they can be expensive, especially in developing regions (Sanchez, 2002) and may have adverse influences on the environment and on soil microbial communities (Pan et al., 2014; Luo et al., 2015; Ikoyi et al., 2018). Thus, alternative fertilizers such as controlled-release fertilizers or slow-released fertilizers have been used to minimize the negative consequences of supplying unnecessarily high levels of soluble P (Hagin and Harrison, 1993; Shaviv, 2001; Van Geel et al., 2016). Slow-release fertilizers have been defined as classes of fertilizers that contain moderately soluble components regardless of the properties of the reaction products in the soil (Hagin and Harrison, 1993). The release pattern of P is important to control the concentration of phosphate ions in the soil solution and to minimize environmental loss (von Sperber et al., 2017; Fischer et al., 2018).

A range of fertilizers, including organic P-based fertilizers, can regulate the P status of agricultural soils (McLaughhlin et al., 2011). Phosphate rocks are often a major component of slow- or controlled-release fertilizers (Bolan et al., 1990; Goh et al., 1990; Reijnders, 2014). Unlike annual crops which need high levels of P over a short period of rapid growth, pastures (especially legumes) require sustained sparingly soluble P sources and hence, phosphate rocks have been used extensively in grass-clover fertilization in temperate regions (van Diest, 1981; Sinclair et al., 1993). The use of rock phosphates in combination with biostimulants such as phosphate solubilizing bacteria and arbuscular mycorrhizal (AM) fungi has been introduced and marketed as a low-cost and low-energy mechanism to promote the agronomic effectiveness of rock phosphate fertilizers (Richardson, 2001; Gyaneshwar et al., 2002).

Pasture plant species differ in their capacity to exploit limiting nutrients (Hill et al., 2006) and this can influence their relative abundance within a sward (Driscoll and Strong, 2017). Long-term application of fertilizers, including slow-release fertilizers, can influence soil microbial communities (Zhao et al., 2014; Pan et al., 2016; Xun et al., 2016), and this in turn may influence the capacity of plants to access nutrients. With increasing interest in and availablility of commercial microbial inoculants marketed as biostimulants there is a need to understand potential modes of action and predictability of their efficacy (Abbott et al., 2018). Therefore, a glasshouse experiment was conducted to assess the impact of a multispecies microbial biostimulant on soil microbial communities in rhizospheres of two annual pasture species in comparison with application of fertilizers of different elemental solubilities, especially P.

While there is potential to improve nutrient use efficiency in pastures with application of slow-release fertilizers (Smith et al., 2018), benefits of inclusion of microbial biostimulants within management systems is less predictable because of inconsistency in responses (Qiu et al., 2019). Management of mixed annual pasture swards in south-western Australia has potential to benefit from incorporation of biostimulants due to relatively low fertilizer input (Bolland et al., 2011; Weaver and Wong, 2011; Gourley et al., 2017) and dependence on microbial processes associated with decomposition of organic matter, symbiotic nitrogen fixation and activities of soil microbial communities including phosphate solubilizing bacteria (Hinsinger et al., 2015). Therefore, we chose two common pasture species (subterranean clover and annual ryegrass) to investigate the efficacy of a multispecies microbial biostimulant and P fertilizers that differed in their solubilities in a glasshouse experiment based on previous studies of the same multispecies microbial inoculant with wheat (Assainar et al., 2018, 2020). The hypotheses were: (i) a multispecies microbial biostimulant will alter the rhizosphere bacterial community associated with two pasture species with different rooting structures (an annual legume and an annual grass) and (ii) a fast-release soluble P fertilizer will decrease rhizosphere soil bacterial diversity to a greater extent than a slow-release P fertilizer. The specific aim of this experiment was to identify soil bacterial community responses to microbial

inoculants that may have potential as biostimulants in order to contribute to the longer-term aim of understanding and predicting modes of action of microbial biostimulants.

MATERIALS AND METHODS

A glasshouse experiment was conducted to evaluate the effects of a multispecies microbial biostimulant, in comparison to a rock mineral and a chemical fertilizer which differed in P solubility, on soil bacterial communities in the rhizospheres of two annual pasture plants that are commonly grown in south-western Australia.

Soil Sampling

The soil was collected at a depth of 0–10 cm from an annual pasture at The University of Western Australia's farm Ridgefield, Pingelly, WA (S 32°30′23″, E 116°59′31″, 116°59′48.50″E). A grid 25 × 25 m was used to select the area where the soil was collected using a zig-zag pattern. The soil was air dried and stored in a cool area and additional soil was collected for assessing bulk density. For 10 replicate soil samples, soil was passed through a 2 mm sieve to remove larger rock and plant residue particles. The initial characteristics of the soil were: 13.6% clay, 12% silt, 76% sand, pH 5.45 in H_2O, pH 4.93 in $CaCl_2$, and electrical conductivity (EC) 163 μS/cm. Available nutrients were assessed to as: Colwell P 65.8 mg kg^{-1} soil, NO_3^- 1.8 mg kg^{-1} soil and NH_4^+ 19.52 mg kg^{-1} soil with a bulk density of 1.24 g/cm^3 using methods described by Rayment and Higginson (1992).

Experimental Design

The soil was potted into non-draining plastic pots (2 kg soil per pot). Four treatments were applied: (i) no soil amendment (control), (ii) a multispecies microbial biostimulant (Mic), (iii) a rock mineral fertilizer (MnF) at a rate of 75 kg ha^{-1} (∼5.6 kg P ha^{-1}), and (iv) a chemical fertilizer (CF) at a rate of 43 kg ha^{-1} (∼5.6 kg P ha^{-1}). There were two pasture species, subterranean clover (*Trifolium subterraneum* L. cv. "Dalkeith") and Wimmera ryegrass (*Lolium rigidum* Gaudin), with four replicate pots of each treatment. Plants were harvested after 10 weeks.

The microbial inoculant used as a biostimulant consisted of a proprietary combination of various bacteria and fungi applied at a rate of 1 g per pot mixed in the top 30 mm of soil. Its trade name is Ag Blend Plus (produced by Australian Mineral Fertilizers Pty Ltd.). According to the distributor, it was a talc-based formulation containing (per g) isolates of *Agrobacterium rhizogenes* (1×10^9), *Azotobacter* spp. (1.2×10^9), *Azospirillum brasilense* (1.1×10^9), *Bacillus subtilis* (112×10^9), *Pseudomonas fluorescens* (2.3×10^9), *Streptomyces* spp. (1×10^9), *Trichoderma harzianum* (8×10^9), and *Rhizophagus irregularis* (75 spores) (see also Assainar et al., 2018, 2020).

The composition of the fertilizers is listed in **Table 1**. The water solubility of P (assessed according to Chien, 1993) was 821 mg kg^{-1} in the chemical fertilizer and 657 mg kg^{-1} in the rock mineral fertilizer. The rock mineral fertilizer (from Australian Mineral Fertilizers Pty Ltd. called NPK Crop Plus) was a poorly soluble fertilizer consisting of a proprietary combination of various fine mineral ores. The ores include micas, alkali feldspars, soft rock phosphate, iron man gypsum (a byproduct from mineral sand processing, containing gypsum, iron and manganese), dolomite, basalt, granite and crystalline silica, and are blended with sulfate of ammonia and sulfate of potash, manganese sulfate, copper sulfate, and zinc sulfate (Storer and Devlin, 2012). The chemical fertilizer was a relatively soluble fertilizer from Summit Fertilizers Australia called Gusto Gold. The fertilizer treatments were added to the soil surface 1 day prior sowing and mixed with the soil. The microbial biostimulant was added as a powder to the seeds before sowing.

TABLE 1 | Characterization of the rock mineral fertilizer (Ag Blend Plus from Western Mineral Fertilizers Pty Ltd.) and chemical fertilizer (Gusto Gold from Summit Fertilizer Australia).

Characteristics	Rock mineral fertilizer (Ag Blend Plus)	Chemical fertilizer (Gusto Gold)
N%	7.5	10.2
P%	7.5	13.1
K%	4.5	12
Ca%	5	0
S%	8	7.2
Mg%	0.9	0
Fe%	2.6	0
Si%	6.7	0
Mn, mg/kg	4,000	0
Zn, mg/kg	430	1,300
Cu, mg/kg	430	900
B, mg/kg	17	0
Ni, mg/kg	30	0
Mo, mg/kg	1.5	0
Bulk density, g/cm^3	1.1	1.1

Data supplied by the manufacturers.

Subterranean clover and Wimmera ryegrass (5 plants per pot) were grown in separate pots for 10 weeks. Water content for all pots was maintained at 70% of field capacity by regular monitoring and addition of water to weight. Plants were grown in a glasshouse at The University of Western Australia under ambient light with a temperature range of 18/10°C (day/night). At harvest, the plants were lifted from the pots and shaken gently to remove soil. Soil subsamples were taken from the rhizosphere for DNA analysis (Mickan et al., 2017). Shoots and roots were dried at 60°C for 72 h then weighed to assess dry weight (DW). Total N and P from plant tissue were determined using a Kjeldahl digest (Bremner and Mulvaney, 1982). Total N was determined using an ammonium N in green method Na-Nitroprusside, and total P was calculated using molybdenum blue colorimetry (Blakemore et al., 1972).

Available N, Extracted P and Soil Acidity

Soil mineral N (NO_3^-) and exchangeable N (NH_4^+) were measured following extraction of 20 g soil with 80 mL 0.5 M K_2SO_4 and analysis of the extracts colorimetrically for exchangeable NH_4^+ using the salicylate–nitroprusside method (Searle, 1984) and NO_3^- concentration using the hydrazine

TABLE 2 | Two-way ANOVA results showing *P*-values for dry shoot and root biomass per pot.

	Treatment	Degrees of freedom	Sum of squares	Mean squares	F. model	*P*
Shoot mass	Soil amendment	3	61.5	20.5	90.9	**<0.001**
	Pasture	1	6.15	6.15	27.2	**<0.001**
	Soil amendment: Pasture	3	12.3	4.12	18.2	**<0.001**
	Residuals	24	5.41	0.22		
Root mass	Soil amendment	3	0.32	0.10	0.62	0.603
	Pasture	1	29.2	29.2	168.9	**<0.001**
	Soil amendment: Pasture	3	1.61	0.53	3.10	0.045
	Residuals	24	4.15	0.17		

Treatments consisted of "Soil amendment" and "Pasture." Significant P-values are indicated in bold.

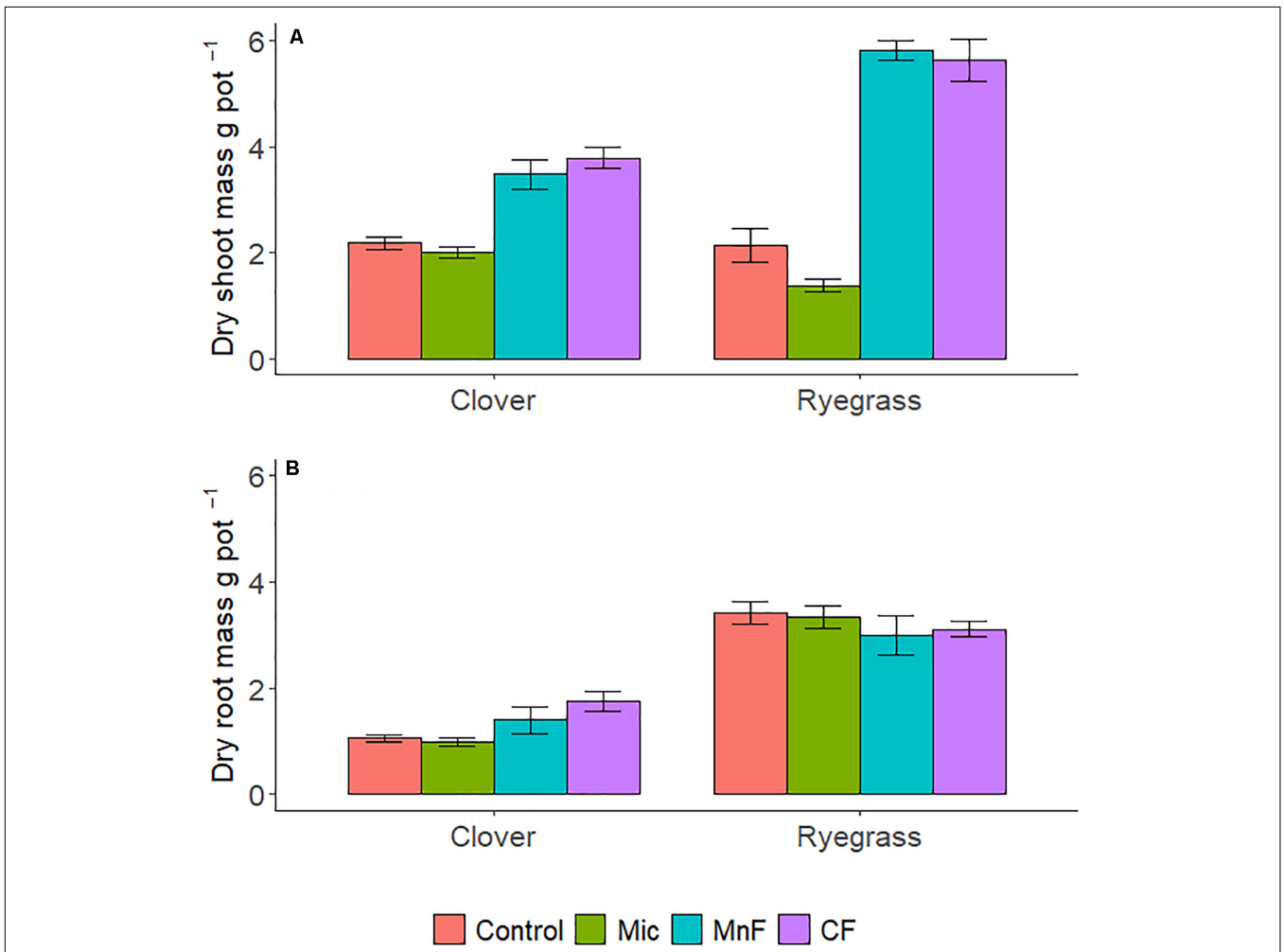


FIGURE 1 | Dry shoot mass **(A)**, dry root mass **(B)** per pot for soil amendment treatments; Mic (microbial biostimulant), MnF (rock mineral fertilizer), and CF (chemical fertilizer), control (no amendment). Plant species were subterranean clover (*Trifolium subterraneum*) and Wimmera ryegrass (*Lolium rigidum*). Bars represent the mean, and error bars are the standard error of the mean (n = 4).

reduction method (Kempers and Luft, 1988) on an automated flow injection Skalar AutoAnalyser (San plus, Skalar Analytical, Netherlands). Extractable Collwell P was determined for air-dried soils in 0.5 M sodium bicarbonate solution at pH 8.5 using the colorimetric methods of Rayment and Higginson (1992). Air dried soils were used to determine soil pH and EC. To determine pH in water suspension, 5 g of air-dried soil was suspended in 25 mL deionized water (1:5) and shaken for 1 h. For pH in $CaCl_2$, 5 g of air-dried soil was suspended in 0.01 M of $CaCl_2$ (Rayment and Higginson, 1992).

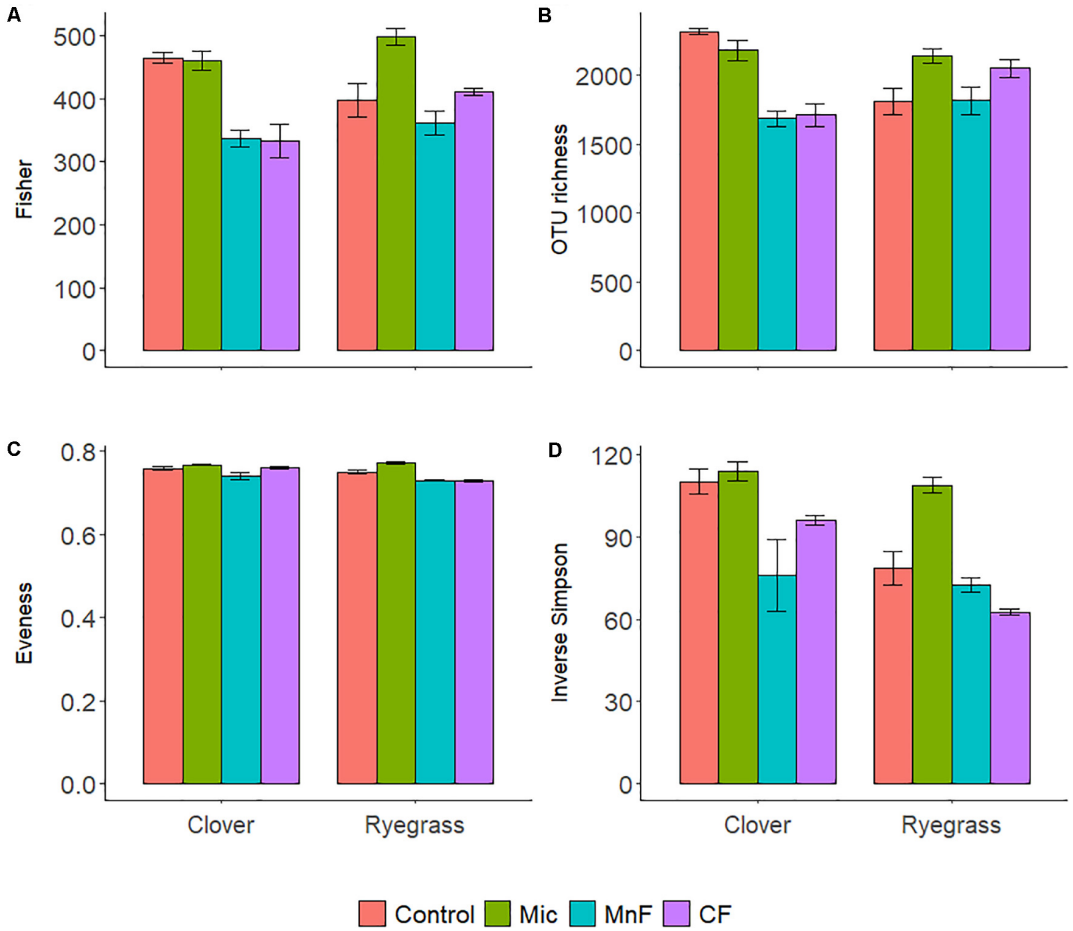


FIGURE 2 | Alpha diversity calculators for soil bacteria at the 97% OTU level; Fisher's **(A)**, richness **(B)**, Evenness **(C)**, Inverse Simpson **(D)**. For soil amendment treatments: Mic (microbial biostimulant), MnF (rock mineral fertilizer), and CF (chemical fertilizer), control (no amendment). Plant species were subterranean clover (*Trifolium subterraneum*) and Wimmera ryegrass (*Lolium rigidum*). Bars represent the mean, and error bars are the standard error of the mean (n = 4).

DNA Extraction and Sequencing

Sub-samples of rhizosphere soil were used to extract bacterial DNA. DNA was extracted using the MoBio Powersoil DNA isolation kit (Geneworks, Australia) and quantified prior to storage at −20°C. Polymerase chain reaction (PCR) was then performed to amplify bacterial 16S rRNA genes from the DNA samples using Golay barcoded primers and PCR conditions described previously (Mickan et al., 2018). The amplification of the target 16S rRNA genes followed (Mickan et al., 2018) using 27F and 519R bacterial primers (Caporaso et al., 2010; Mori et al., 2014) amended by the barcodes of Golay (Caporaso et al., 2012) with negative controls. DNA sequencing was performed on the MiSeq platform at the Australian Genome research facility, Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2014).

Bioinformatics

The primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010). Usearch (version 8.0.1623; Edgar, 2010; Edgar et al., 2011) and UPARSE software. Using Usearch tools DNA sequences were quality-filtered, and full-length duplicate sequences were removed and sorted according to abundance, singletons or unique reads in the data set were subsequently discarded. Sequences were clustered according to a chimera that was filtered using the "rdp_gold" database as a reference. To obtain the number of reads in each operational taxonomic unit (OTU), the reads were mapped back to the OTUs with a minimum identity of 97%. QIIME taxonomy was assigned using the Greengenes database (version 13_8, Aug 2013; DeSantis et al., 2006).

Statistics

The experiment was set up as a bi-factorial design with the first factor being: "soil amendment" (control, microbial biostimulant, mineral fertilizer, chemical fertilizer), and the second factor being: "pasture type" (subterranean clover, Wimmera ryegrass). The interaction between "soil amendment" with "pasture type" was assessed using an two way ANOVA within the R environment. Data were checked for normality as part of the

TABLE 3 | Soil bacteria two-way ANOVA results showing *P*-values for alpha diversity calculators.

Alpha diversity calculators	Treatment	Degrees of freedom	Sum of squares	Mean squares	F. model	*P*
Shannon	Soil amendment	3	0.78	0.26	29.0	**<0.001**
	Pasture	1	0.06	0.06	7.15	**0.013**
	Soil amendment: Pasture	3	0.09	0.03	3.34	**0.036**
	Residuals	24	0.22	0.01		
inverse Simpson	Soil amendment	3	6,718.7	2,239.5	16.9	**<0.001**
	Pasture	1	2,728.3	2,728.2	20.7	**<0.001**
	Soil amendment: Pasture	3	1,602.5	534.1	4.05	**0.018**
	Residuals	24	3,163.5	131.8		
Fisher	Soil amendment	3	82,957.0	27,652.5	22.4	**< 0.001**
	Pasture	1	2,625.0	2,625.1	2.13	0.158
	Soil amendment: Pasture	3	22,187.0	7,395.8	5.99	**0.003**
	Residuals	24	29,616.0	1,234.0		
Richness	Soil amendment	3	821,831.0	273,944.0	12.6	**<0.001**
	Pasture	1	2,757.0	2,757.0	0.13	0.725
	Soil amendment: Pasture	3	771,849.0	257,283.0	11.8	**<0.001**
	Residuals	24	521,137.0	21,714.0		
Evenness	Soil amendment	3	0.01	0.00	21.7	**<0.001**
	Pasture	1	0.00	0.00	13.7	**0.001**
	Soil amendment: Pasture	3	0.00	0.00	5.54	**0.005**
	Residuals	24	0.00	0.00		

Treatments consisted of "Soil amendment" and "Pasture." Significant P-values are indicated in bold.

statistical analysis. The significance of "soil amendment" and "pasture type" driving bacteria community was assessed with PERMANOVA using distance matrices (Adonis function) and square root-transformed OTU relative abundance data in the R environment. A canonical correspondence correlation analysis (CCA) was used to explore the relationship between bacterial taxa with "soil amendment" and "pasture type" at the 97% OTU level with soil chemical and plant growth data. The variance inflation factor (VIF) was calculated for multiple regression models using the R package Vegan version 2.3.0 (Oksanen et al., 2010) and was used to evaluate if the variables should be included in the subsequent CCA. We used the criterion VIF < 3 to select suitable variables in the best multiple regression models to remove strongly multicollinear variables (Yang et al., 2017). The treatment means were compared using least significant differences (LSD). The analyses were performed using R version 3.4.3 (R Core Development Team, 2015, Austria, 2017) and Vegan version 2.3.0 (Oksanen et al., 2010) and GenStat V.12.1.5.3.

RESULTS

Plant Biomass

Shoot Biomass

There were distinct changes in shoot biomass with both one way and two way ANOVA interactions for "soil amendment" and "pasture type" (**Table 2** and **Figure 1A**). Where applicable, subsequent *post hoc* Tukey *T*-test within the plant species, subterranean clover dry shoot biomass was unaffected by seed inoculation with the microbial biostimulant. However, an increase in shoot biomass was observed for both rock mineral ($P = 0.014$) and chemical ($P < 0.001$) fertilizers compared to the untreated control soil (**Figure 2A**). Inoculation with the microbial biostimulant did not achieve the same level of shoot biomass as the rock mineral ($P = 0.004$) and chemical ($P < 0.001$) fertilizers. For ryegrass the microbial biostimulant did not increase shoot biomass ($P = 0.355$), but both the rock mineral ($P < 0.001$) and chemical ($P < 0.001$) fertilizers increased shoot biomass in comparison to the control soil (**Table 2** and **Figure 2A**).

Root Biomass

Root biomass was not influenced by soil amendment ($P = 0.603$), although pasture species ($P < 0.001$) and the interaction between "soil amendment" and "pasture type" were significant ($P = 0.045$) (**Table 2** and **Figure 1B**).

Soil Bacterial Community Assemblages

Alpha Diversity

There were distinct changes to all alpha diversity calculators for both one way and two-way ANOVA interactions for both "soil amendment" and "pasture type" (**Table 3** and **Figure 2**). Subsequent *post hoc* Tukey *T*-test revealed no changes in Fisher's alpha diversity for the microbial biostimulant applied for subterranean clover, but there was a reduction in Fisher's alpha diversity with application of the rock mineral ($P < 0.001$) and chemical ($P < 0.001$) fertilizers (**Figure 2A**).

In contrast, for ryegrass there was a significant increase in Fisher's alpha diversity when the microbial biostimulant was

TABLE 4 | Soil bacteria two-way ANOVA results showing *P*-values fixed at Phylum resolution of relative abundance.

Taxon	Treatment	Degrees of freedom	Sum of Squares	Mean squares	F. model	*P*
Actinobacteria	Soil amendment	3	35.8	11.9	5.6	**0.005**
	Pasture	1	339.8	339.8	160.2	**<0.001**
	Soil amendment: Pasture	3	97.6	32.6	15.3	**<0.001**
	Residuals	24	50.9	2.1		
Proteobacteria	Soil amendment	3	35.3	11.8	5.1	**0.007**
	Pasture	1	75.3	75.3	32.7	**<0.001**
	Soil amendment: Pasture	3	9.3	3.1	1.3	0.285
	Residuals	24	55.3	2.3		
Firmicutes	Soil amendment	3	114.1	38.0	4.8	**0.010**
	Pasture	1	3.0	3.0	0.4	0.548
	Soil amendment: Pasture	3	36.3	12.1	1.5	0.236
	Residuals	24	191.5	8.0		
Acidobacteria	Soil amendment	3	16.7	5.6	9.9	**<0.001**
	Pasture	1	27.5	27.5	48.9	**<0.001**
	Soil amendment: Pasture	3	7.9	2.6	4.7	**0.010**
	Residuals	24	13.5	0.6		
Chloroflexi	Soil amendment	3	1.7	0.6	1.8	0.180
	Pasture	1	7.8	7.8	25.1	**<0.001**
	Soil amendment: Pasture	3	1.3	0.4	1.3	0.284
	Residuals	24	7.5	0.3		
Gemmatimonadetes	Soil amendment	3	22.5	7.5	17.8	**<0.001**
	Pasture	1	3.0	3.0	7.3	**0.013**
	Soil amendment: Pasture	3	8.4	2.8	6.7	**0.002**
	Residuals	24	10.1	0.4		
Planctomycetes	Soil amendment	3	1.2	0.4	27.7	**<0.001**
	Pasture	1	0.5	0.5	37.8	**<0.001**
	Soil amendment: Pasture	3	0.2	0.1	5.2	**0.006**
	Residuals	24	0.3	0.0		

Treatments consisted of "Soil amendment" and "Pasture." Significant P-values are indicated in bold.

applied (P = 0.009), but there were no effects of the rock mineral and chemical fertilizers (**Figure 2A** and **Table 3**). For subterranean clover, the microbial biostimulant had no effect on bacterial OTU richness, but it was decreased by both the rock mineral ($P < 0.001$) and chemical ($P < 0.001$) fertilizers (**Figure 2B**). Evenness was increased with application of the microbial biostimulant (P = 0.03) (**Figure 2C**) and the inverse Simpson index was affected by all treatments, "soil amendment" ($P < 0.001$), "pasture type" ($P < 0.001$), and there was an interaction between "soil amendment" and "pasture type" (P = 0.018) (**Table 3** and **Figure 2D**). Addition of the microbial biostimulant, reduced inverse Simpson index for subterranean clover (P = 0.006), but increased it for ryegrass (P = 0.019) (**Figure 2D**).

Soil Bacteria Phylum Level Relative Abundance

The relative abundance of bacteria at the phylum level in soil across all treatments was dominated by Actinobacteria (35%), Proteobacteria (27%), and to a lesser extent Acidobacteria (10%), Firmicutes (8%), Chloroflexi (7%), and Gemmatimonadetes (4%). There were changes to the relative abundance at the phylum level for "soil amendment" and "pasture type" with interactions between treatments (**Table 4** and **Figure 3**).

For subterranean clover, the relative abundance of Actinobacteria was not affected by inoculation with the microbial biostimulant (P = 0.251), the rock mineral fertilizer (P = 0.869) or the chemical fertilizer (P = 0.075) (**Figure 3A**). For ryegrass, the relative abundance of Actinobacteria decreased with application of the microbial biostimulant (P = 0.008) but increased in soil amended with the rock mineral fertilizer ($P < 0.001$) and was unaffected by the chemical fertilizer (P = 0.673) (**Figure 3B**).

For subterranean clover, the relative abundance of Proteobacteria was unaffected by the microbial biostimulant (P = 0.852), rock mineral fertilizer (P = 0.914) and chemical fertilizer (P = 0.999) (**Figure 3A**). There were no differences in relative abundance of Proteobacteria following application of the microbial biostimulant compared with the rock mineral (P = 0.999) and chemical (P = 0.728) fertilizers (**Figure 3A**). For ryegrass, the relative abundance of Proteobacteria was not affected by any treatment (**Figure 3B**). For the Firmicutes, there were minor changes in relative abundance associated with soil amendment (P = 0.009), but there was no effect of pasture species (P = 0.541) and there were no interactions between these treatments (**Table 4**).

For subterranean clover, the relative abundance of Acidobacteria was unaffected by the microbial biostimulant

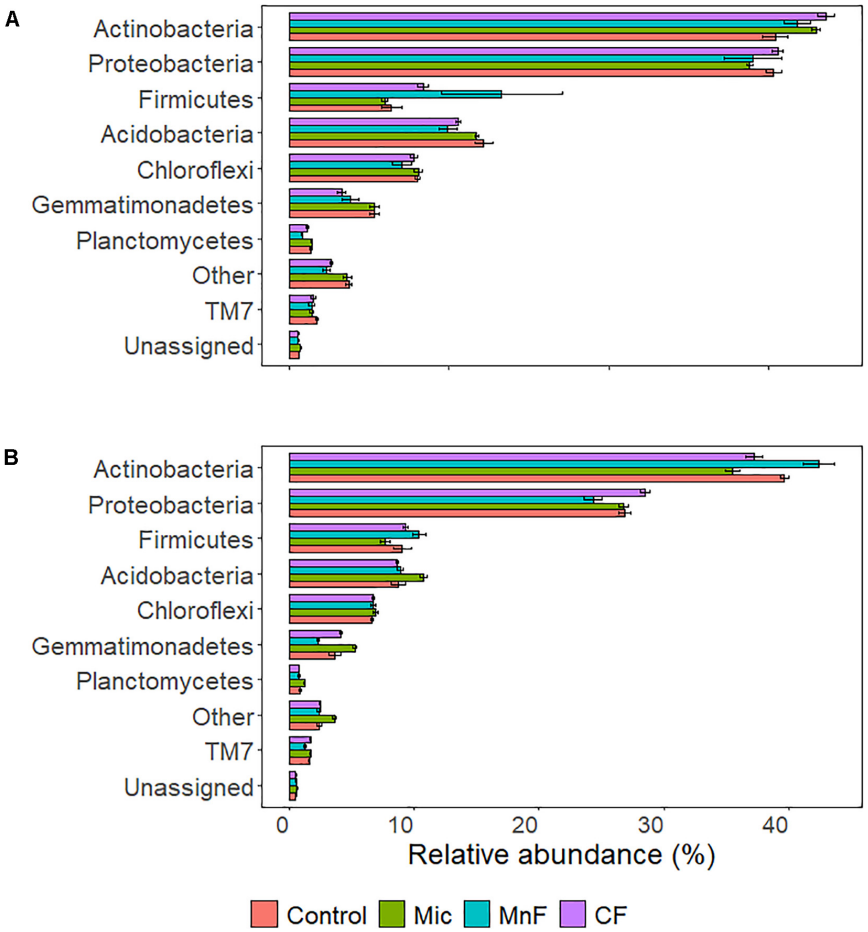


FIGURE 3 | Soil bacterial relative abundance at the phylum level for plant species **(A)** subterranean clover (*Trifolium subterraneum*) and **(B)** Wimmera ryegrass (*Lolium rigidum*). For soil amendment treatments: Mic (microbial biostimulant), MnF (rock mineral fertilizer), and CF (chemical fertilizer), control (no amendment). Bars represent the mean, and error bars are the standard error of the mean (n = 4).

(P = 0.981), decreased with the rock mineral fertilizer (P = 0.005), and was unaffected by the chemical fertilizer (P = 0.095) (**Figure 3A**). The microbial biostimulant increased the relative abundance of Acidobacteria compared to the rock mineral fertilizer (P = 0.042) but not compared to the chemical fertilizer (P = 0.413) (**Figure 3A**). For ryegrass, the relative abundance of Acidobacteria increased with application of the microbial biostimulant (P = 0.017) but was not affected by the mineral fertilizer (P = 0.991) or the chemical fertilizer (P = 0.991) (**Figure 3B**). The microbial biostimulant increased the relative abundance of Acidobacteria under ryegrass compared with both the rock mineral (P = 0.034) and chemical (P = 0.011) fertilizers (**Figure 3B**).

The relative abundance of Chloroflexi was not affected by soil amendment (P = 0.180) but was affected by pasture species (P < 0.001), with no interactions between these treatments (**Table 4** and **Figure 3**). For subterranean clover, the relative abundance of Gemmatimonadetes was not influenced by the microbial biostimulant (P = 0.991) or the rock mineral fertilizer (P = 0.058) but decreased with the chemical fertilizer (P = 0.004) (**Figure 3A**). The microbial biostimulant increased the relative abundance of Gemmatimonadetes compared to the mineral fertilizer (P = 0.004) but was unaffected by the chemical fertilizer (P = 0.413) (**Figure 3A**). For ryegrass, the relative abundance of Gemmatimonadetes increased with the microbial biostimulant (P = 0.035) but was unaffected by either the rock mineral (P = 0.102) or chemical (P = 0.971) fertilizer (**Figure 3B**). The microbial biostimulant increased the relative abundance of Gemmatimonadetes compared to the rock mineral fertilizer (P < 0.001) but not the chemical fertilizer (P = 0.249) (**Figure 3B**).

For subterranean clover, the relative abundance of Planctomycetes was unaffected by the microbial biostimulant (P = 0.996), but it decreased with the rock mineral fertilizer (P < 0.001) and was unaffected by the chemical fertilizer (P = 0.250) (**Figure 3A**). The microbial biostimulant increased the relative abundance of Planctomycetes compared to the rock mineral fertilizer (P < 0.001) but not the chemical fertilizer (P = 0.060) (**Figure 3A**). For ryegrass, the relative abundance of Planctomycetes increased with application of the microbial

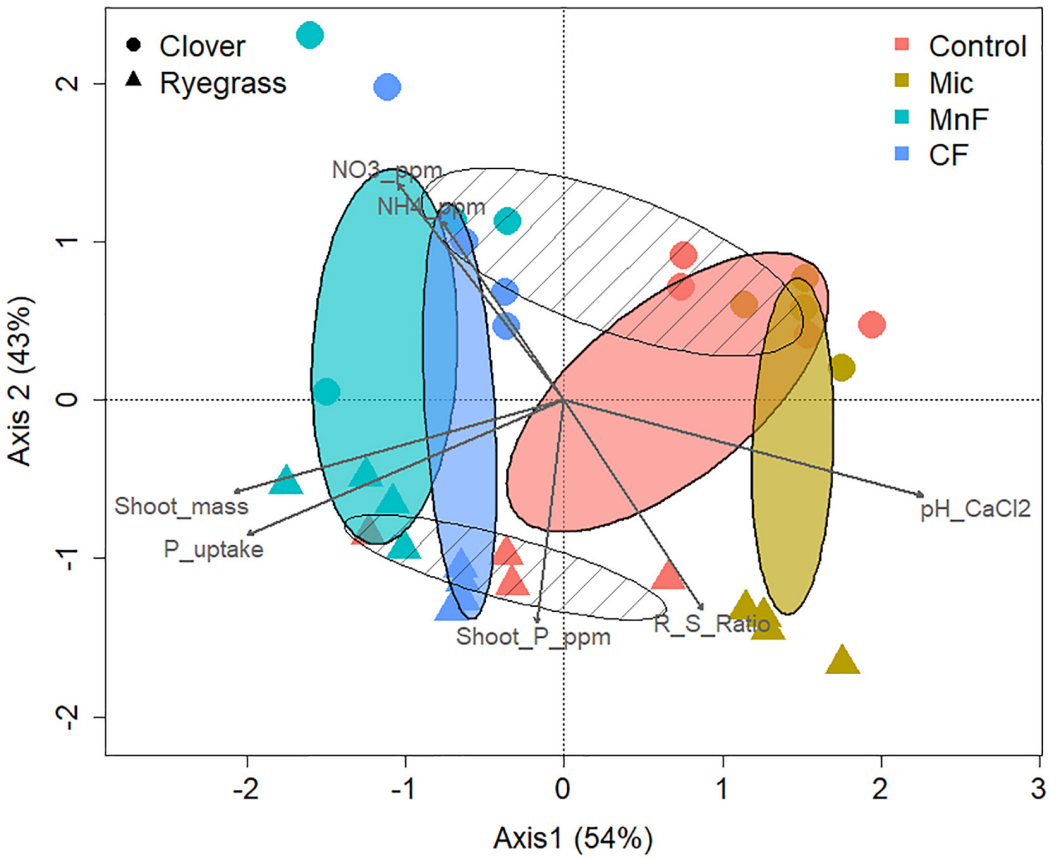


FIGURE 4 | Canonical correspondence analysis of rhizosphere bacterial OTUs (97%) for soil amendment treatments: Mic (microbial biostimulant), MnF (rock mineral fertilizer), and CF (chemical fertilizer), control (no amendment). Plant species were clover (*Trifolium subterraneum*) and Wimmera ryegrass (*Lolium rigidum*). Shaded ellipses represent 95% confidence intervals of the soil amendment treatments, with hatched ellipses displaying plant species clusters.

TABLE 5 | Soil bacterial community analysis by PERMANOVA results based on 97% similarity OTU abundance data (square root transformed), using 999 permutations.

Treatment	Degrees of freedom	Sum of squares	Mean squares	F. model	R^2	*P*
Soil amendments	3	0.296	0.098	4.88	0.253	**0.001**
Pasture	1	0.166	0.166	8.23	0.142	**0.001**
Soil amendment: Pasture	3	0.219	0.073	3.60	0.187	**0.002**
Residuals	24	0.486	0.020	0.41		
Total	31	1.169	1			

Treatments consisted of "Soil amendment" and "Pasture." Significant P-values are indicated in bold.

biostimulant ($P = 0.013$) but not the rock mineral ($P = 0.898$) or chemical ($P = 0.751$) fertilizers (**Figure 3B**). The microbial biostimulant increase the relative abundance of Planctomycetes compared to the rock mineral fertilizer ($P = 0.001$) but not the chemical fertilizer ($P < 0.001$) (**Figure 3B**).

Beta Diversity OTU Level Community Analysis

To investigate the effects of the two pasture species and the form of fertilizer applied on the composition of the soil bacterial community at the 97% OTU level, a canonical correspondence analysis (CCA) was performed to determine which significantly correlated environmental variables with a VIF score < 3 (pH, NO^{3-}–N, NH^{4+}–N, shoot mass, P uptake, P concentration) best explained changes in bacterial community composition as assessed by a variation in inflation factor (**Figure 4**). Further analysis of community composition by PERMANOVA indicated significant community separation due to soil amendment ($P < 0.001$), pasture type ($P < 0.001$) and the interaction of soil amendment × pasture type ($P < 0.002$) (**Table 5**). The largest separation of OTU occurred along axis 1 (**Figure 4**) where there was a distinct clustering for the control samples that clearly separated from the soil amendments, with the microbial biostimulant clustering on the side most distant to the mineral and chemical fertilizer treatments. There

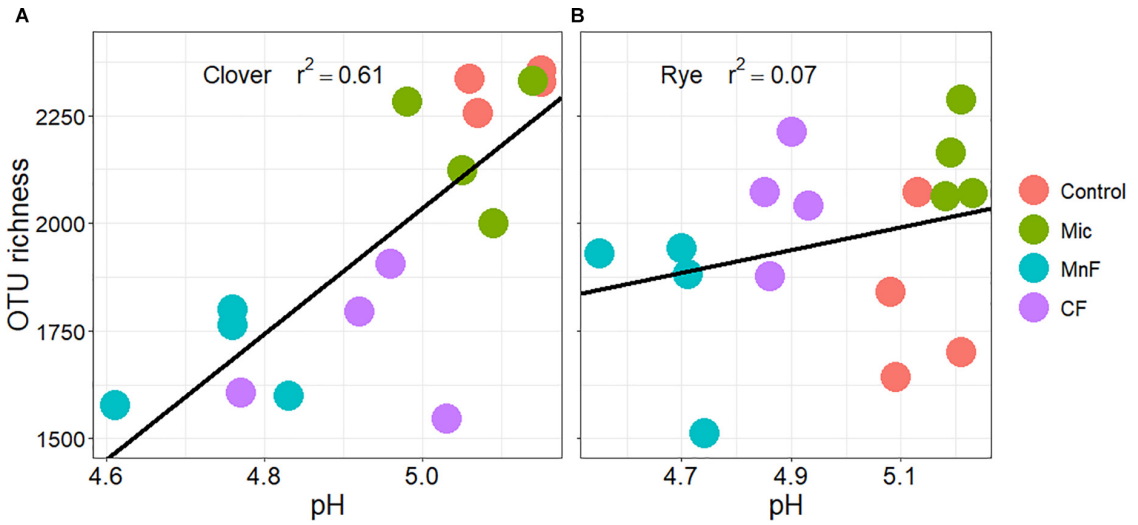


FIGURE 5 | Correlation regression between OTU richness (97%), with soil pH for soil amendment treatments: Mic (microbial biostimulant), MnF (rock mineral fertilizer), and CF (chemical fertilizer), control (no amendment). Plant species were **(A)** subterranean clover (*Trifolium subterraneum*) and **(B)** Wimmera ryegrass (*Lolium rigidum*).

was a distinct treatment effect on the bacterial community composition, with the communities from the untreated soil and soil treated with fertilizers clearly separating along axis 1 whilst the pasture species treatments separated along axis 2 as distinct communities (**Figure 4**).

DISCUSSION

Shoot dry weight was unaffected by inoculation with the microbial biostimulant and did not reach the levels achieved with either the rock mineral or conventional fertilizers, aligning with trends reported previously (e.g., Assainar et al., 2018, 2020). Root dry weight was less influenced by soil amendment, although there are morphological differences among these pasture species (Gilbert and Robson, 1984; Reid et al., 2015; Guy et al., 2018). This is consistant with previous studies showing a greater capacity for ryegrass to access soil P than subterranean clover (Barrow, 1975). This related to the greater area surface area of annual ryegrass roots compared with subterranean clover, and to its ability to decrease P concentration at the root surface to lower threshold concentration level (Barrow, 1975), which differ among plant species (Barber, 1980). These responses can also be related to root hair morphology, which can be longer and more dense when plants are growing in P-deficient soil (Foehse and Jungk, 1983; Bates and Lynch, 1996; Gahoonia and Nielsen, 1997; Ma et al., 2001). Nevertheless, there was no difference between the effect of the rock mineral and chemical fertilizers on root and shoot dry weight, probably reflecting the relatively high initial level P in this agricultural soil.

Although the two commercial fertilizers used in this study differed in their P solubilities, no difference in soil P concentration was measured for subterranean clover and annual ryegrass at the end of the experiment. Soil pH that can affect the solubility and availability of P from fertilizers in soil either directly (Hinsingher and Gilkes, 1996; Manning, 2008) or indirectly by decreasing the exudation of carbohydrates from roots (Graham et al., 1981).

Inoculation with the microbial biostimulant induced plant dependent responses to the diversity indices of soil bacteria including alpha diversity of bacterial OTUs richness (Shannon, Fisher, Richness, and Evenness). Some decreases in alpha diversity indices were similar to those reported previously by Assainar et al. (2020), and in soils with higher fertilizer inputs (Dai et al., 2018). In our experiment, most alpha diversity indices were less influenced by the microbial biostimulant in soil where subterranean clover was grown, and more influenced in soil where annual ryegrass was grown. However, decreases in alpha diversity observed were less consistent; some conventional fertilizers and microbial inoculants lead do increases in alpha diversity calculators (Assainar et al., 2018). Incorporation of composts applied to agricultural soil can also lead to substantial increases in alpha diversity calculators (Mickan et al., 2018), especially toward the latter phase of the plant growth cycle for organic material consisting of microbial residues (Zarezadeh et al., 2019).

Both soil amendment and plant were major drivers of bacterial community structure in our study. The observed richness of OTUs (97%) was lower when the chemical fertilizer was applied to soil sown with subterranean clover than with annual ryegrass, but there was no effect of the microbial biostimulant. There was a highly significant correlation between soil bacterial OTU richness and soil pH for subterranean clover ($R^2 = 0.61$) but no correlation for annual ryegrass ($R^2 = 0.07$) (**Figure 5**). This plant dependent response could indicate direct relationships between soil bacterial community composition and plant-induced changes in soil pH, especially in the rhizosphere (Hinsinger et al., 2003). Alterations in soil pH may also be associated with microbial activity

responsible for plant access to less available forms of P by reducing soil pH via production of organic acids (Marschner et al., 2011; Lagos et al., 2016). Community assemblages of soil bacteria have been demonstrated to be correlated with soil pH (O'Brien et al., 2019). Over longer time frames, the addition of fertilizer can also have a direct influence on soil bacteria by altering pH, with implications on nutrient cycling and P availability (Zhang et al., 2017). Whilst short term responses of bacterial communities can be associated with differences in plant species composition, this can be greater than those in response to fertilizer application (Bardgett et al., 1999). Our study demonstrates that a short-term response to both fertilizer and plant species can be related to either a direct influence of fertilizer or to indirect influences associated with soil microbial processes.

CONCLUSION

The mechanisms contributing to an influence of plant species on soil bacterial community structure were highlighted in this study where seeds of two pasture plant species were inoculated with a multispecies microbial biostimulant. Although there was no beneficial effect of the microbial biostimulant on plant growth, there were significant influences on the soil bacterial community. The potential for interactions between biostimulants and the soil bacterial community provides scope for selection of plant-specific bacterial biostimulants in relation to either direct (fertilizer) and indirect (bacterial) localized changes to soil pH which could contribute to dissolution of poorly soluble forms of fertilizer, especially P fertilizers. The two commercial fertilizers investigated here differed in their P solubility and shoot biomass of subterranean clover and annual ryegrass both responded to their application in this agricultural soil. Fertilizers which varied in P solubility were associated with plant species dependent changes in naturally occurring soil bacterial communities. Further investigation could involve consideration of effects on soil microbial communities when selecting optimum rates of fertilizer, especially those that include poorly soluble P sources. Fertilizer application to mixed pasture communities should support soil microbial diversity and function involved in mineral dissolution processes at rates that meet plant requirements. Thus, further studies could consider the impacts of microbial biostimulants on soil bacterial communities, even when there are no plant growth responses to inoculation.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI database repository, accession number PRJNA638485.

AUTHOR CONTRIBUTIONS

AA and ZS conceived the original experimental design. AA conducted the experiment. BM initiated and completed the molecular analysis and led the interpretation of the data. All authors contributed to data interpretation and scientific writing.

FUNDING

Support from Australian Mineral Fertilizers Pty Ltd. in supplying rock mineral fertilizer and a multispecies microbial inoculant is gratefully acknowledged. The Committee for Education Development in Iraq provided a Ph.D. scholarship for AA. An Australian Research Council Linkage Project (LP140100046) with industry partner Australian Mineral Fetilizers Pty Ltd. supported the salary of ZS.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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