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Multilateral interactions in the rhizosphere

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Multilateral interactions in the rhizosphere

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Table of contents

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Editorial: Multilateral Interactions in the Rhizosphere

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Keywords: rhizosphere, plant-microbe interaction, PGPR—plant growth-promoting rhizobacteria, induced systemic resistance, quorum sensing (QS)

Editorial on the Research Topic

Multilateral Interactions in the Rhizosphere

Interactions between plants and diverse microorganisms colonizing their rhizosphere play a central role in determining nature of the relationship. The plant host fitness as well as the microorganisms are influenced by the outcome of such interactions. Environmental and ecological factors leading to perturbations or disruption of this balanced relationship have also a significant impact. The plant rhizosphere is a complex ecosystem serving as a niche for diverse microorganisms (bacteria, archaea, fungi), nematodes and other organisms. Within the rhizosphere the root exudates have a dual function, influencing nutrient availability and organisms in the vicinity of the root, on one hand. On the other, many microorganisms produce phytohormones that alter the root architecture or other compounds, which affect nutrient availability and thereby the competition between neighboring plants. Sometimes their presence can be beneficial for their host plant since they suppress the growth of phytopathogenic microorganisms. Some other rhizosphere microorganisms such as rhizobacteria and some fungi promote directly plant growth or stimulate the plant immune system. All these phenomena have potential practical applications in agriculture.

Even though we know a lot about the direct impact of individual microbial molecules on the plant itself, the exact mechanisms underpinning the action of complex microbial inoculants are not yet completely understood. It is however widely accepted that both below- and aboveground plant native microbiomes will have impact on the outcome of such interaction (Vishwakarma et al.). Nowadays, the microbial inocula used in order to prevent soil-borne diseases become rather complex and products based on single-strains are being replaced by biological consortia (Niu et al.). The effect on the plant immune system was very often in focus of the application of such complex microbial inoculants. Induced Systemic Resistance and priming for enhanced resistance have been practiced in agriculture for many years, and today the application of novel biotechnological advances is helping to provide better insights into the functional and ecological aspects. Nonetheless, how complex situations, e.g., multiple bacterial molecules influence the plant, remains unclear. It seems though, that complex bacterial quorum sensing molecules from the *N*-acyl homoserine lactones group induce priming for enhanced resistance (Shrestha et al.). An impact on the resistance to pathogens was observed also in the tripartite interaction between potato, arbuscular mycorrhiza fungi and potato virus Y (Deja-Sikora et al.). Interestingly, the presence of mycorrhiza reduced the production of free oxygen radicals, otherwise induced by the virus, even in PVY-infected plants. These results suggest that mycorrhizal fungi could mask the viral infection and promote asymptomatic growth. In addition to the impact of microbial partners on

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plant physiology, the effect of the plant's altered physiological response on the microbial community was also observable. This phenomenon was studied in drought-tolerant transgenic sugarcane, which can attract a different microbiome than its wild type parental line, illustrating also how the genotype of the host plant influences the microorganisms in its rhizosphere (Zhao et al.). Another example of a very specific and close association between plant and microorganisms, is the canonical symbiosis between legumes and rhizobia. These very specific associations depend on a selective rhizosphere communication between the bacterium and the legume plant. This communication includes multiple levels and both partners can influence the outcome. The constantly increasing knowledge of molecular mechanisms employed by both associates provides us with new opportunities to use and to understand this phenomenon (Walker et al.).

When discussing interactions in the rhizosphere, not only interactions between the plant and microorganisms seem important, also the structure and diversity of soil microbial community, which acts as the reservoir of microorganisms, are important because, beneficial or antagonistic interactions among microorganisms themselves are crucial. So does the edaphic factor. This was illustrated by the observation that in some cases the growing site and the agricultural practice are two major driving forces, which shape the rhizosphere community. Namely, in an experiment focused on *Brassica napus* only one bacterium was common among different experimental setups (Floc'h et al.). Several plant protection products have been developed using different *Bacillus* species. The performance however, largely depends on their interactions with competing rhizosphere microorganisms. How such plant-beneficial inocula react to other microorganisms is not always understood, and bridging this knowledge gap would improve the product development (Andrić et al.). A good example of how one strain may actually inhibit the function of another beneficial bacterium, is the quorum quenching activity of *Bacillus subtilis* on the N-fixing *Ensifer meliloti*, which diminishes its symbiotic activity (Rosier et al.). Particular bacterial community members, as well as the availability of nutrients, especially that of phosphorous, were also key factors in the tripartite association between plant, truffles and the soil microbiota (Zhang et al.), reflecting in addition the role of edaphic factors.

The complex systems assessed in the above-mentioned studies require a good experimental design and possibilities to control many different variables. This is possible in growth chamber studies under controlled conditions, the choice of an appropriate growing chamber seems therefore crucial and has been addressed in this issue in the study of Yee et al. Similarly, the colonization of the host plant by beneficial microorganisms necessitates quantification and documentation possibilities. Standardized frameworks assessing colonization efficacy and patterns could allow the comparison of different studies (Carroll et al.).

Much remains to be revealed about the multifunctionality of the belowground interplay among soil microorganisms and plant roots and the influence on the aboveground plant parts. Only through the understanding of these interactions will we be able to manage processes in this highly dynamic compartment to our benefit and enhance sustainable ecosystem functioning and crop production. Learning from natural ecosystems and employing targeted approaches which lead to enhanced plant productivity in agroecosystems will be of tremendous importance in the coming years and decades.

AUTHOR CONTRIBUTIONS

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

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Exogenous Nitric Oxide and Phosphorus Stress Affect the Mycorrhization, Plant Growth, and Associated Microbes of *Carya illinoinensis* Seedlings Colonized by *Tuber indicum*

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In the artificial cultivation of truffles, ectomycorrhizal colonization level, host plant quality, and the associated microbes in the rhizosphere soil are vitally important. To explore the effects of nitric oxide (NO) and phosphorus (P) stress on the early symbiosis of truffles and host plants, different concentrations of exogenous NO donor sodium nitroprusside (SNP) and P were applied to *Carya illinoinensis* seedlings inoculated with the Chinese black truffle (*Tuber indicum*). The growth of *T. indicum*-mycorrhized seedlings and their mycorrhizal colonization rate were investigated. Additionally, the denitrifying bacterial community harboring NO reductase (*norB*) genes and the fungal community in the rhizosphere of the host were analyzed by high-throughput sequencing. The results showed that the colonization rate of *T. indicum* was significantly influenced by SNP treatments and P stress, with the highest level being obtained when the SNP was 100 μmol/L under low P stress (5 μmol/L). Treatment with 100 μmol/L SNP alone also increased the colonization rate of *T. indicum* and had positive effects on the plant height, stem circumference, biomass, root-shoot ratio and root POD activity of the seedlings at different times after inoculation. Under low P stress, the 100 μmol/L SNP increased the richness of the *norB*-type denitrifying bacterial community. Interestingly, the diversity and richness of *norB*-type denitrifying bacteria were significantly positively correlated with the colonization rate of *T. indicum*. SNP treatments under low P stress altered the abundance of some dominant taxa such as *Alphaproteobacteria*, *Gammaproteobacteria*, *Pseudomonas*, *Ensifer*, and *Sulfitobacter.* Evaluation of the fungal community in the rhizosphere revealed that 100 μmol/L SNP treatment alone had no noticeable effect on their richness and diversity, but it did shape the abundance of some fungi. *Buellia*, *Podospora, Phaeoisaria, Ascotaiwania,* and *Lophiostoma* were more abundant following exogenous NO application, while the abundance of *Acremonium, Monographella,* and *Penicillium* were decreased. Network analysis indicated that *T. indicum* was positively and negatively correlated with some

fungal genera when treated with 100 μmol/L SNP. Overall, these results revealed how exogenous NO and P stress influence the symbiosis of truffles and host plants, and indicate that application of SNP treatments has the potential for ectomycorrhizal synthesis and truffle cultivation.

Keywords: *Tuber indicum*, ectomycorrhizae, nitric oxide, phosphorus stress, truffle

INTRODUCTION

Tuber spp., commonly known as truffles, are ascomycete fungi that form ectomycorrhizae in a symbiotic relationship with plant roots and are prized for their hypogeous, edible fruiting bodies which adds a unique flavor to dishes (Kues and Martin, 2011; Vahdatzadeh et al., 2015). The interest in artificial cultivation of truffles has increased because of both the scarcity of truffle resources and reports of them being a source of polysaccharides with antitumor activity (Luo et al., 2011; Pereira et al., 2013; Zhao et al., 2014; Schmidberger and Schieberle, 2017). At present, synthesis of truffle-colonized seedlings and establishment of truffle plantations are the main methods of truffle cultivation (Deng et al., 2014). *Tuber indicum,* which is morphologically and phylogenetically similar to *T. melanosporum,* is the major commercial species of black truffle in China (Geng et al., 2009; Liu et al., 2011). *Carya illinoinensis* is an economically important nut tree native to North America, which is now cultivated worldwide (Benucci et al., 2012; Marozzi et al., 2017). Accordingly, utilizing *C. illinoinensis* as the host plant for *T. indicum* would have many practical and economic benefits and it has been shown that the ectomycorrhizae of *T. indicum* have now been successfully cultivated with *C. illinoinensis* (Bonito et al., 2011). Although the mycorrhization of these two organisms has been successfully accomplished, determining how to increase the ectomycorrhizal colonization levels and physiological and molecular mechanisms in response to this symbiont formation requires further exploration.

Ectomycorrhizae play an important role in P cycling of the rhizosphere (Cumming et al., 2015; Liu et al., 2018). Many studies have shown that ectomycorrhizae can contribute to the P absorption of plant roots, especially when P is scarce in the rhizosphere of the soil (Núñez et al., 2008; Bortier et al., 2018; Köhler et al., 2018). The content of P in the environment has been verified to impact the growth of ectomycorrhizal fungi and their colonization (Jones et al., 1990; Xue et al., 2008; Kluber et al., 2012). NO can affect the growth and development of plants as a signaling molecule that participates in many physiological processes, including seed germination, leaf growth, lateral root growth, stomatal movement and response to various biotic and abiotic stresses (Baudouin, 2011; Corpas and Barroso, 2015). Some reports have shown that NO can be produced in much higher levels in plants roots after colonization by arbuscular mycorrhizal fungi (Calcagno et al., 2012; Corpas and Barroso, 2015), which indicates that NO is important during the process of mycorrhizal colonization. In addition, evidence of the involvement of NO in plant responses to low P stress has been obtained (Simontacchi et al., 2015). As an exogenous NO donor, sodium nitroprusside (SNP) has commonly been used to explore the effects of NO on the physiology of many plants. However, it is still not known if SNP can affect the colonization levels of ectomycorrhizal fungi such as truffles on host plant root systems. Moreover, the effects of exogenous NO on the ectomycorrhizal synthesis of *T. indicum* and on the symbiotic system of *C. illinoinensis* with *T. indicum* under P stress conditions have not yet been reported.

In terrestrial ecosystems, ectomycorrhizal fungi including truffles have an important ecological function. A variety of microbial communities are involved in the lifecycle of truffles, and these play important roles in the truffle ectomycorrhizae and ascocarp formation, while also contributing to their aroma (Splivallo et al., 2015; Vahdatzadeh et al., 2015). Moreover, truffles have been predicted to influence soil microbial communities because of the formation of a brûlé (an area devoid of herbaceous cover) (Streiblova et al., 2012; Mello et al., 2013; Li et al., 2018). Our previous studies have also indicated that *T. indicum* shapes the bacterial and fungal communities in the ectomycorrhizosphere of *P. armandii* and *Q. aliena* (Li et al., 2017, 2018). Denitrification is a key nitrogen removal process that can produce NO, N_2O, and N_2, and different bacteria including bacteria harboring NO reductase (*norB*) genes can perform this process (Yunfu et al., 2017). Considering that NO may play an important role in the mycorrhizal colonization process of truffles and exogenous NO treatment was provided in this study, bacteria harboring *norB*-genes were selected for analysis, rather than 16S rRNA genes. Although many previous studies have investigated the microbial communities associated with truffles (Antony-Babu et al., 2014; Benucci and Bonito, 2016; Deveau et al., 2016; Fu et al., 2016), the specific roles of these microbial communities and the interaction between these microbes and truffle ectomycorrhizae is unclear, as are the conditions that occur under exogenous NO and P stress.

In this study, different concentrations of exogenous NO and P were provided to the *C. illinoinensis* seedlings inoculated and uninoculated with *T. indicum*. The colonization rate and the host plant growth and physiology were assessed. Additionally, high-throughput sequencing was used to analyze the *norB*-type denitrifying bacterial community of the rhizosphere soil. Next, to further explore the effects of NO alone on this symbiotic system, a suitable concentration of NO was selected for application to the colonized seedlings. The colonization rate, seedling growth and fungal communities of the rhizosphere soil were subsequently investigated from month 1 to 6 after inoculation. To our knowledge, this is the first study to explore the effects of exogenous NO and P stress on the ectomycorrhizal colonization of truffles and on the symbiotic system of host plants with truffles, with the goal of learning more about the physiological and molecular mechanism response to this

symbiont formation under different conditions to improve the artificial cultivation of truffles.

MATERIALS AND METHODS

C. illinoinensis Seedling Cultivation and *T. indicum* Inoculation

Carya illinoinensis seeds obtained from Yangbi County, China were first sterilized by soaking in 0.1% potassium permanganate solution for 2 h. Next, washed seeds were sown in sterilized nursery substrate composed of vermiculite, perlite, and water (volume ratio of 1:1:1) (Li et al., 2017). After 3 months, seedlings that were growing well were selected for transplantation into separate plastic containers filled with 1 L of sterilized cultivation substrate. There were two kinds of cultivation substrate prepared, Substrate I and Substrate II. Substrate I was composed of nutrient-poor sand, and Substrate II consisted of organic soil, vermiculite and water (volume ratio 1:1:0.5). The two cultivation substrates were autoclaved for 90 min at 121°C before use (Li et al., 2017, 2018). Truffle inoculation was performed when the seedlings were transplanted.

Tuber indicum was from Yanbian County, China. The truffle inoculum was prepared as previously described (Li et al., 2018). Briefly, 75% alcohol was used to disinfect the surface of the truffle ascocarps, after which they were pulverized and blended to spore powder. Next, 2 and 1 g of spore powder was inoculated into Substrate I and Substrate II, respectively, surrounding the roots of each *C. illinoinensis* seedling. There were 36 and 42 inoculated *C. illinoinensis* seedlings cultivated in Substrate I and Substrate II, respectively. Additionally, 3 uninoculated seedlings were cultivated in Substrate I. All seedlings were cultivated in a greenhouse under the same conditions with a clean environment and appropriate temperature and moisture content of the substrate.

Experimental Design

Exogenous NO Treatment Combined With P Stress

Seedlings cultivated in Substrate I were subjected to exogenous NO treatment combined with P stress (**Supplementary Table S1a**).

After *T. indicum* inoculation, the exogenous NO donor SNP with four different concentrations (0, 10, 100, and 1000 μmol/L) was applied to the inoculated *C. illinoinensis* seedlings every 15 days, while uninoculated seedlings were treated with 0 μmol/L SNP. Samples were treated with 100 mL SNP per pot at each treatment time, half of which was applied to the cultivation substrate, while the remainder was sprayed on the leaf surface. The inoculated seedlings treated with 0, 10, 100, and 1000 μmol/L SNP were denoted S0, S1, S2, and S3, respectively.

When the seedlings were treated with different concentrations of SNP, they were also treated with different levels of P. Briefly, modified Hoagland nutrient solution containing three different concentrations of P was prepared (0, 5, and 2000 μmol/L). The P originated from KH_2PO_4 and the final concentration of each element in the nutrient solution except P is shown in **Supplementary Table S2**. The inoculated *C. illinoinensis* seedlings that were irrigated with 0, 5, and 2000 μmol/L P nutrient solution were denoted as the no P treatment (P_0), low P treatment (P_5), and high P treatment group (P_{2000}), respectively. Uninoculated seedlings treated with 0 μmol/L SNP were only irrigated with 0 μmol/L P nutrient solution and were assigned to CK group. Overall, there are 12 treatments (excluding CK): P_0S_0, P_0S_1, P_0S_2, P_0S_3, P_5S_0, P_5S_1, P_5S_2, P_5S_3, $P_{2000}S_0$, $P_{2000}S_1$, $P_{2000}S_2$, and $P_{2000}S_3$. Each treatment contained at least three *C. illinoinensis* seedlings, all of which were timely irrigated with corresponding modified Hoagland nutrient solution, while sterile water was periodically applied to keep the cultivation substrate moist.

Exogenous NO Treatment Only

Exogenous treatment of only NO was applied to seedlings that were cultivated in Substrate II (**Supplementary Table S1b**). To further investigate the effects of only NO on the growth of *C. illinoinensis* seedlings with *T. indicum* colonization in the early symbiotic stage, the appropriate concentration (100 μmol/L) of SNP was applied alone as described above. Overall, half of the inoculated seedlings cultivated in Substrate II were treated with 100 mL of 100 μmol/L SNP every 15 days after inoculation of *T. indicum* until day 90 (SNP treatment), while the remaining inoculated seedlings were treated with an equal amount of water at the same time (Control-M treatment). The seedlings in these two treatments were irrigated with water every 2–3 days to keep the cultivation substrate moist.

Sampling Strategy and Analysis

After 4 months from inoculation, seedlings cultivated in Substrate I were observed, and samples were collected. The ectomycorrhizae of *C. illinoinensis* seedlings colonized by *T. indicum* were successfully detected by morphological analysis using a microscope. In each treatment, seedlings and their root system were harvested. Moreover, the rhizospheres soil of seedlings in CK and low P treatments (P_5S_0, P_5S_1, P_5S_2, and P_5S_3) were also collected aseptically. The mycorrhizal colonization rate was determined by counting the number of root segments colonized by *T. indicum* under a stereomicroscope based on the mycorrhizal fungal structures, with 30 root segments randomly selected in total for each seedling, which was finally expressed as: (root segments colonized by *T. indicum*/total observed root segments) × 100% (Andres-Alpuente et al., 2014). The plant morphology and physiology was determined immediately after the collection of seedlings and their roots. The rhizosphere soil samples were stored at −80°C prior to high-throughput sequencing of the *norB*-type denitrifying bacterial community.

For the seedlings of two treatments cultivated in Substrate II, their root systems and rhizosphere soil were collected every month after inoculation and used to determine the plant morphology and physiology, as well as for high-throughput sequencing of the fungal communities. Samples harvested at 0, 1, 2, 3, 4, 5, and 6 months after inoculation were denoted M0, M1, M2, M3, M4, M5, and M6, respectively. The ectomycorrhizae in samples from each month were detected and the colonization rate of *T. indicum* was calculated at month 6.

The whole experimental design and sampling strategy in this study can be seen in **Supplementary Table S1**. Three biological samples in each treatment were used for analysis, including the analysis of plant physiology, colonization rate and microbial communities.

Determination of Plant Morphology and Physiology

The measured plant morphological and physiological indices included the plant height, stem circumference, root-shoot ratio, biomass, root activity, and superoxide dismutase (SOD) activity in roots and peroxidase (POD) activity in roots.

The plant height and stem circumference of *C. illinoinensis* seedlings were measured using a ruler and vernier caliper. Next, the seedlings were put in 100°C water for 20 min to halt respiration, then they were oven-dried at 75°C until constant weight for determination of the dry-weight, which was taken as the biomass. Next, the seedlings were divided into their underground and aboveground parts and the dry weights of the two parts were determined. The root-shoot ratio was expressed as the ratio of the dry weight of underground to aboveground parts of the seedlings (Maunoury-Danger et al., 2010).

The root activity was determined by the triphenyl tetrazolium chloride method as previously described (Zhang et al., 2012). The root SOD activity was determined by their ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) under light, and a unit of enzyme activity (U) was expressed as 50% inhibition of the NBT photoreduction (Fridovich, 2011). The determination of root POD activity was based on the theory that H_2O_2 can oxidize guaiacol under the catalysis of POD and form a tawny substance that can be detected using a spectrophotometer (Meloni et al., 2003). A unit of POD activity (U) was expressed as an absorbance change of 0.01 per minute at 470 nm.

Soil DNA Extraction and PCR Amplification of *norB* Genes and ITS Genes

Total genomic DNA of the rhizosphere soil samples was extracted using a Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, United States) according to the manufacturer's instructions. The extracted DNA was detected by 0.8% agarose gel electrophoresis and quantified by ultraviolet spectrophotometry.

For the DNA extracted from the soil samples of the P_5S_0, P_5S_1, P_5S_2, P_5S_3, and CK treatments (each treatment was performed in triplicate), the *norB* gene was amplified with the universal primers cnorB2F (5′-GACAAGNNNTACTGGTGGT-3′) and cnorB6R (5′-GAANCCCCANACNCCN GC-3′). The PCR reaction mix was 25 μL, which included the DNA template (2 μL), reaction buffer (5 μL), GC buffer (5 μL), 2 μL dNTPs (2.5 mmol L^{-1}), 1 μL forward primer (10 μmol L^{-1}), 1 μL reverse primer (10 μmol L^{-1}), 0.25 μL Q5 DNA polymerase, and 8.75 μL ddH_2O. The cycling conditions were as follows: initial denaturation at 98°C for 2 min, followed by denaturation at 98°C for 15 s, and annealing at 55°C for 30 s, extension at 72°C for 30 s, after which samples were subjected to final extension at 72°C for 5 min. For the DNA extracted from the soil samples in Substrate II, the ITS1 region was amplified using primers ITS1 (5′-GGAAGTAAAAGTCGTA ACAAGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR products were checked by 2% agarose gel electrophoresis and the target fragments were recovered using an Axygen Axy Prep DNA Gel Extraction kit (AxyGen Biosystems, United States). The recovered PCR products obtained from three technical replicates were combined in equidense ratios for each sample and purified with a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The PCR products were then quantified using a Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen). An Illumina TruSeq Nano DNA LT Sample Prep Kit (Illumina, San Diego, CA, United States) was used to generate PCR amplicon libraries, after which the library quality was assessed with Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc., United States).

Illumina MiSeq High-Throughput Sequencing and Data Analysis

High throughput sequencing was conducted by Personal Biotechnology, Co., Ltd. (Shanghai, China) on an Illumina MiSeq sequencing platform. The overlapping paired-end reads were assembled using PEAR software and poor-quality sequences were removed using QIIME (v1.8.0) and USEARCH (v5.2.236) (Caporaso et al., 2010; Luo et al., 2017). High-quality sequences with 97% similarity were assigned to operational taxonomic units (OTUs) using UCLUST. The taxonomic information of *norB*-denitrifying bacteria was obtained using Ribosomal Database Project (RDP) (Release 11.1[1]) and the fungal sequences were taxonomically classed using UNITE database (Release 5.0[2]) (Wang et al., 2007; Edgar, 2010). The alpha and beta diversity of *norB*-type denitrifying bacterial and fungal communities were respectively analyzed using QIIME (v1.8.0). The alpha diversity of the species complexity of each sample was determined using the Chao1, ACE, Shannon, and Simpson indices. The beta diversity was determined by non-metric multidimensional scaling (NMDS) using R software, which reflects the differences in microbial communities among groups. Permutational multivariate analysis of variance (PERMANOVA) was performed by QIIME accompanied with NMDS. Linear discriminant analysis effect size analysis was used to respectively reveal the bacterial and fungal taxa at all taxonomic levels with significantly differential abundance between groups, which was carried out by Galaxy online analysis platform[3]. Network analysis for investigation of the interactions between the dominant genera was also performed using Mothur software (Schloss et al., 2009).

All of the raw sequencing data used in this study were submitted to the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA544895/SRP199549.

Statistical Analyses

Statistical analyses were performed using SPSS v22.0 (IBM, Inc., Armonk, NY, United States). The data were analyzed by one-way analyses of variance (ANOVAs) and independent *t*-tests,

[1]http://rdp.cme.msu.edu/

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and the results reported were the means ± standard deviation (SD) of three biological replicates for each treatment. The least significant difference (LSD) test was performed using $P < 0.05$ as the threshold. Spearman's correlation coefficient (rho) was calculated using SPSS 22.0.

RESULTS

Effects of *T. indicum* Colonization on *C. illinoinensis* Seedlings

Four months after inoculation, mycorrhization was successfully detected on the inoculated *C. illinoinensis* seedlings, while other uninoculated seedlings (CK) had not been colonized by truffles based on morphological evidence (**Figure 1**). Comparison of the P_0S_0 treatment and CK revealed that *T. indicum* inoculation significantly increased the plant height and POD activity in roots, but significantly decreased the root SOD activity ($P < 0.05$) (**Table 1**). The root activity and stem circumference were higher in the P_0S_0 treatment than in CK, but not significantly. *T. indicum* inoculation had no noticeable effect on the root-shoot ratio and biomass.

Effects of Exogenous NO Combined With P Stress on *C. illinoinensis* Seedlings Colonized by *T. indicum*

The colonization rate of *T. indicum* and the physiological indices of *C. illinoinensis* seedlings were significantly affected by different concentrations of exogenous NO and P (**Table 1**). Under the same P concentration, the colonization rate of *T. indicum* on *C. illinoinensis* seedlings significantly increased as the SNP concentration increased from 0 to 100 μmol/L ($P < 0.05$), but when the SNP concentration increased to 1000 μmol/L, there were no ectomycorrhizae successfully detected. Under the same

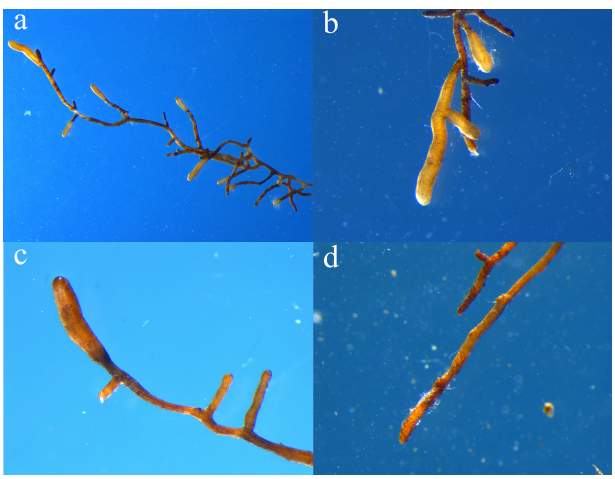

FIGURE 1 | Ectomycorrhizae of *Carya illinoinensis* seedlings with *Tuber indicum* **(a–c)** and the roots of *C. illinoinensis* seedlings that were not colonized by *T. indicum* **(d)**.

TABLE 1 | The colonization rate of *T. indicum* and the growth of *C. illinoinensis* seedlings under different concentrations of exogenous NO donor SNP and P treatments.

Treatments	Colonization rate	Root activity μg/(g·h) FW	SOD activity U/g FW	POD activity U/(g·min) FW	Root-shoot ratio	Biomass (g)	Plant height (mm)	Stem circumference (mm)
CK	–	45.15 ± 2.95de	5.10 ± 0.11abc	148.16 ± 4.53e	1.91 ± 0.10a	3.56 ± 0.38abcd	294.67 ± 7.77e	4.19 ± 0.20bcd
P_0S_0	0.37 ± 0.02a	67.78 ± 4.68cd	4.26 ± 0.52d	462.66 ± 86.38a	1.65 ± 0.33ab	3.53 ± 0.56abcd	364.33 ± 35.72abcd	4.66 ± 0.83abcd
P_0S_1	0.42 ± 0.03b	61.49 ± 10.86cd	3.47 ± 0.51f	304.00 ± 42.56bc	1.23 ± 0.35b	2.54 ± 1.34cd	334 ± 60.70cde	4.39 ± 0.26abcd
P_0S_2	0.57 ± 0.03c	147.93 ± 39.85a	4.19 ± 0.28de	135.66 ± 58.7e	1.42 ± 0.16ab	3.06 ± 0.39abcd	351.67 ± 22.59bcde	4.12 ± 0.32cd
P_0S_3	–	120.47 ± 23.57b	3.62 ± 0.29ef	152.00 ± 42.56e	1.87 ± 0.15a	3.52 ± 0.79abcd	318 ± 28.16de	4.33 ± 0.20abcd
P_5S_0	0.44 ± 0.01b	68.26 ± 6.09cd	5.50 ± 0.54a	149.33 ± 24.58e	1.90 ± 0.28a	3.77 ± 0.56abc	386.33 ± 48.23abc	4.56 ± 0.06abcd
P_5S_1	0.56 ± 0.02c	74.71 ± 10.75c	5.51 ± 0.17cd	143.66 ± 38.17e	1.70 ± 0.44ab	3.83 ± 0.75abc	407 ± 17.44ab	4.76 ± 0.31ab
P_5S_2	0.81 ± 0.03d	82.81 ± 2.99e	5.39 ± 0.20a	356.66 ± 139.01b	1.44 ± 0.28ab	2.36 ± 0.14d	359.67 ± 17.01abcd	4.44 ± 0.20abcd
P_5S_3	–	28.89 ± 3.58cd	5.15 ± 0.27ab	242.66 ± 20.59cde	1.42 ± 0.27ab	3.13 ± 0.30abcd	412.67 ± 17.67a	4.72 ± 0.17abc
$P_{2000}S_0$	0.19 ± 0.03e	66.15 ± 12.2cd	5.30 ± 0.38ab	186.66 ± 22.03de	1.46 ± 0.57ab	2.89 ± 0.48bcd	412.67 ± 56.22a	4.08 ± 0.24d
$P_{2000}S_1$	0.23 ± 0.04f	69.78 ± 6.65cd	5.24 ± 0.34ab	210.33 ± 47.01cde	1.53 ± 0.56ab	4.32 ± 1.63a	310.33 ± 31.37de	4.89 ± 0.47a
$P_{2000}S_2$	0.63 ± 0.02g	67.47 ± 21.54cd	5.26 ± 0.20ab	290.00 ± 112.2bcd	1.86 ± 0.63a	2.76 ± 1.12cd	321.33 ± 26.73de	4.11 ± 0.58cd
$P_{2000}S_3$	–	62.04 ± 10.40cd	4.73 ± 0.46bcd	181.00 ± 49.15de	1.81 ± 0.02ab	4.13 ± 0.45ab	347 ± 39.51cde	4.31 ± 0.21abcd

Each value is the mean of three replicates (± SD). Values followed by different lowercase letters indicate significant differences ($P < 0.05$) between samples in a row. NO, nitric oxide; SNP, exogenous NO donor sodium nitroprusside; P, phosphorus; FW, fresh weight. CK, uninoculated C. illinoinensis seedlings with 0 μmol/L SNP and P supply. P_0, P_5, and P_{2000} represent C. illinoinensis seedlings colonized by T. indicum treated with 0, 5, and 2000 μmol/L P, respectively. S_0, S_1, S_2, and S_3 represent C. illinoinensis seedlings colonized by T. indicum treated with 0, 10, 100, and 1000 μmol/L SNP, respectively.

concentration of SNP (except 1000 μmol/L), the colonization rate was significantly higher when the P concentration was 5 μmol/L ($P < 0.05$) and reached a maximum of 81 ± 3% in P_5S_2 treatment.

The different concentrations of P had no noticeable effect on the plant height, stem circumference, root-shoot ratio or biomass of seedlings (**Table 1**). In the P_0 and P_5 treatments, no differences in these four indices under different SNP concentrations were observed, while in the P_{2000} treatments, the stem circumference and biomass were significantly higher when the SNP concentration was 10 μmol/L compared to when SNP was 0 and 100 μmol/L ($P < 0.05$).

The highest root activity was observed in the P_0S_2 treatment. Specifically, no significant differences were observed among the three different P levels when the SNP concentration was 0 or 10 μmol/L, but at 100 μmol/L, the root activity significantly decreased as the P concentration increased ($P < 0.05$). In the P_0 and P_5 treatments, root activity was significant higher when SNP was 100 μmol/L ($P < 0.05$), and there were no significant differences in P_{2000} treatments. The SOD activity of roots in the P_0 treatments were significantly lower than in the P_5 and P_{2000} treatments ($P < 0.05$). There was no noticeable effect of different SNP concentrations on root SOD activity. The maximum POD activity was observed in the P_0S_0 treatment, followed by the P_5S_2 treatment. In the P_5 treatments, POD activity was significantly higher when the SNP was 100 μmol/L ($P < 0.05$) and there were no significant differences between P_{2000} treatments. Overall, the colonization rate and the physiological indices of the root system were higher when the SNP concentration was 100 μmol/L and the P content was low.

Effects of Solely Exogenous NO (100 μmol/L SNP) on the Growth of *C. illinoinensis* Seedlings Colonized by *T. indicum*

The morphology of the inoculated seedlings and their root systems are shown in **Figure 2**. The seedlings supplied by SNP grew better and had more lateral roots. The ectomycorrhizae of the two different treatments both occurred on the third month after inoculation, but the indicators of *T. indicum* colonization were more noticeable in the SNP treatment. On the sixth month after inoculation, the colonization rate of *T. indicum* reached 88 ± 2% on the seedlings to which 100 μmol/L SNP were applied, which was significantly higher than that of the seedlings in the Control-M treatment (62 ± 3%) ($P < 0.05$).

Sodium nitroprusside treatment significantly increased the plant height from the second month to the sixth month compared with the Control-M ($P < 0.05$), and the stem circumference became significantly thicker in the SNP treatment from the third to the sixth month ($P < 0.05$) (**Figure 3**). The biomass was higher in the SNP treatment, and significant differences were observed between the treatments on the third and fourth month ($P < 0.05$). SNP treatment also significantly increased the root-shoot ratio on the third, fifth and sixth month ($P < 0.05$). The POD activity in roots differed significantly between the two treatments on the first and second month, and was also higher in response to SNP treatment ($P < 0.05$). However, SNP treatment had no noticeable effect on the SOD activity in roots because there were no significant differences between the two treatments after inoculation. Root activity was significantly lower in response to SNP treatment during the fourth and fifth month ($P < 0.05$).

Analyses of *norB*-Type Denitrifying Bacterial Communities

Alpha Diversity of *norB*-Type Denitrifying Bacteria in Rhizosphere Soil

Sequencing of the rhizosphere soils of the CK treatment and those treated with different concentrations of SNP under low P stress were yielded 947,615 high-quality sequences from all 15 samples after quality control, which were clustered into 4,387 OTUs (**Supplementary Figure S1a**). The Venn diagram revealed 469 shared OTUs among the samples of the five different treatments (**Figure 4**), with the number of unique OTUs in treatment P_5S_2 being highest, followed by that in the P_5S_3 treatment. The unique number of OTUs in P_5S_0 and P_5S_1 was even lower than that in CK.

The two diversity indices (Shannon and Simpson) showed no significant differences among the five treatments (**Table 2**), indicating that the effects of treatment with different concentrations of SNP on the diversity of *norB*-type denitrifying bacteria were not significant. The estimated richness indices (Chao1 and ACE) revealed that the *norB*-type denitrifying bacterial community richness was highest in the P_5S_2 treatment, and was significantly higher than that in the P_5S_0 and CK treatments ($P < 0.05$).

Taxonomic Composition of *norB*-Type Denitrifying Bacterial Communities

In the 15 samples from the five different treatments, a total of 10 phyla, 16 classes, 35 orders, 56 families, and 95 genera of *norB*-type denitrifying bacterial communities were detected. At the phylum level, *Proteobacteria* was the most abundant phylum, accounting for 98.33% (**Supplementary Figure S2**), followed by *Actinobacteria* (1.07%) and *Acidobacteria* (0.42%). The relative abundance of these three phyla showed no significant differences among the five treatments.

At the class level (**Figure 5A**), *Alphaproteobacteria* (75.70%), *Gammaproteobacteria* (20.07%), and *Betaproteobacteria* (2.50%) were the dominant taxa. Under low P stress, the abundance of *Alphaproteobacteria* gradually increased as the SNP concentrations increased from 0 to 100 μmol/L and then decreased when the SNP concentration was 1000 μmol/L. *Alphaproteobacteria* was significantly more abundant in the CK and P_5S_2 groups than in the P_5S_0 and P_5S_3 groups ($P < 0.05$). The abundance of *Gammaproteobacteria* was lowest in the CK and P_5S_2 groups, while the P_5S_3 treatment contained significantly more *Gammaproteobacteria* than the CK ($P < 0.05$). *Betaproteobacteria* was more abundant in the P_5S_1 group.

At the genus level (**Figure 5B**), the most abundant genera were *Pseudomonas* (19.97%), *Sinorhizobium* (16.27%), *Rhizobium* (13.20%), *Ensifer* (12.80%), *Rhodobacteraceae_unidentified*

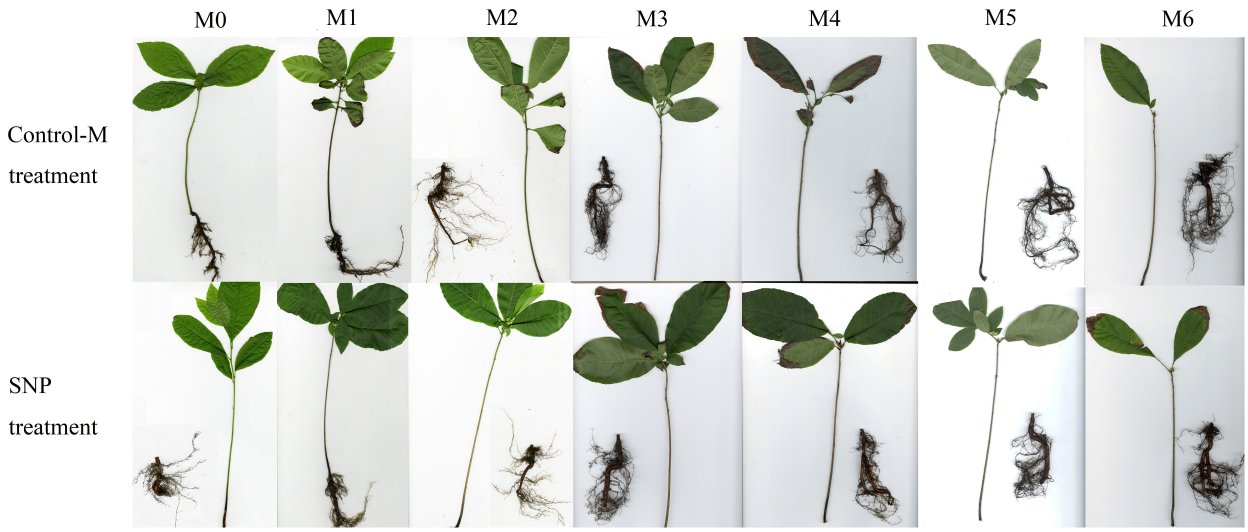

FIGURE 2 | Morphology of inoculated *C. illinoinensis* seedlings and their root system with or without exogenous NO (100 μmol/L SNP). Control-M treatment, inoculated seedlings that did not receive the 100 μmol/L SNP treatment; SNP treatment, inoculated seedlings treated with 100 μmol/L SNP. M0, M1, M2, M3, M4, M5, and M6 represent seedlings harvested at month 0, 1, 2, 3, 4, 5, and 6, respectively, after *T. indicum* inoculation.

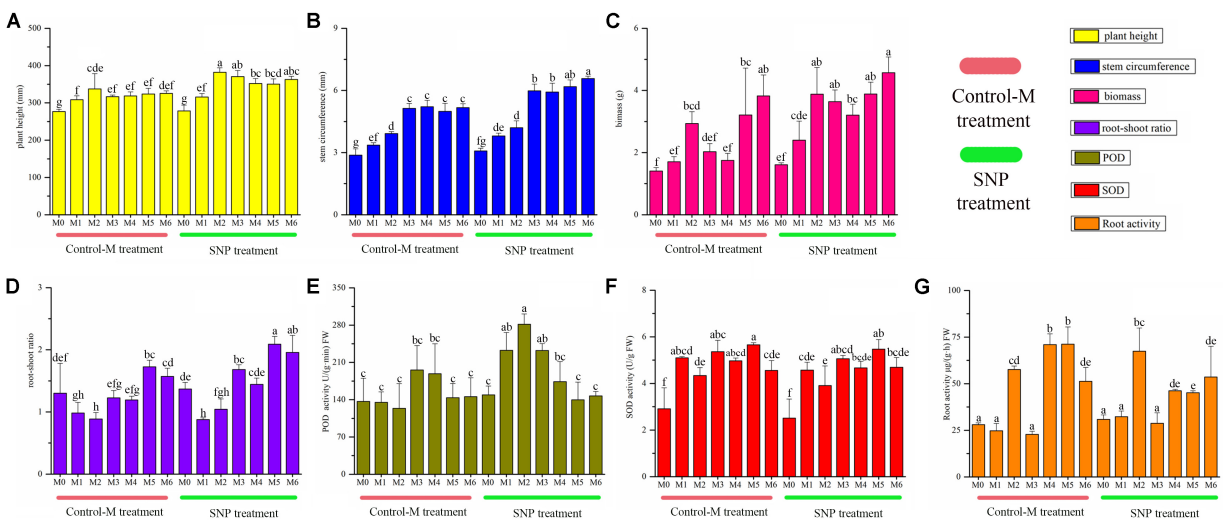

FIGURE 3 | Growth indicators of inoculated *C. illinoinensis* seedlings with or without exogenous NO (100 μmol/L SNP) at different growth months after inoculation. **(A)** plant height, **(B)** stem circumference, **(C)** biomass, **(D)** root-shoot ratio, **(E)** POD activity in roots, **(F)** SOD activity in roots, **(G)** root activity. Each value is the mean of three replicates (±SD) in a treatment. Different lowercase letters indicate significant differences between all of the treatments in different months after inoculation ($P < 0.05$ in the LSD test of ANOVA). Control-M treatment: inoculated seedlings that did not receive 100 μmol/L SNP; SNP treatment: inoculated seedlings treated with 100 μmol/L SNP. M0, M1, M2, M3, M4, M5, and M6 represent seedlings harvested at month 0, 1, 2, 3, 4, 5, and 6, respectively, after *T. indicum* inoculation.

(8.70%), *Polymorphum* (5.96%), *Bradyrhizobium* (3.52%), and *Sulfitobacter* (2.88%). Under low P stress, *Pseudomonas* abundance gradually decreased as the SNP concentrations increased from 0 to 100 μmol/L, then increased to the maximum when the SNP concentration was 1000 μmol/L. The abundance of *Pseudomonas* in the P_5S_3 group was significantly higher than in the CK ($P < 0.05$) (**Figure 5C**). *Sinorhizobium* and *Rhizobium* showed no significant differences among the five treatments. *Ensifer* was significantly more abundant in the CK ($P < 0.05$) (**Figure 5C**), and the change in its abundance was contrary to that of *Pseudomonas*. *Polymorphum* was also significantly more abundant in CK ($P < 0.05$), while *Sulfitobacter* was significantly more abundant in the P_5S_1 group ($P < 0.05$) than in the other groups (**Figure 5C**).

Structural Differentiation and Network Associations of *norB*-Type Denitrifying Bacterial Communities

The differences in the *norB*-type denitrifying bacterial community structure among the five treatments were visualized

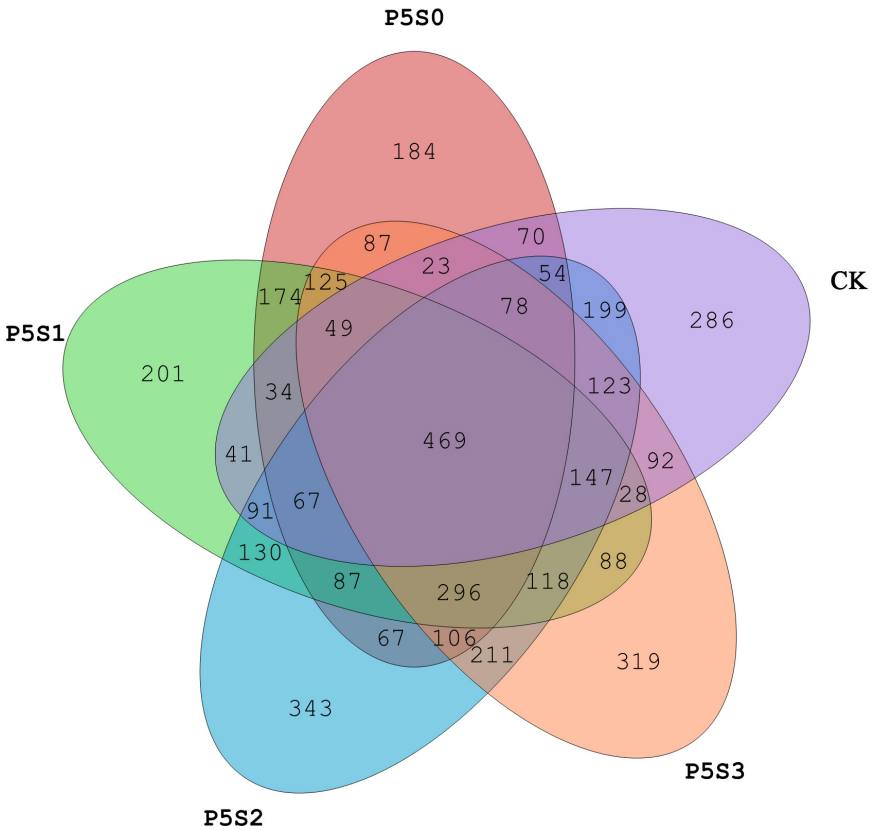

FIGURE 4 | Shared and unique *norB*-type denitrifying bacterial operational taxonomic units (OTUs) among rhizosphere soil samples in CK treatment and in different SNP treatments under low P stress. CK, the rhizosphere soil of uninoculated *C. illinoinensis* seedlings that received 0 μmol/L SNP and P application. P_5S_0, P_5S_1, P_5S_2, and P_5S_3 represent the rhizosphere soil of *C. illinoinensis* seedlings colonized by *T. indicum* treated with 0, 10, 100, and 1000 μmol/L SNP, respectively, under low P stress (5 μmol/L).

TABLE 2 | The richness and diversity indices of *norB*-type denitrifying bacteria in rhizosphere soil of *C. illinoinensis* seedlings with different concentrations of exogenous NO donor SNP treatment under low P stress.

Treatments	Simpson	Shannon	Chao1	ACE
CK	0.99 ± 0.00a	7.76 ± 0.21a	1106.46 ± 224.79a	1115.67 ± 232.67a
P_5S_0	0.90 ± 0.13a	6.42 ± 1.52a	1142.76 ± 94.78a	1141.64 ± 110.57a
P_5S_1	0.94 ± 0.07a	6.87 ± 1.19a	1330.28 ± 173.28ab	1334.32 ± 168.06ab
P_5S_2	0.98 ± 0.00a	8.06 ± 0.03a	1641.35 ± 55.19b	1666.54 ± 66.02b
P_5S_3	0.93 ± 0.09a	6.93 ± 1.14a	1325.11 ± 359.71ab	1366.19 ± 362.22ab

Each value is the mean of three replicates (± SD). Values followed by different lowercase letters indicate significant differences ($P < 0.05$) between samples in a row. NO, nitric oxide; SNP, exogenous NO donor sodium nitroprusside; P, phosphorus. CK, rhizosphere soil of uninoculated C. illinoinensis seedlings with 0 μmol/L SNP and P application. P_5S_0, P_5S_1, P_5S_2, and P_5S_3 represent the rhizosphere soil of C. illinoinensis seedlings colonized by T. indicum that were treated with 0, 10, 100, and 1000 μmol/L SNP, respectively, under low P stress (5 μmol/L).

by NMDS analysis (PERMANOVA, $P = 0.001$) (**Figure 5D**). The *norB*-type denitrifying bacterial community structures of the P_5S_0 and P_5S_1 treatment were similar and differed significantly from those of the other treatments. The community structure of the P_5S_2 treatment also differed from that of other treatments.

Among the top 50 genera of *norB*-type denitrifying bacterial communities, 36 showed correlations with others (**Supplementary Figure S4a**). *Pseudomonas* was negatively correlated with *Ensifer* and *Paracoccus*. *Sulfitobacter* was positively correlated with *Pseudogulbenkiania*, *Chromobacterium*, and *Anaeromyxobacter*, while it was negatively correlated with *Methylobacterium*.

Correlation Analysis Between *norB*-Type Denitrifying Bacterial Community and Colonization Rate of *T. indicum*

There were significant correlations between the colonization rate of *T. indicum* and the richness and diversity of *norB*-type denitrifying bacterial communities ($P < 0.05$) (**Supplementary Table S3**). Based on the Chao1 and ACE indices, colonization

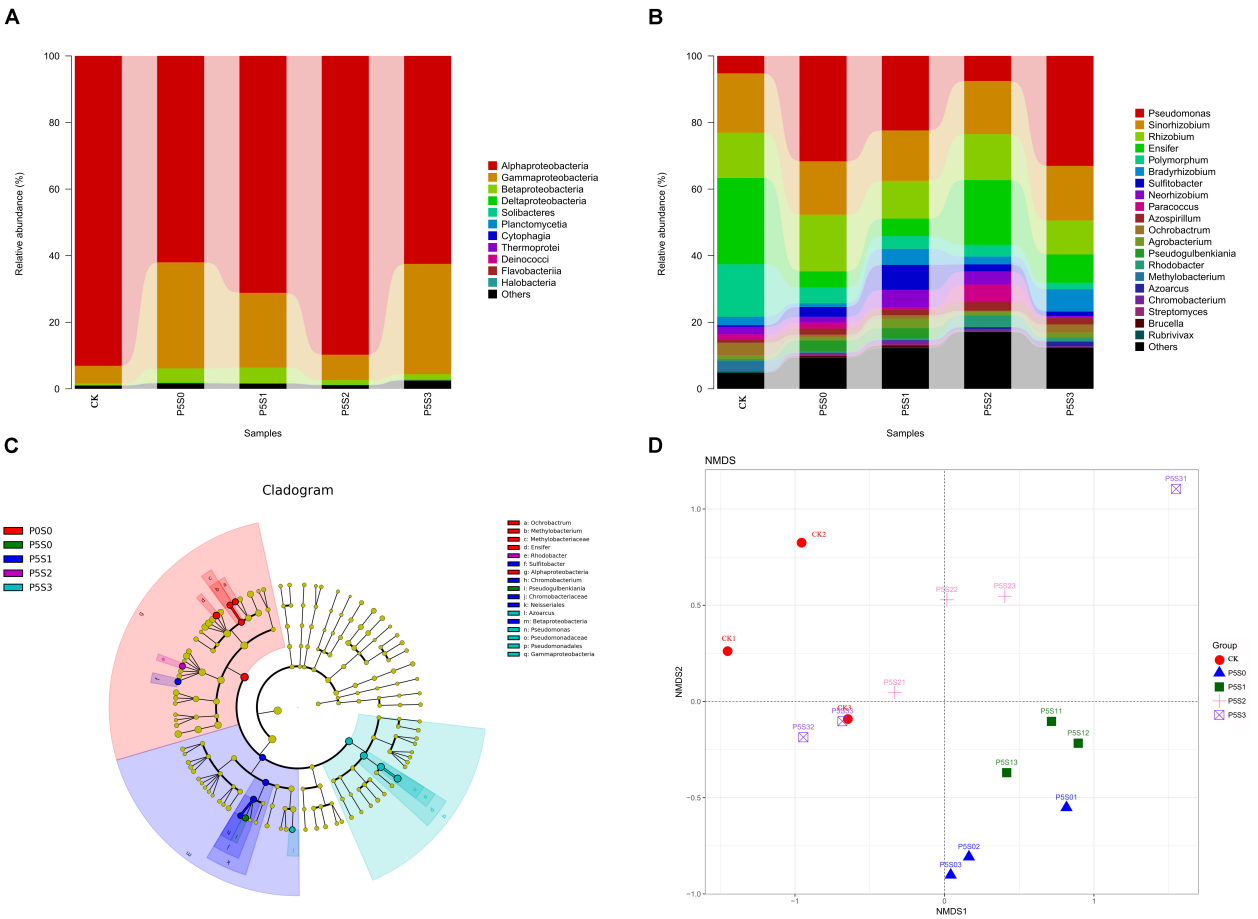

FIGURE 5 | Taxonomic composition of *norB*-type denitrifying bacterial communities at the **(A)** class and **(B)** genus levels in rhizosphere soil of *C. illinoinensis* seedlings in CK treatment and in different SNP treatments under low P stress. **(C)** Cladogram based on linear discriminant analysis effect size (LEfSe) analysis ($P < 0.05$, LDA score > 2) showing significant differences in abundance of *norB*-type denitrifying bacterial taxa in different groups; **(D)** non-metric multidimensional scaling (NMDS) analysis of *norB*-type denitrifying bacterial communities in rhizosphere soil of *C. illinoinensis* seedlings with different treatments. All treatments were had three replicates. CK, rhizosphere soil of uninoculated *C. illinoinensis* seedlings that received 0 μmol/L SNP and P application. P_5S_0, P_5S_1, P_5S_2, and P_5S_3 represent the rhizosphere soil of *C. illinoinensis* seedlings colonized by *T. indicum* and treated with 0, 10, 100, and 1000 μmol/L SNP, respectively, under low P stress (5 μmol/L).

rate was positively correlated with the richness of the *norB*-type denitrifying bacterial communities. Additionally, the Shannon and Simpson indices indicated that colonization rate was positively correlated with the diversity of *norB*-type denitrifying bacterial communities.

Analyses of Fungal Communities

Fungal Alpha Diversity in Rhizosphere Soil

Overall, 1,824,064 high-quality sequences were obtained from the 42 samples collected during different months after quality control procedures. These sequences were clustered into 1,452 OTUs in all, and the rarefaction curves of the fungal OTUs in different samples are shown in **Supplementary Figure S1b**. The Venn diagram displays the degree of overlap of the fungal OTUs between the samples in the two treatments (**Figure 6**). The number of the unique OTUs in the SNP treatment was 166, which was twofold lower than that in the Control-M treatment.

Based on the Chao1 and ACE indices, fungal community richness of the rhizosphere soil did not differ significantly between the SNP treatments and Control-M treatments during each month (**Table 3**). Additionally, the Simpson index indicated that fungal diversity did not differ significantly between the two different treatments during each month. The Shannon index indicated that the fungal diversity was lowest in the fourth month in the two different treatments, but was significantly higher in the Control-M treatment in the third month compared with the SNP treatment ($P < 0.05$). In general, the SNP treatment did not have any noticeable effect on the diversity and richness of fungal communities in rhizosphere soil at different growth times.

Taxonomic Composition of Fungal Communities

Among the 42 samples of SNP and Control-M treatments, a total of 9 phyla, 30 classes, 84 orders, 177 families, and 291 genera were detected. At the phylum level, *Ascomycota* (80.26%) was the dominant fungal phylum, followed by *Basidiomycota* (10.41%)

TABLE 3 | The richness and diversity indices of fungal communities in rhizosphere soil of inoculated *C. illinoinensis* seedlings with or without exogenous NO (100 μmol/L SNP) application during different growth months.

Treatments		Simpson	Shannon	Chao1	ACE
M0	Control-M	0.91 ± 0.05a	4.95 ± 0.39ab	266.20 ± 26.72bc	264.06 ± 24.70a
	SNP	0.92 ± 0.02a	5.11 ± 0.63ab	301.52 ± 80.34abc	284.47 ± 53.80abc
M1	Control-M	0.90 ± 0.06ab	4.69 ± 0.26abc	262.35 ± 11.56bc	262.71 ± 10.93a
	SNP	0.87 ± 0.11ab	4.78 ± 0.81abc	299.83 ± 50.00abc	302.59 ± 52.85abc
M2	Control-M	0.93 ± 0.02a	5.10 ± 0.48ab	382.64 ± 28.95a	388.59 ± 26.33c
	SNP	0.84 ± 0.11ab	4.20 ± 0.91abcd	303.92 ± 45.85abc	310.10 ± 50.84abc
M3	Control-M	0.90 ± 0.07a	5.18 ± 1.56a	321.62 ± 152.39abc	320.60 ± 142.49abc
	SNP	0.85 ± 0.03ab	3.79 ± 0.48bcde	263.76 ± 78.18bc	267.17 ± 81.44a
M4	Control-M	0.64 ± 0.28c	3.07 ± 1.44de	254.77 ± 70.15bc	262.49 ± 70.52a
	SNP	0.64 ± 0.06c	2.76 ± 0.44e	244.25 ± 105.38c	243.88 ± 96.14a
M5	Control-M	0.85 ± 0.01ab	4.05 ± 0.15abcde	287.90 ± 43.41abc	288.71 ± 44.29abc
	SNP	0.88 ± 0.04ab	4.58 ± 0.33abc	369.21 ± 19.64ab	379.63 ± 24.03bc
M6	Control-M	0.78 ± 0.15abc	3.85 ± 1.00abcde	262.81 ± 43.27bc	271.03 ± 49.00ab
	SNP	0.72 ± 0.13bc	3.56 ± 0.75cde	307.52 ± 66.21abc	318.07 ± 67.84abc

Each value is the mean of three replicates (± SD). Values followed by different lowercase letters indicate significant differences ($P < 0.05$) between samples in a row. NO, nitric oxide; SNP, exogenous NO donor sodium nitroprusside. Control-M treatment, the rhizosphere soil of inoculated C. illinoinensis seedlings that had no SNP application; SNP treatment, the rhizosphere soil of inoculated C. illinoinensis seedlings treated with 100 μmol/L SNP. M0, M1, M2, M3, M4, M5, and M6 represent rhizosphere soil harvested on month 0, 1, 2, 3, 4, 5, and 6, respectively, after T. indicum inoculation.

and *Zygomycota* (4.08%) (**Figure 7A**). The relative abundance of these three phyla showed no significant differences between the SNP treatments and Control-M treatments in each month.

At the class level, *Pezizomycetes* (41.06%), *Sordariomycetes* (18.32%), *Agaricomycetes* (10.53%), and *Saccharomycetes* (8.58%) were the dominant taxa (**Supplementary Figure S3**). The relative abundance of *Pezizomycetes* did not differ between the SNP treatments and Control-M treatments during each month. However, the SNP treatment significantly decreased the abundance of *Sordariomycetes* on the first month and decreased that of *Saccharomycetes* on the second month when compared with the Control-M treatment ($P < 0.05$).

At the genus level, the top 10 of the most abundant genera were *Tuber* (32.68%), *Rhizoctonia* (5.64%), *Phaeoacremonium* (4.93%), *Pichia* (3.81%), *Basidiobolus* (2.53%), *Fusarium* (2.27%), *Acremonium* (1.71%), *Buellia* (1.44%), *Bullera* (1.30%), and *Monographella* (0.98%) (**Figure 7B**). *Tuber* abundance was higher in the SNP treatment, but this difference was not significant. The relative abundance of *Tuber*, *Rhizoctonia*, *Phaeoacremonium,* and *Basidiobolus* did not differ significantly between the two treatments during each month. However, *Pichia* abundance was significantly lower in the SNP group than in the Control-M on the second month ($P < 0.05$). The abundance of *Fusarium* was also significantly lower in the SNP treatment on the first and second month ($P < 0.05$). In the SNP groups, *Tuber* showed significantly greater abundance from the second to the sixth month compared with month 0 and 1 ($P < 0.05$). However, in Control-M treatment, *Tuber* abundance increased from the fourth month, and was significantly more abundant compared with months 0, 1, 2, and 3 ($P < 0.05$).

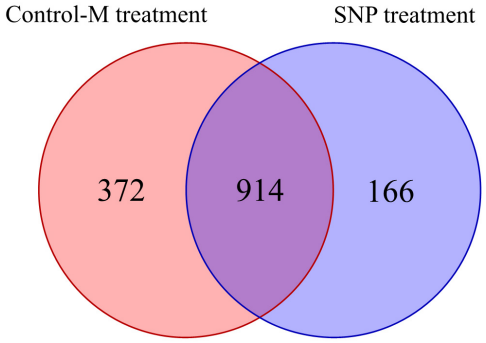

FIGURE 6 | Shared and unique fungal operational taxonomic units among rhizosphere soil samples of inoculated *C. illinoinensis* seedlings with or without exogenous NO (100 μmol/L SNP) application. Control-M treatment, inoculated seedlings that did not receive 100 μmol/L SNP; SNP treatment, inoculated seedlings that received 100 μmol/L SNP.

Differentially Abundant Taxa and Network Associations of Fungal Communities

Linear discriminant analysis effect size (LEfSe) analysis was used to reveal the fungal taxa that showed significantly different abundance between the SNP and Control-M treatments ($P < 0.05$) (**Figure 7C**). At the phylum level, there were no differentially abundant phyla between treatments. At the class level, the samples of the SNP treatments contained significantly more *Orbiliomycetes* and *Lecanoromycetes*. At the family level, the relative abundances of *Physciaceae, Orbiliaceae, Boletaceae, Lophiostomataceae, Inocybaceae,* and *Cortinariaceae* were significantly higher in the SNP treatments, while the abundances of *Dothioraceae* and *Kickxellaceae* were significantly higher in the Control-M treatments. At the genus level, among the top 50 genera, *Buellia*, *Podospora*, *Phaeoisaria*, *Ascotaiwania,* and *Lophiostoma* were significantly more abundant in the SNP treatments while *Acremonium, Monographella,* and *Penicillium* were significantly more abundant in the Control-M treatments.

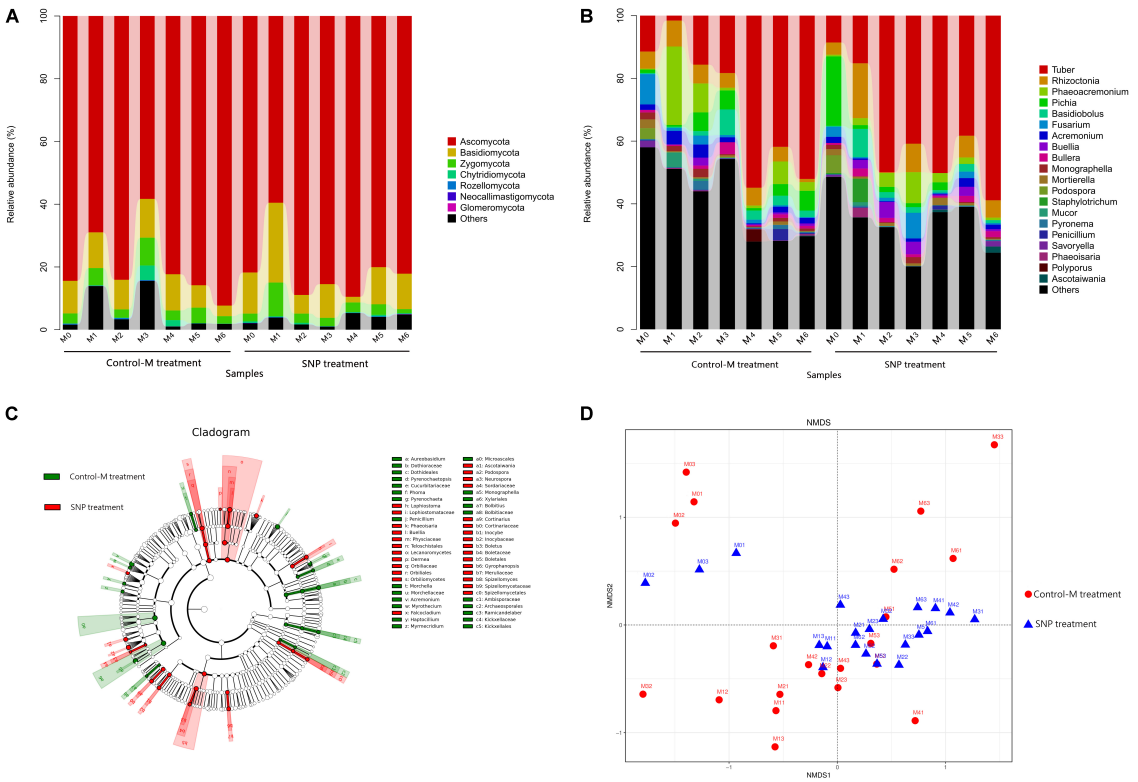

FIGURE 7 | Taxonomic composition of fungal communities at the **(A)** phylum and **(B)** genus levels in rhizosphere soil of inoculated *C. illinoinensis* seedlings with or without 100 μmol/L SNP during different growth months. **(C)** Cladogram based on linear discriminant analysis effect size (LEfSe) analysis ($P < 0.05$, LDA score > 2) showing the significantly differentially abundant fungal taxa in the rhizosphere soil of inoculated *C. illinoinensis* seedlings with or without SNP application. **(D)** Non-metric multidimensional scaling analysis of fungal communities in rhizosphere soil of inoculated *C. illinoinensis* seedlings with or without SNP application during different growth months. All of the treatments were conducted with three replicates. Control-M treatment, inoculated seedlings that did not receive 100 μmol/L SNP application; SNP treatment, inoculated seedlings that received 100 μmol/L SNP. M0, M1, M2, M3, M4, M5, and M6 represent seedlings harvested at month 0, 1, 2, 3, 4, 5, and 6, respectively, after *T. indicum* inoculation.

Among the top 50 genera, 39 showed correlations with others (**Supplementary Figure S4b**). *Tuber* was negatively correlated with *Archaeorhizomyces*, *Podospora* and *Penicillium*, but positively correlated with *Tricholoma*.

Structural Differentiation of Fungal Communities

Differences in fungal community structure among samples were visualized by NMDS analysis (PERMANOVA, $P = 0.008$) (**Figure 7D**). In SNP treatments, the fungal community structure of samples at month 0 differed obviously from those of other months. Analogously, in Control-M treatments, the fungal community structure of the samples at month 0, from month 0 to month 5, and at month 6 differed from each other. Comparison of the SNP treatments and Control-M treatments revealed that the fungal community structures of the two treatments differed in the same month, indicating that exogenous NO can shape the fungal community structure to a certain degree.

DISCUSSION

As ectomycorrhizal fungi, the successful and efficient synthesis of ectomycorrhizae is the basis for the artificial cultivation of truffles (Li et al., 2017). The ability of truffles to colonize plant roots and successfully form ectomycorrhizae can be affected by various abiotic and biotic factors such as soil properties, soil fertility, soil microorganisms, and vegetation (Slankis, 1974). Thus, the surrounding environment and management measures are important to the symbiosis of truffles and host plants. In this study, different concentrations of exogenous NO donor SNP and P were provided to the *C. illinoinensis* seedlings. The shifts in the colonization levels of *T. indicum*, in the growth of host plants, and in the associated microbes of rhizosphere soil were then investigated during the early symbiotic stage.

The colonization rate of truffles reflects the degree of mycorrhization (García-Montero et al., 2008). In our study, different concentrations of exogenous NO and P had significant effects on the colonization rate of *T. indicum* with *C. illinoinensis* seedlings. The colonization rate reached a high level (81 ± 3%) when SNP was 100 μmol/L under low P stress (5 μmol/L). Previous research showed that plants could be less dependent on ectomycorrhizae for P absorption when more soil P is available, and that ectomycorrhizal colonization may be greater under P-limited conditions (Kluber et al., 2012). When compared with the high P treatments (2000 μmol/L) in the present study, the colonization levels of *T. indicum* significantly increased under

low P stress. However, excessive P deficiency (no P treatments) did not contribute to colonization of *T. indicum*. The trend in colonization levels was $P_5 > P_0 > P_{2000}$. These findings indicated that the plants could adjust their root architecture in response to low P conditions (Niu et al., 2013). The growth of primary roots was inhibited and the development of lateral roots, cluster roots and root hair was promoted to improve the P uptake. This adjustment seemed to provide more attached sites for ectomycorrhizal fungi, which was beneficial to the colonization of truffles. NO in plants was demonstrated to participate in the response to low P conditions (Niu et al., 2013; Simontacchi et al., 2015). P deficiency enhanced NO accumulation in primary and lateral roots. Previous studies confirmed that the appropriate concentration of SNP could promote cluster roots proliferation and lateral root development (Lira-Ruan et al., 2013; Corpas and Barroso, 2015; Sun et al., 2015). In the present study, 100 μmol/L SNP was found to be optimal for *T. indicum* colonization under different P concentrations, and high concentrations of SNP could completely inhibit *T. indicum* colonization. Treatment of *C. illinoinensis* seedlings with 100 μmol/L SNP alone also significantly increased the colonization rate of *T. indicum* (88 ± 2%). In previous studies, the colonization rate of truffles was between approximately 40 and 60%, depending on the truffle species species and host plant (García-Montero et al., 2008; Geng et al., 2009; Benucci et al., 2012; Li et al., 2018). Therefore, this increase in colonization rate caused by 100 μmol/L SNP could be applied to the ectomycorrhizal synthesis of truffles and material exchange between mycorrhizal fungi and host plants, which may be useful in the artificial cultivation of truffles.

In addition to the colonization rate, the quality of the host plant also contributes to the success or failure of truffle crops (Andres-Alpuente et al., 2014). If plant growth was improved while the ectomycorrhizal level was not affected, the truffle yields may be better or earlier (Bonet et al., 2006). *T. indicum* inoculation significantly increased the plant height and root POD activity of *C. illinoinensis* seedlings in this study, but had negative effect on the root SOD activity. Previous research showed that the growth of *Pinus halepensis* seedlings could be improved by *T. melanosporum* inoculation and that the nutrient uptake of the seedlings was also improved (Dominguez et al., 2012). *T. indicum* colonization on several Chinese indigenous trees could also lead to better growth of the host, showing higher ground diameter increases, plant height, and biomass compared with the uninoculated seedlings (Hu, 2004), which was similar with our results. However, further analysis is needed to explain the decrease in root SOD activity. Under low P stress, the maximum root and POD activity was obtained when the SNP concentration was 100 μmol/L, which was consistent with the colonization rate. Evaluation of various abiotic stresses revealed that SNP with appropriate concentration could enhance the activity of the antioxidant system in plants, such as SOD and POD (Yang et al., 2012; Arora and Bhatla, 2015). The increase in POD activity indicated that 100 μmol/L SNP improved the ability of the host plants to cope with stress. However, under low P stress, SOD activity was highest at 10 μmol/L SNP, and improvement of SOD activity in response to exogenous NO was not as great as the improvement of POD activity. Many studies have shown that exogenous NO application promoted plant growth under various stresses; however, the effects of exogenous NO on plants that formed symbiotic relationships with ectomycorrhizal fungi have rarely been reported (Dong et al., 2014; Liu et al., 2014; Kaya and Ashraf, 2015). In the present study, application of only 100 μmol/L SNP to inoculated *C. illinoinensis* seedlings induced positive effects on plant height, stem circumference, biomass, root-shoot ratio, and POD activity of seedlings, but the variations in these indicators were not synchronous during the 7 months after inoculation. Therefore, treatment with 100 μmol/L SNP could improve the growth of host plants colonized by truffles to a certain degree; however, the effect of SNP on the artificial cultivation of truffles and fructification requires further verification in the field.

Rhizosphere soil microbes play important roles in ecological environments associated with truffles, contributing to ectomycorrhizae synthesis and truffle production, as well as the formation of truffle aroma (Splivallo et al., 2015; Vahdatzadeh et al., 2015). Moreover, microbes in rhizosphere soil participate in plant growth as well as the plant tolerance to disease and abiotic stress (Choudhary, 2012). Using high-throughput sequencing, the effects of exogenous NO at different concentrations under low P stress on bacteria harboring *norB*-type genes in rhizosphere soil were analyzed in this study. Some studies have reported that NO was frequently involved in the early basal signaling of interactions between plant roots and bacteria, which greatly influenced the root growth patterns and the accumulation of major nutrients (Simontacchi et al., 2015; Vaishnav et al., 2018). NO was also found to promote the formation of biofilms in bacteria (Vaishnav et al., 2016). Under low P stress, the exogenous NO did not influence the diversity of *norB*-type denitrifying bacteria in the present study, but did increase their richness when 100 μmol/L SNP was applied. Interestingly, the diversity and richness of *norB*-type denitrifying bacteria were significantly correlated with the colonization rate of *T. indicum*. This indicated that an interactive network may exist among the NO, *norB*-type denitrifying bacteria community and the colonization of truffles. Many studies have investigated the role of NO in symbiotic interactions, and exogenous NO has been reported to promote the establishment of the PGPR, i.e., *Pseudomonas simiae*, strain, which contributed to better colonization and plant growth under saline conditions (Vaishnav et al., 2016). However, the role of NO in symbiotic interactions of ectomycorrhizal fungi is not clear. In the present study, exogenous NO affected some dominant populations of *norB*-type denitrifying bacteria under low P stress. *Alphaproteobacteria* was more abundant while *Gammaproteobacteria* was less abundant when SNP was applied at 100 μmol/L. *Alphaproteobacteria* and *Gammaproteobacteria* comprised the predominant components of the bacterial communities of truffles (Barbieri et al., 2007; Li et al., 2017). The abundance of the *Pseudomonas* genus was $P_5S_3 > P_5S_0 > P_5S_1 > P_5S_2$, which was contrary to the colonization rate. However, *Pseudomonas* was reported to play a role in ectomycorrhizal symbiosis and *P. fluorescens* is believed to be important to growth and truffle mycorrhizal synthesis (Dominguez et al., 2012; Li et al., 2017). However, further study is needed to explain these phenomena and the

interactions of exogenous NO and the denitrifying bacteria associated with truffles.

To date, the effects of exogenous NO on fungal communities in rhizosphere soil have rarely been reported to date. In this study, the effects of only exogenous NO (100 μmol/L SNP) on soil rhizosphere fungi associated with truffles were investigated. Not surprisingly, *Tuber* was the dominate genus, accounting for 32.36%. These results indicated that exogenous NO did not significantly influence the abundance of *T. indicum* mycelia during the first 7 months after inoculation. However, the significant increase in *Tuber* abundance occurred earlier in exogenous NO treatments, which seems to be beneficial to truffle colonization. Previous studies showed that truffle inoculation reduced fungal richness and diversity in the roots and surrounding soil (Li et al., 2017, 2018). No significant effects of exogenous NO on the fungal richness and diversity in rhizosphere soil were observed in this study. NO has been shown to protect roots against further aggression from phytopathogens (Compant et al., 2010). In addition, *Buellia*, *Podospora, Phaeoisaria, Ascotaiwania,* and *Lophiostoma* were found to be more abundant because of exogenous NO application, while the abundance of *Acremonium, Monographella,* and *Penicillium* decreased. Network analysis provides an understanding of the potential interactions in microbial communities, and may identify keystone populations (Gu et al., 2018). During the early symbiotic stage, *Tuber* was positively correlated with *Tricholoma*, but negatively correlated with *Archaeorhizomyces*, *Podospora,* and *Penicillium* when 100 μmol/L SNP was provided. These fungal communities may be closely related to the growth of truffles under NO application.

CONCLUSION

Both exogenous NO and P stress affected the ectomycorrhizal synthesis of *T. indicum* and the growth of host seedlings, with the shift of colonization rate, plant physiology, and some microbial communities in the rhizosphere, which could have potential application in the artificial cultivation of truffles in the future. Also, the mechanism of how exogenous NO and P stress affect the symbionts of truffles and the host also needs to be further explored.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA544895/SRP199549.

AUTHOR CONTRIBUTIONS

XL, XZ (first author), and XZ (last author) conceived and designed the experiments. CW, ZK, and LY performed the experiments. XZ (first author) and XL wrote and revised the manuscript. All of the authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02634/full#supplementary-material

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Arbuscular Mycorrhiza Changes the Impact of Potato Virus Y on Growth and Stress Tolerance of *Solanum tuberosum* L. *in vitro*

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Under the field conditions crop plants interact with diverse microorganisms. These include beneficial (symbiotic) and phytopathogenic microorganisms, which jointly affect growth and productivity of the plants. In last decades, production of potato (*Solanum tuberosum* L.) suffers from increased incidence of potato virus Y (PVY), which is one of most important potato pests. Arbuscular mycorrhizal fungi (AMF) are common symbionts of potato, however the impact of mycorrhizal symbiosis on the progression of PVY-induced disease is scarcely known. Therefore, in the present study we investigated the effect of joint PVY infection and mycorrhizal colonization by *Rhizophagus irregularis* on growth traits of the host potato plant (cv. Pirol). The tested PVY isolate belonged to N-Wilga strain group, which is considered to be predominant in Europe and many other parts of the world. The viral particles were concentrated in the leaves, but decreased the root growth. Furthermore, the infection with PVY evoked prolonged oxidative stress reflected by increased level of endogenous H_2O_2. AMF alleviated oxidative stress in PVY-infected host plants by a substantial decrease in the level of shoot- and root-derived H_2O_2, but still caused asymptomatic growth depression. It was assumed that mycorrhizal symbiosis of potato might mask infection by PVY in field observations.

Keywords: *Solanum tuberosum* L., potato virus Y (PVY), *Rhizophagus irregularis*, arbuscular mycorrhiza, oxidative stress

INTRODUCTION

Plant growth and physiology are affected by both symbionts and phytopathogens co-infecting the same host. These tripartite biotic interactions (involving antagonistic, protective, exclusive, or synergistic effects) are of particular interest with regard to crop plants, since they strongly influence the crop productivity. It is documented that under the field conditions the majority of crop plants establishes symbiotic association between their roots and arbuscular mycorrhizal fungi (AMF) being an inherent component of each agricultural ecosystem (Smith and Smith, 2011; Van Geel et al., 2016). In this endomycorrhizal relationship both partners benefit from one another. AMF

hyphae act as root system extension and explore the soil outside the rhizosphere. Host plant, due to high absorptive capacity of extraradical mycelium, gains an easier acquisition of soil water and slowly diffusing mineral compounds (in particular phosphorus and nitrogen ions), which results in the improved plant nutritional status and fitness (Bitterlich and Franken, 2016). In exchange, plants furnish a habitat (as physical and favorable physiological support) that allows AMF to uptake energy (i.e., carbohydrates and lipids) in order to complete their life cycle (Mercy et al., 2017; Rich et al., 2017; Wang et al., 2017). Furthermore, mycorrhizal plants often display enhanced tolerance to abiotic stress factors (e.g., drought or salinity) and increased resistance to both phytopathogen attack and development of phytopathogen-induced disease (Bücking et al., 2016; Deja-Sikora et al., 2019). These nutritional and non-nutritional (bioprotective) benefits of endomycorrhiza contribute to the improved crop yields and encourage the wide application of AMF-based natural biofertilizers to support the sustainable agriculture systems (Hart et al., 2015; Rouphael et al., 2015; Basu et al., 2018; Bitterlich et al., 2018). Recently, AMF are even perceived a key factor for optimization of crop productivity, especially in the low-input agriculture (Verbruggen et al., 2013).

Potato (*Solanum tuberosum* L.) belongs to the most meaningful horticultural species grown worldwide for food and industrial purposes. Numerous experiments conducted under greenhouse, shade house and field conditions showed that potato roots are prone to establish endomycorrhiza with several AMF species, including *Rhizophagus intraradices* (formerly *Glomus intraradices*), *Rhizophagus irregularis* (formerly *Glomus irregulare*), *Funneliformis mosseae* (formerly *Glomus mosseae*), or *Gigaspora* sp. (Douds et al., 2007; Gallou et al., 2011; Lone et al., 2015; Hijri, 2016). Mycorrhizal potato plants were reported to display improved growth, pathogen resistance, and productivity compared to non-mycorrhizal ones (Douds et al., 2007; Bharadwaj et al., 2008). The results of large scale field trials indicated that inoculation of potato with *R. irregularis* DAOM 197198 caused significant increase in tuber yield, and the effect was cultivar independent (Hijri, 2016). The positive effect of *R. intraradices* and *F. mosseae* on the host morphological parameters was found for two potato cultivars (Jyoti and TPS) (Lone et al., 2015). Root colonization with AMF improved fresh and dry matter of both plant shoot and root, increased the chlorophyll content and tuber yield. This observation was in line with the other study that demonstrated the enhancement of potato (cv. Yungay) growth parameters upon mycorrhization with *R. intraradices* due to greater uptake of P, Fe, and Mg as well as higher efficiency of P utilization (Davies et al., 2005). Furthermore, colonization with AMF was linked to the lower incidence of infection with some potato pathogens or reduced disease severity. *G. etunicatum* and *R. intraradices* were associated with milder symptoms of *Rhizoctonia solani*-induced disease in potato (cv. Goldrush) (Yao et al., 2002). AMF were indicated to have bio-protective function against leaf pathogen *Phytophthora infestans*, as mycorrhizal potato plants (cv. Bintje) showed decreased progress of disease resulting from activation of plant systemic resistance to pathogen attack (Gallou et al., 2011). Nevertheless, the results of investigations on the bio-control of potato viruses by endomycorrhiza are less conclusive.

Potato virus Y (PVY) is an extremely devastating pathogen of *S. tuberosum* that dramatically reduces tuber yield and quality causing huge economical losses worldwide (Funke et al., 2016). PVY causes foliar and/or tuber disease with variable symptoms depending on virus strain, host growth stage and susceptibility, and environmental conditions (Fox et al., 2017). Currently, PVY^{N} and recombinant PVY^{NTN} and $PVY^{N\text{-}Wi}$ strains largely predominate under field conditions, accounting for > 90% of all PVY cases (Davie et al., 2017). PVY is transmitted non-persistently by aphids (e.g., *Myzus persicae*) being so far the only identified vectors for this pathogen. However, the application of insecticides seems to be ineffective in PVY infection control. New, potentially successful strategies to manage the virus may involve the utilization of microbiological (biocontrol) agents comprising bacterial and fungal species (Al-Ani et al., 2013). These plant-associated microorganisms can alleviate the negative impact of virus, e.g., by modulating the level of plant stress response. The treatment of potato tubers with either *Pseudomonas fluorescens* or *Rhodotorula* sp. was found to reduce the severity of PVY-induced disease (Al-Ani et al., 2013). Unfortunately, the interaction between PVY and AMF (known for their bio-protective function against different potato pathogens) is poorly characterized. The worsening of growth parameters in *R. irregularis*-inoculated potato plants along with an increase in the symptoms of PVY-induced disease were previously showed in the pot experiment (Sipahioglu et al., 2009). However, no data for potato plants grown *in vitro* are available.

Plants induce H_2O_2 signaling in response to both pathogen attack and symbiosis establishment, e.g., during initial stage of endomycorrhiza development (Pozo and Azcon-Aguilar, 2007; Nath et al., 2016). The specific plant-AMF interaction resulting in H_2O_2 synthesis was previous indicated in several articles (Puppo et al., 2013; Nath et al., 2016; Kapoor and Singh, 2017), which can be related to the temporal and spatial control of plant root colonization (Salzer et al., 1999). It was indicated that H_2O_2 accumulated in root cortical cells, in the vicinity of arbuscules, as well as around the intraradical hyphal tips penetrating the host cells (Salzer et al., 1999). Furthermore, H_2O_2 was reported to act as long-distance signal molecule for activation of biotic stress adaptation mechanisms (Sewelam et al., 2016). Among reactive oxygen species (ROS), membrane-permeable H_2O_2 is thought to be key player involved in regulation of many biological reactions, e.g., stress response (Saxena et al., 2016).

The goal of this investigation was to examine the effect of (i) PVY infection, (ii) AMF inoculation, and (iii) PVY-AMF co-infection on both vegetative growth parameters and stress response in potato host plants grown *in vitro*. Since plant cells regulate oxidative metabolism in response to pathogen attack we analyzed the level of hydrogen peroxide (H_2O_2) in shoots and roots of PVY-infected mycorrhizal plants. We hypothesized that *R. irregularis* can improve the growth of PVY-infected potato plants by alleviating the negative impact of virus. By verification of this hypothesis we wanted to check the role of AMF in biocontrol of PVY.

MATERIALS AND METHODS

Biological Material

Potato virus Y-infected and virus-free plantlets of *S. tuberosum* cvs. Pirol, Delikat and Schubert were *in vitro* subcultured on the standard Murashige and Skoog (MS) medium without growth regulators (pH 5.8). Single-node cuttings were aseptically transferred into MS medium (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 30 g l^{-1} sucrose and solidified with 7 g l^{-1} agar (Sigma-Aldrich, St. Louis, MO, United States). The plants were cultured in growth chamber under the continuous white fluorescent light (45 μmol m^{-2} s^{-1}) at 26°C ± 1°C.

Virus-positive *in vitro* plantlets used in this study were infected with the same strain of PVY before the experiment was started. Inoculation with PVY was done with carborundum (silicon carbide) method using 2-week plantlets as recipients. Leaf of donor PVY-positive potato plant was homogenized in cold 10 mM potassium phosphate buffer pH 7.0 (ratio 1:10) using sterilized mortar and pestle. The homogenate was gently rubbed with swab into the recipient leaves, that were previously dusted with 600-mesh carborundum. The inoculated plants were cultured for 3 weeks at 26°C. The infection was checked with PVY-AgriStrip tests (Bioreba AG, Reinach, Switzerland). Virus-infected plantlets were *in vitro* subcultured for several weeks to confirm the stable infection.

Rhizophagus irregularis line QS81 (provided by INOQ, GmbH, Schnega, Germany) was cultured on dual-compartment plates using Ri T-DNA transformed carrot roots (*Daucus carota* L.) as a host for the fungus. Both AMF and roots were grown at 23–25°C on the modified Strullu and Romand (MSR) medium solidified with 3 g l^{-1} Gelrite (Duchefa Biochemie, Haarlem, Netherlands). MSR lacked sucrose and vitamins in the fungus compartment.

Experimental Design

The presented study consisted of two stages: the selection of potato cultivar having the lowest concentration of PVY in the plant roots (**Figure 1A**), and the examination of AMF effect on the growth and stress response of PVY-infected plants (**Figure 1B**).

PVY Strain Identification

Potato virus Y strain in virus infected potato plantlets was identified using multiplex reverse-transcriptase PCR (RT-PCR) assay as described by Lorenzen et al. (2006). The protocol was based on the usage of 12-primer set targeted at specific recombination junctions within PVY genomes to discriminate between different strains.

Total RNA from plant tissue was isolated using RNA Extracol Reagent (EURx, Gdańsk, Poland). DNase I-treated RNA samples were reverse transcribed into cDNA using smART Reverse Transcriptase Kit (EURx, Gdańsk, Poland) with random hexamers. Multiplex RT-PCR for PVY identification was performed according to Lorenzen's protocol. Amplicons were analyzed on 2% agarose gel and sequenced with Sanger method using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, United States). Sequencing reactions were analyzed with ABI3730 Genetic Analyzer (Oligo IBB PAS, Warsaw, Poland). Reads were quality checked with MEGA X software and sequences were deposited in GenBank under accession numbers MK455818 and MK455819. The phylogenetic tree for reference PVY strains (according to Glais et al., 2017) was generated with ML algorithm in MEGA X (Kumar et al., 2018).

TAS-ELISA for PVY Concentration

Potato virus Y concentration in virus infected potato cultivars was examined with TAS-ELISA using ELISA Reagent Set for Potato virus Y (Agdia, Inc., Elkhart, IN, United States). For each of three biological repeats, leaves, stems and roots were collected separately from three 4-week old plantlets (per cultivar) grown *in vitro* under conditions described above. 30–50 mg of fresh plant tissue was homogenized in general extract buffer (GEB) at a 1:10 ratio as recommended by the manufacturer. The assay was performed according to the manufacturer's protocol. Positive controls for potato virus Y (Agdia, Inc., Elkhart, IN, United States) were processed along with the analyzed samples to validate the measurements. Three virus-free plantlets of each cultivar were included as negative controls. The buffer wells were prepared to subtract the background absorbance. The sample was regarded PVY infected if its absorbance value was greater than three-times the average value of negative control. PVY concentration in the sample was calculated in relation to positive control.

Inoculation of Potato Plantlets With AMF

In total 15 single-node cuttings were transferred to glass tubes (ø 25 mm) with 10 ml of liquid (non-solidified) MSR medium without plant regulators. Tubes contained also 1.5 g of perlite in order to maintain the shoot in a vertical position. After 6–7 days of culturing (26 ± 1°C, 16/8 h L/D), when adventitious roots were observed (c.a. 10 mm), plantlets were inoculated with spores of *R. irregularis*. The pieces of solid MSR medium containing c.a. 50 spores were placed directly on the emerging roots. Then the lower parts of the tubes were covered with aluminum foil to prevent the light access. The plantlets were maintained for 8 weeks under conditions described above. Mycorrhiza development in roots was confirmed at the end of incubation period by standard Trypan blue staining (Phillips and Hayman, 1970) and microscopic analysis (**Figure 2**). The experiment was performed in duplicate (two biological repeats). Each replicate consisted of 10–12 plants (technical repeats), that were selected for the parameters examination.

Plant Growth Parameters and the Measurement of H_2O_2 Level

Eight-week-old potato plants were removed from the experimental medium and the following parameters were measured: shoot height, number of nodes, root length, and root and shoot fresh weight.

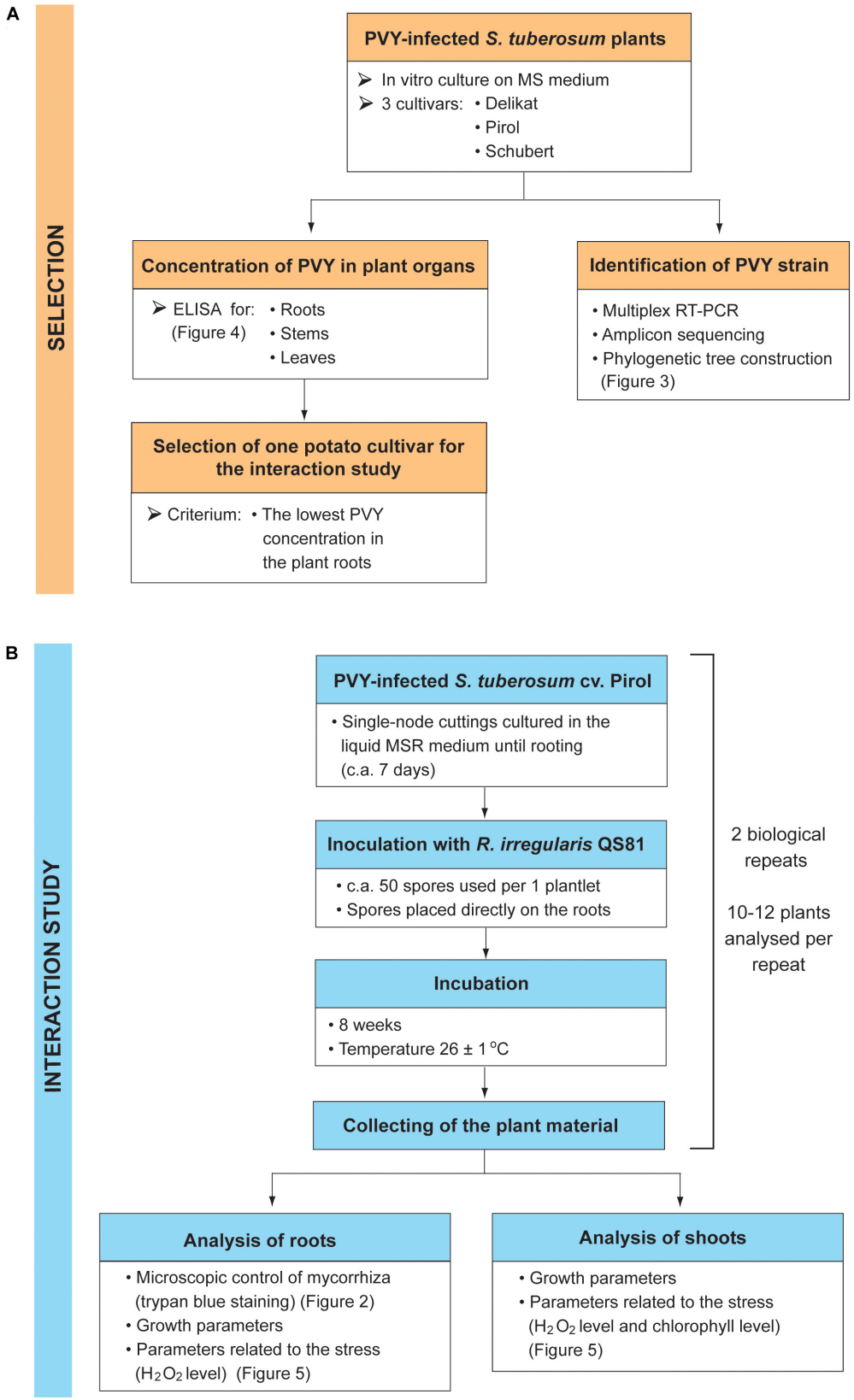

FIGURE 1 | Design of the experiment. The study consisted of two stages: the selection of potato cultivar based on the PVY concentration in the plant roots **(A)**, and the examination of AMF effect on the growth and stress response of PVY-infected plants **(B)**.

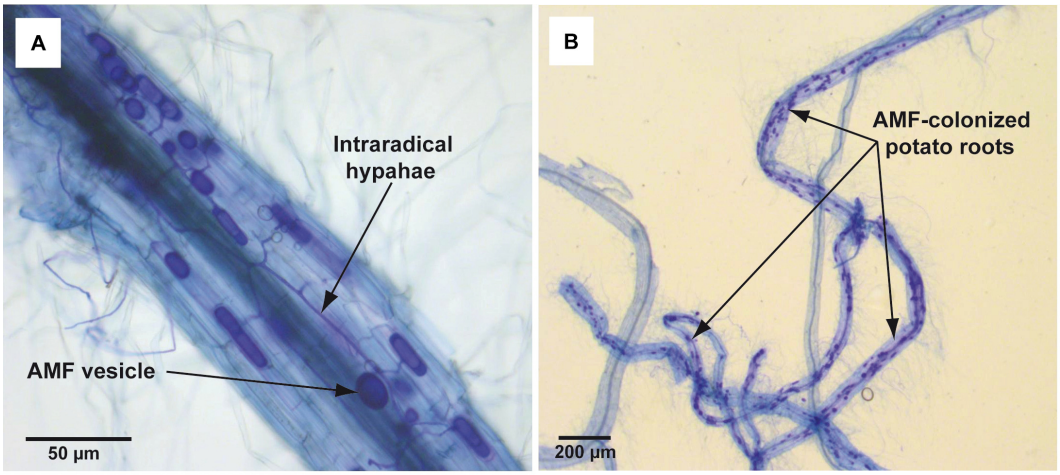

FIGURE 2 | Trypan blue-stained mycorrhizal structures in potato roots cv. Pirol colonized with *Rhizophagus irregularis*. The mycorrhization was performed in liquid *in vitro* system and the microscopic analysis was done in 8 week post-inoculation. Panel **(A)** shows dark blue-stained AMF structures (vesicles and intraradical hyphae) inside the potato root (100x magnification). Panel **(B)** shows dark blue-stained fragments of potato roots strongly colonized with AMF (12.5x magnification).

Total content of chlorophyll in fresh leaves (expressed in µg g^{-1} FW) was determined using the method by Lichtenthaler and Buschmann (2001). Photosynthetic pigments including chlorophyll were extracted from 20 mg of homogenized tissue by incubating in 10 ml of 95% (v/v) ethanol for 72 h under dark conditions at 4°C. The absorbance of the supernatant was measured at wavelengths of 664.2 nm ($A_{664.2}$) and 648.6 nm ($A_{648.6}$) using UV-VIS Spectrophotometer UV-1601PC (Shimadzu, Kyoto, Japan). All measurements were performed in triplicate. Total chlorophyll content was calculated from the following formula: total volume of chlorophyll = 5,24*($A_{664.2}$) + 22,24*($A_{648.6}$).

H_2O_2 level in plant tissue (root and shoot) was measured with colorimetric method using potassium iodide (Velikova et al., 2000). Briefly, 100 mg of lyophilized plant tissue powder was treated with 1 ml of 0.1% trichloroacetic acid (TCA) and incubated on ice for 20 min with agitation. The homogenate was centrifuged (10,000 × *g*; 10 min, 4°C) and 0.5 ml of supernatant was added to 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide. The mixture was incubated in darkness for 1 h at room temperature. The absorbance was measured at 390 nm. The samples were measured in triplicate against standard curve. The concentration of H_2O_2 was calculated from the equation: $C_{H2O2} = (C_{total} * V_{total})(V * w)$; C_{total} – nanomolar concentration of H_2O_2 determined from standard curve; V_{total} – total volume of supernatant (1 ml); V – volume of supernatant in the reaction mixture (0.5 ml); w – sample weighting.

Statistical Analyses

Observations lying beyond 75th percentile (outliers) were detected using Outliers package in R and removed from dataset. Normality of data distribution was checked with Shapiro–Wilk *W*-test. Levene's test was used to check the homogeneity of variance. The results of the experiment were analyzed using Student's *t*-test (for equal variances) or Welch's *t*-test (for unequal variances) to evaluate the differences in studied parameters between control (non-inoculated) and mycorrhizal potato plants. Two-way ANOVA was calculated to examine the effect of AMF-inoculation on the parameters of virus-free (healthy) and PVY-infected plants. Statistical analyses were performed in Statistica 7.0 (StatSoft, Inc., Tulsa, OK, United States).

RESULTS

PVY Identification and Distribution in Plant Tissues

Multiplex RT-PCR assay revealed that analyzed potato cultivars, i.e., Pirol, Delikat and Schubert, were infected with N:O/N-Wi type A recombinant variant of PVY. Sequencing of PVY genome fragments containing specific recombination junctions confirmed the result of PCR assay. Identified PVY strain, denoted as $PVY^{N:O}$-T1, was closely related to PVY^{N-Wi} (N-Wilga) and $PVY^{N:O}$ genotypes (above 99% of similarity). $PVY^{N:O}$-T1 was placed within $PVY^{N:O/N-Wi}$ group in the phylogenetic tree (**Figure 3**). Potato cultivars infected with $PVY^{N:O}$-T1 were asymptomatic, since no foliar disease was noticed.

Potato virus Y distribution in different organs of virus-positive potato plantlets grown *in vitro* was examined with TAS-ELISA method. The analysis indicated that all tested cultivars were systemically infected with PVY (**Figure 4**). Irrespectively of potato cultivar, virus preferentially accumulated in leaves. Decreasing concentration of PVY was detected in stems, however in Schubert and Pirol the lowest titer of virus was observed in roots. Due to the highest difference between leaf and root PVY concentration in Pirol (ratio 6.7 compared to 1.5 in Schubert and 1.4 in Delikat), the roots of this potato cultivar could be least impacted by the virus. Since physiological condition of the root is essential for successful establishment of mycorrhiza, *S. tuberosum* cv. Pirol was chosen for studying the interaction

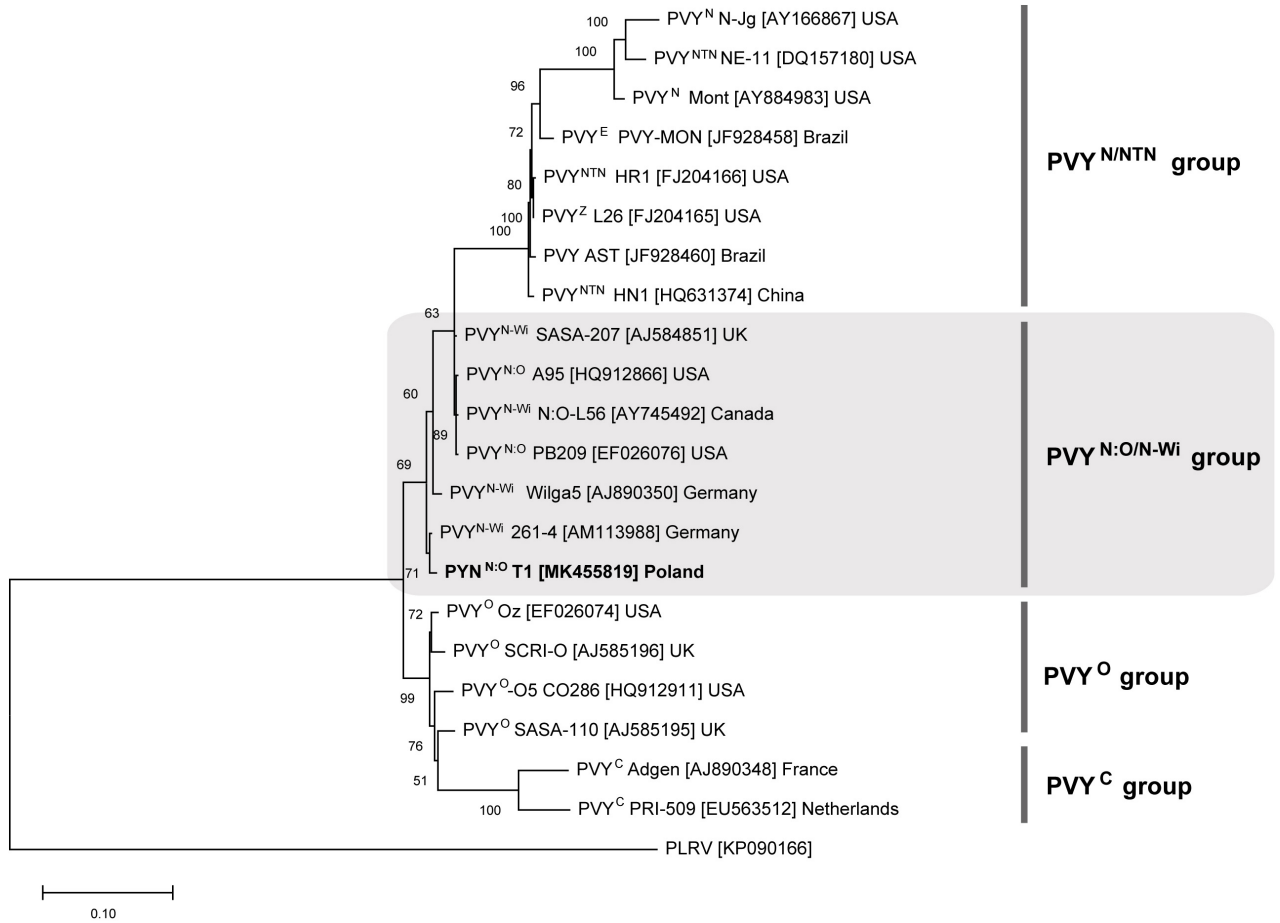

FIGURE 3 | Phylogenetic analysis of reference PVY isolates belonging to different strain groups. The tree was generated with ML method. PVY isolate identified in this study ($PVY^{N:O}$-T1) is most closely related to PVY^{N-Wi} (N-Wilga) strain group (shaded). Potato leafroll virus (PLRV) was used as an outgroup.

between PVY and *R. irregularis* in tripartite (host plant-PVY-AMF) biosystem.

The Effect of PVY on Plantlet Growth Parameters and H_2O_2 Level

Although undetectable by visual inspection, $PVY^{N:O}$-T1 isolate noticeably changed growth parameters and H_2O_2 level in potato cv. Pirol. The average length of shoot significantly decreased in PVY-positive plantlets compared to virus-free (control) ones (by above 25%; from 112.6 to 83.8 mm) (**Supplementary Table 1**). Virus-caused reduction in average length of root was even more dramatic reaching nearly 68% (from 87.4 to 27.6 mm). Furthermore, PVY infection significantly influenced the host biomass. The virus provoked a decrease in fresh weight of shoot (by 25%; from 172.8 to 129 mg) and root (by nearly 61%, from 47.8 to 18.7 mg), resulting in total plantlet biomass reduction by 33%. Moreover, PVY significantly affected H_2O_2 level in host tissues. H_2O_2 concentration was 1.9-fold higher in shoots (0.81 vs. 1.51 μmol g^{-1} FW) and 3.1-fold higher in roots (0.87 vs. 2.73 μmol g^{-1} FW) of virus-positive plantlets compared to the control ones (**Supplementary Table 2**). The virus exerted the influence neither on the number of nodes that invariably was 8 (data not shown) nor the content of chlorophyll.

The Effect of *R. irregularis* on Growth Parameters and H_2O_2 Level in Healthy and PVY-Infected Plantlets

Inoculation of potato cv. Pirol with *R. irregularis* seemed to have no effect on the length of shoot and root, as well as fresh weight of shoot, irrespectively of PVY infection (**Figure 5**). The roots colonized by *R. irregularis* were noticeably longer in both the virus-free (87.4 mm in control vs. 107.2 mm after inoculation) and the virus-positive plantlets (27.6 vs. 32.1 mm), however no statistical significance of this result was found (**Supplementary Table 1**). Furthermore, *R. irregularis* significantly increased the root biomass of healthy plantlets (by nearly 74%; 47.8 vs. 83 mg), but the fungus exerted no influence on the roots of PVY-infected ones. Similar result was observed for chlorophyll content that was considerably higher after inoculation, but only in leaves of virus-free hosts (407 vs. 564 μg g^{-1} FW). The presence of PVY masked the effect of mycorrhiza and the chlorophyll content was at the level of control plants (**Supplementary Table 2**). Colonization by

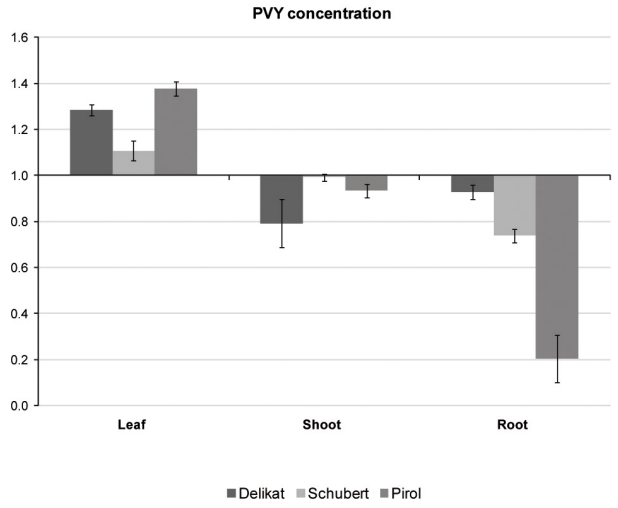

FIGURE 4 | Relative concentrations of PVY measured with TAS-ELISA in different organs (leaves, shoots, and roots) of three PVY-positive potato cultivars, i.e., Pirol, Delikat and Schubert. The values are expressed as relative to commercial positive control for PVY representing the axis of value 1.

R. irregularis exerted the strongest effect on the level of H_2O_2 in host tissues. In the absence of PVY infection the level of H_2O_2 in plant shoot remained unchanged upon mycorrhization, however the level of H_2O_2 in plant root was significantly raised (from 0.87 to 1.19 μmol g^{-1} FW). The result was different in the PVY-infected plants, because the interaction with *R. irregularis* induced dramatic decrease in H_2O_2 level in plantlet shoot (by 48%; from 1.51 to 0.78 μmol g^{-1} FW) and root (by 39.5%; from 2.73 to 1.65 μmol g^{-1} FW).

DISCUSSION

Virus-positive potato cultivars used in this study, i.e., Pirol, Delikat and Schubert, were systemically infected with N:O recombinant variants of PVY. Such mosaic genotypes phylogenetically originate from an ancestral genome consisting of fragments exchanged between PVY^{N} (necrotic) and PVY^{O} (ordinary) strain (Lorenzen et al., 2006; Karasev et al., 2011). Monitoring of PVY strain incidence conducted during last decades showed the common shift from PVY^{O} strains towards recombinant ones (Crosslin et al., 2006; Davie et al., 2017). This trend is observed worldwide and according to current estimation recombinant PVY^{N} strains may account for up to 90% of all PVY cases found under field conditions (Davie et al., 2017). Based on sequence similarity analysis our PVY isolate (denoted as $PVY^{N:O}$-T1) was identified to be closely related to $PVY^{N\text{-}Wi}$ (N-Wilga) strain group. Wide distribution of $PVY^{N\text{-}Wi}$ strains becomes more and more apparent nowadays (Visser et al., 2012; Quenouille et al., 2013; Green et al., 2017). The study by Yin et al. (2012) demonstrated that recombinant PVY belonging to the $PVY^{N\text{-}Wi}$ along with PVY^{NTN} are predominant variants of the virus infecting potato crops in Poland. Similar findings were previously reported for the other parts of the world, e.g., the United States, Canada and potato-producing regions in South Africa (Crosslin et al., 2006; Visser and Bellstedt, 2009; Gray et al., 2010). Although the members of $PVY^{N:O/N\text{-}Wi}$ share the properties of both parental strains, they tend to induce barely detectable disease that due to mild symptoms remains unnoticed during visual inspection (Gray et al., 2010; Funke et al., 2016; Glais et al., 2017). Furthermore, these strains can also remain latent (symptomless) in potato plants (Glais et al., 2005; Kamangar et al., 2014), which is in line with results of our studies. *S. tuberosum* L. plantlets cvs. Pirol, Delikat and Schubert infected with $PVY^{N:O}$-T1 were asymptomatic under *in vitro* conditions, however the virus was detectable in all examined organs, i.e., roots, stems, and leaves. Since viral infection caused neither foliar nor tuber disease it can be concluded that host-PVY interaction was compatible, and tested cultivars were susceptible but tolerant to these PVY isolates. Measured concentrations of $PVY^{N:O}$-T1 differed across the studied cultivars and plant organs. It is not surprising as PVY isolates, in spite of close phylogenetic relatedness, may behave in a contrasting way and accumulate to different level in the same host cultivar (Davie et al., 2017). The highest titer of PVY was found in the leaves of potato plantlets, irrespectively of analyzed cultivar. Our result is partially in opposition to the study by Kogovšek et al. (2011) showing different distribution of PVY^{NTN} strain within potato plants maintained in growth chamber. The authors reported high accumulation of the virus in symptomatic leaves and stems of sensitive potato cv. Igor, while virus amount in asymptomatic leaves was low or even undetectable. Nevertheless, similarly to Kogovšek et al. (2011) we also found the lowest concentration of PVY in roots of two tolerant potato cultivars (Pirol and Schubert). This partial discrepancies in results can be explained by variable distribution pattern of different PVY strains depending on individual virus characteristics, cultivar susceptibility and specific environmental conditions. Our observation can be also supported by results described by Mehle et al. (2004) indicating different kinetics of PVY multiplication and accumulation in organs of sensitive, tolerant and resistant potato cultivars.

Our study demonstrated that infection with $PVY^{N:O}$-T1, although symptomless, inhibited the vegetative growth of tolerant potato cv. Pirol, causing reduction in plantlet total biomass by 33%, which was explicitly visible by root and shoot length shortening. PVY infection was previously found to be associated with axillary growth retardation (measured as shoot length decrease) in potato plantlets cvs. Desirée, Igor and Pentland Squire maintained under *in vitro* culture conditions (Anžlovar et al., 1996). However, the effect was more pronounced in sensitive cultivars (Desirée and Igor) than tolerant one (Pentland Squire). Additionally, Anžlovar et al. (1996) showed that virus exerted no influence on the number of nodes. This observation is in agreement with the results of our study, since we found invariable number of nodes in control and PVY-positive plantlets. We demonstrated that the presence of PVY most negatively impacted the development of plantlet roots, causing dramatic decrease in their length (by c.a. 68%) and fresh weight (by c.a. 61%). Similarly, Dolenc and Dermastia (1999) indicated that PVY strongly reduced growth capacity of primary

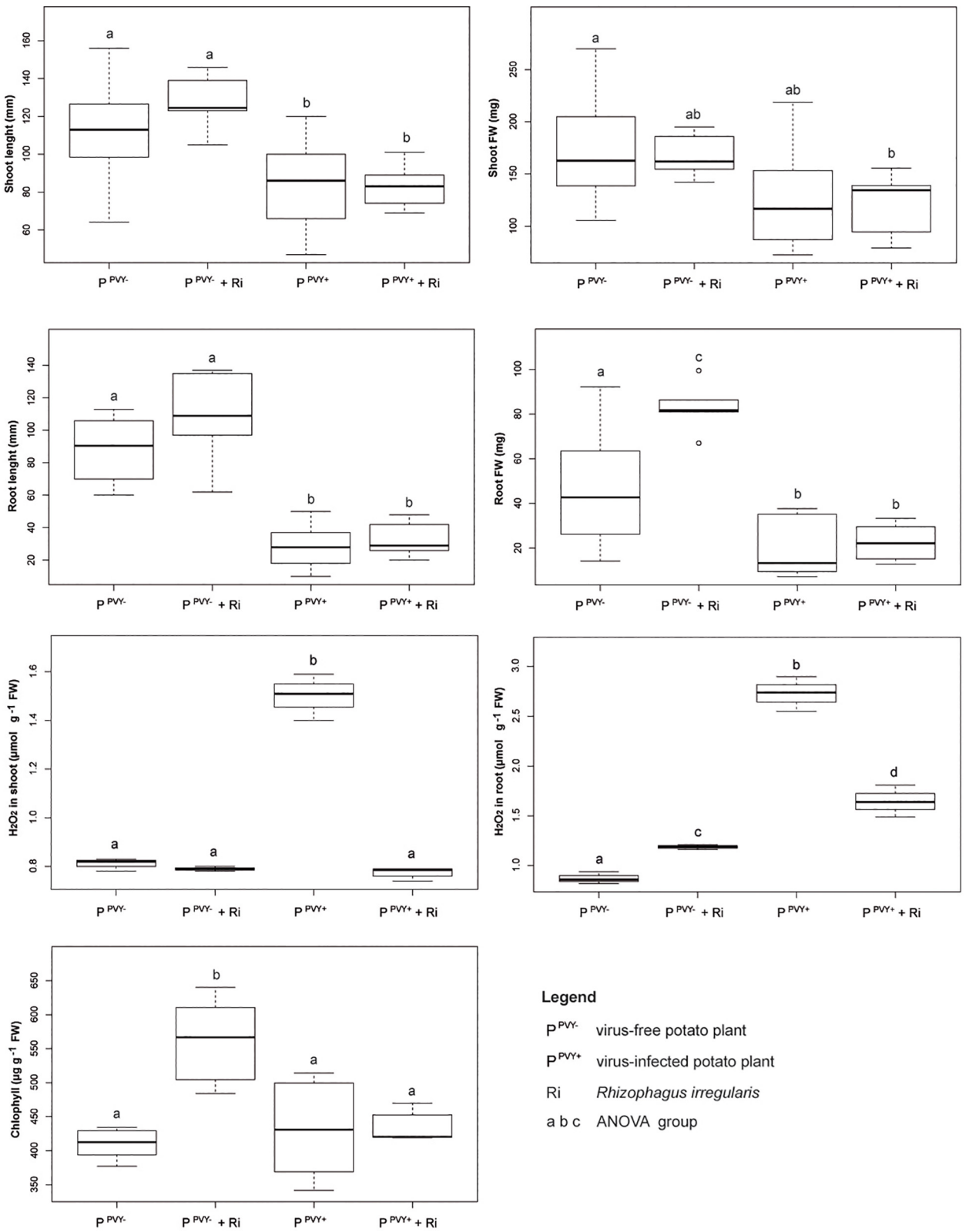

FIGURE 5 | The impact of *R. irregularis* inoculation on growth parameters (shoot length and fresh weight, root length and fresh weight), stress response (H_2O_2 level in shoot and root) and chlorophyll content in virus-free and PVY-infected plantlets of potato cv. Pirol.

and secondary roots in potato cv. Igor, due to pronounced histological changes in the root apical meristems. Within last years, adverse effect of PVY infection on growth parameters (including shoot and root length) of Chinese potato cv. Zihuabai was reported by Li et al. (2013).

Apart from changed growth parameters, potato cv. Pirol infected with $PVY^{N:O}$-T1 displayed also strongly elevated endogenous level of H_2O_2. Additionally, comparison of H_2O_2 concentration in plantlet shoot and root, showed that the second one was more severely impacted by the virus. Previously, Thiem et al. (2014), using the same potato cv. Pirol grown in pots, found the long-term increase in amount of endogenous H_2O_2 to be associated with PVY presence. Furthermore, accumulation of H_2O_2 in response to viral infection was also shown for other host plant and pathogen (i.e., tobacco and M strain of Cucumber mosaic virus, M-CMV) (Lei et al., 2016). According to literature data, oxidative metabolism, involving utilization of ROS as signal factors, is associated with plant defense response to the pathogen invasion (Shetty et al., 2008; Saxena et al., 2016; Gonzalez-Bosch, 2018). H_2O_2 may play a pivotal roles in pathogen control comprising (i) induction of the oxidative burst in hypersensitive response (HR) in order to inhibit pathogen infection development and (ii) activation of biotic stress response mechanism, e.g., SAR (systemic acquired resistance) pathway (Gilroy et al., 2016; Hernández et al., 2016). On the other side, constantly maintained high concentration of endogenous H_2O_2 may exert toxic effect on plant development and contribute to the biomass reduction (Gapper and Dolan, 2006), which is suggested in this and previous studies (Thiem et al., 2014).

Current study examined the interaction between PVY and *R. irregularis* sharing the same host plant. The knowledge on the way how pathogen-symbiont interplay shape the host plant condition is still scarce. Plant root growth capacity is essential for successful establishment of mycorrhiza. Therefore, we used potato cv. Pirol (having the lowest concentration of PVY in the roots) to minimize negative effect of the virus on the symbiosis development. We noticed that healthy plantlets colonized with *R. irregularis* displayed growth parameters similar to the control, with the only significant differences found in the higher root biomass (but not length) and higher chlorophyll content upon mycorrhization. Beneficial effect of AMF on root biomass production was already described for potato (Davies et al., 2005; Thiem et al., 2014) as well as for the other plant species (Saia et al., 2015; Chen M. et al., 2017; Jacott et al., 2017; Shao et al., 2018). We cannot exclude that enhanced biomass (but not the length) of root system could be associated with some structural changes that are known to be induced by AMF (e.g., enlargement of root cortex due to an extra cell layer development for accommodation of fungal structures) (Dreyer et al., 2014). However, it is even more probable that improved nutritional status of *R. irregularis*-inoculated potato cv. Pirol, due to more efficient acquisition of water and nutritional compounds, contributed to the changes in root biomass, which was previously noticed (Lekberg and Helgason, 2018). The other growth parameters of plantlets (i.e., shoot and root length, shoot fresh weight) remained unchanged upon mycorrhization. Furthermore, it is also possible that raised content of chlorophyll in leaves of healthy mycorrhizal potato plantlets could be associated with increased photosynthetic rate, as it was demonstrated for potato cv. Marfona (Sipahioglu et al., 2009) and other hosts, e.g., maize (Zare-Maivan et al., 2017), cucumber (Chen S. et al., 2017), or pepper (Beltrano et al., 2013).

Positive effects of mycorrhiza described above were masked in the presence of PVY, thus growth capacity of virus-positive *R. irregularis*-potato plantlets did not differ from the control ones. Nevertheless, mycorrhizal fungi strongly influenced the endogenous level of H_2O_2 in both healthy and PVY-infected potato plants. In the absence of viral pathogen, colonization of potato cv. Pirol roots with *R. irregularis* slightly (but significantly) increased H_2O_2 level in plant root but not shoot. The generation of ROS in plants, as a response to the mycorrhizal colonization of roots, was previously described (Puppo et al., 2013; Nath et al., 2016; Kapoor and Singh, 2017). ROS acting as long distance signal molecules play an important role during plant adaptation to biotic stress (Sewelam et al., 2016). Interestingly, our study revealed prolonged maintenance of elevated H_2O_2 in mycorrhizal potato plantlets, which is in line with the previously described results (Thiem et al., 2014), however the basis of this observation is unclear and requires further consideration. Nevertheless, Hause and Fester (2005) demonstrated that H_2O_2 is produced in arbuscules and the use of ROS scavengers (e.g., ascorbic acid and salicylhydroxamic acid) reduces both H_2O_2 level and mycorrhizal development (Kapoor and Singh, 2017).

Mycorrhizal fungi exerted the most pronounced effect in PVY-infected potato plants, causing considerable reduction of endogenous H_2O_2 content. This effect was stronger in plant shoot where H_2O_2 concentration was restored down to the control level, thus suggesting the protective role of mycorrhiza against PVY-induced oxidative stress. However, in the roots of mycorrhizal plantlets, the level of H_2O_2 was only partially lowered. The maintenance of oxidative stress in plant root means that this organ was the most affected by the interaction between host, PVY and AMF. As it was discussed above, typical plant reaction to pathogen invasion involves increased production of H_2O_2. In our experiment mycorrhiza alleviated stress caused by PVY. The result is contradictive to study by Sipahioglu et al. (2009) suggesting that mycorrhiza enhances the negative impact of the virus and exacerbates disease symptoms in sensitive potato cv. Marfona. This discrepancy may be related to the various characteristics of potato cultivars used in both experiments (tolerant vs. sensitive), since virus behavior may differ depending on the host genotype (Valkonen, 2015; Davie et al., 2017). Nevertheless, the investigation by Maffei et al. (2014), examining different experimental biosystem consisting of tomato, Tomato yellow leaf curl Sardinia virus (TYLCSV) and *Funneliformis mosseae*, indicated attenuation of viral disease symptoms upon symbiosis. More recently, Wang et al. (2018) showed that mycorrhization of tomato affected by *Cladosporium fulvum* (pathogenic mold) caused significant increase in activities of both superoxide dismutase and peroxidase, which correlated with decrease in H_2O_2 level. We suspect that similar biological

processes may explain reduced concentration of H_2O_2 in virus-positive potato host, however molecular studies are necessary to confirm this presumption.

CONCLUSION

Our study demonstrated that infection with $PVY^{N:O}$-T1, although asymptomatic, negatively affected vegetative growth of the Pirol cultivar. Furthermore, the virus induced stress response in plants. *R. irregularis* inoculation had slightly positive effect on plantlets' growth parameters, however mycorrhizal benefits were inhibited by PVY. The processes that cause the effects of PVY infection to be more pronounced over the mycorrhizal benefits are not identified yet.

Interestingly, mycorrhiza modulated plant-pathogen interaction. The effect of PVY infection in potato can be alleviated and masked by mycorrhizal symbiosis. In consequence of this result the molecular mechanism underlying this biotic interactions and the practical consequences for field observations in potato breeding need to be analyzed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the sequences were deposited in GenBank under accession numbers MK455818 and MK455819.

AUTHOR CONTRIBUTIONS

ED-S was responsible for the original draft preparation, review and editing of the manuscript, multiplex RT-PCR, sequencing, data annotation, virus identification, phylogenetic tree, ELISA, and statistical analyses. AK worked on the *in vitro* culture of AMF and mycorrhiza staining. AT worked on the *in vitro* culture of potato and analysis of plant growth parameters. AS-J analyzed the hydrogen peroxide. CB and LM participated in the review and the editing of the manuscript. KH conceptualized the study, supervised, reviewed, and edited the manuscript, and was responsible for the funding acquisition. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02971/full#supplementary-material

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Effects of Drought-Tolerant *Ea-DREB2B* Transgenic Sugarcane on Bacterial Communities in Soil

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Drought is a major abiotic stress affecting plant growth and development. Sugarcane, a sugar crop planted in warmer climate, suffers dramatically from drought stress. Bacterial communities colonizing the rhizosphere, where roots sense drought stress initially, have been well studied for their beneficial effects on plant growth and health. The *Ea-DREB2B* gene cloned from the sugarcane, *Saccharum arundinaceum*, belongs to the DREB2 subgroup of the DREB gene family, which is involved in drought response regulation. Here, we present a detailed characterization of the rhizoplane, rhizosphere, and bulk soil bacterial communities determined using a high-sequencing approach with the transgenic (TG) sugarcane variety GN18 harboring the drought-tolerant *Ea-DREB2B* gene and its isogenic wild-type (WT) variety FN95-1702 under the same environmental conditions. In addition, the total carbon (TC), total nitrogen (TN), and total phosphorus (TP) contents in each soil area were compared to explore the relationship between bacterial alteration in the TG and WT plants and environmental factors (TC, TN, TP, C:N, C:P, and N:P). Our results showed that the bacterial communities in the rhizosphere and rhizoplane of TG sugarcane were more similar and perfectly correlated with the environmental factors than those of the WT. This suggested that the bacterial communities of the TG plants were altered in response to the changes in root exudates. The results of our study suggest that the change in soil environment caused by transgenic sugarcane alters soil bacterial communities.

Keywords: drought-tolerant, *Ea-DREB2B*, sugarcane, bacterial community, environmental factor

INTRODUCTION

Plants are intimately intertwined with the microbial communities living in and around them (Naylor et al., 2017). The rhizosphere is a small compartment of the soil that is adjacent to and directly affected by the plant roots, and it has long been regarded as one of the most important interfaces for life on Earth. The rhizoplane is the root surface that forms the interface between the plant root and rhizosphere soil (Ding et al., 2019). There is a strong relationship between

plant and microbiomes colonized in the rhizosphere. Genetically modified (GM) plants with stress-tolerant ability are prevalent worldwide. Considering the contribution of microbial–plant interactions for plant growth and development, numerous studies have focused on the influence of GM stress-resistant plants on soil- and root-associated bacterial communities (Dunfield and Germida, 2004; Sohn et al., 2016; Ibarra et al., 2020). Some studies also suggest that the modification of certain genes in plants can influence the associated bacterial communities, resulting in a change in the rhizosphere compared with that of the wild-type (WT) plant communities (Brusetti et al., 2005, 2008). Therefore, it is important to assess the specific effect of each GM plant on the soil environment and community.

Drought stress represents one of the most significant obstacles to global crop production and is expected to increase in severity and frequency in the future (Xu L. et al., 2018). As drought severely stunts plant growth and development, several studies have focused on strategies for improving drought resistance from a global perspective. Many genes that play a role in plant responses to drought have been identified, and some of these have been shown to be effective in improving drought tolerance by genetic engineering (Zolla et al., 2013). Sugarcane, an important source of sugar and ethanol, is a relatively high water-demanding crop and its growth is highly sensitive to water deficits (Ferreira et al., 2017). Genetic engineering has been applied in the enhancement of the drought resistance of sugarcane (Ramiro et al., 2016). Genes encoding the dehydration-responsive element-binding (DREB) transcription factors identified in *Arabidopsis thaliana* have been reported to enhance drought resistance in transgenic (TG) plants (Mizoi et al., 2012). *Ea-DREB2B*, cloned from the hardy sugarcane *Saccharum arundinaceum* is a member of the DREB2 family, which is a subfamily of DREB that regulates the expression of several stress-inducible genes and plays a critical role in enhancing the tolerance of plants to drought and salinity (Lata and Prasad, 2011; Augustine et al., 2015). It has been reported that the drought resistance of sugarcane modified by *Ea-DREB2B* was significantly enhanced as compared to that of non-transgenic sugarcane (Xu S. et al., 2018). CBF/DREB regulon is one of the activated regulons of the abscisic acid (ABA)-independent pathway (Saibo et al., 2009). ABA is a plant growth regulator and stress hormone that induces leaf stomata closure to reduce water loss *via* transpiration and decreases the photosynthetic rate to improve the efficiency of water usage by plants (Agarwal et al., 2006). The expression of *SlDREB3* in tomatoes affects several ABA-associated processes by reducing the ABA levels and responses, thereby leading to higher photosynthesis (Upadhyay et al., 2017). The root-associated bacteria are sensitive to even small changes in the pattern of compounds in the rhizosphere (Persello-Cartieaux et al., 2003), which may be altered by genetic modifications for enhancing or conferring specific traits. Although numerous studies have confirmed that DREB2s contribute greatly to the enhanced drought and salinity tolerance of a plant (Chen et al., 2009; Matsukura et al., 2010; Li et al., 2017), few studies have focused on the bacterial communities in the soil of plants modified by DREB2s (Chunmiao et al., 2015). Therefore, it is important to evaluate the influence of plants modified with DREB2 genes on hormone processes that could influence the root-associated bacterial communities.

In this milieu, the main objective of this study was to investigate the effects exerted by the TG sugarcane modified using the *Ea-DREB2B* gene on the root-associated layers of the soil and the bulk soil bacterial communities. The specific aims were to (1) determine the variation in the diversity and composition of TG bacterial communities in the rhizoplane, rhizosphere, and bulk soil compared with those of the non-transgenic WT communities; (2) explore the relationship between the alteration in TG bacterial communities and environmental factors, including total carbon (TC), total nitrogen (TN), and total phosphorus (TP) contents and the C:N, C:P, and N:P ratios; and (3) determine any correlations among the bacterial communities of the rhizoplane, rhizosphere, and bulk soil of TG sugarcane. Our study will provide general insights into the potential effects of genetic modifications on key traits to improve crop production and stress tolerance in a broader ecosystem context and, thus, offer guidance for the development and monitoring of new GM varieties.

MATERIALS AND METHODS

Plants and Field Experiment Design

This study was performed in the forage breeding ground in Quli, Fusui, Chongzuo, China (between 107°31′ and 108°06′E, and 22°17′ and 22°57′ N) of Guangxi University in the summer of 2018. The average annual temperature was 21.3°C. The lowest temperature in the past year was −0.6°C and the highest temperature was 39.5°C. The total annual radiation was 108.4 kcal/cm, with 1,693 h of annual average sunshine, and the frost-free period was up to 346 days. The annual precipitation in the whole region was 1,050–1,300 mm. It was windy and dry in the winter and spring, and rainy and humid in the summer and autumn (**Supplementary Figure S1**). Fields cultivated over the long term with sugarcane had the following properties: lateritic red earth, pH of 5.15, 19.47 g/kg organic matter, 0.84 g/kg TN, 2.98 g/kg TP, 7.11 g/kg total potassium, 136 mg/kg alkaline-hydrolyzed nitrogen, 83 mg/kg available phosphorus, and 77.1 mg/kg available potassium. GN18 is a TG variety that was derived from FN95-1702 as the acceptor parent material using the inducible promoter RD28A and a gene gun for overexpression of the *Ea-DREB2B* gene to confer greater drought resistance (Xu S. et al., 2018). The resistance of GN18 under drought stress and the ability to recover after rehydration were confirmed to be stronger than those of the acceptor parental material FN95-1702 (Xu S. et al., 2018). The experiment consisted of a random block design with six blocks, with each block covering an area of 30 m × 4.2 m. Each block contained both sugarcane varieties (three lines for each plant). The distance between two varieties was 2.1 m and the distance between two

sugarcanes was 30 cm in each block; each line was planted with 46 sugarcanes.

Soil Sample Collection and Physicochemical Analysis

Soil samples were collected in the late jointing stage on November 18, 2018. Bulk soil was taken adjacent to the excavated sugarcane (20 cm from where the stalk had been) from 0- to 20-cm depth using a standard soil corer. Each sampling site consisted of five subsamples collected between two lines of sugarcane (Wang et al., 2016). Three of five points were selected to dig out sugarcane roots. The rhizospheric compartment was separated by thoroughly vortexing the roots for 20 s and collecting the resulting soil precipitation in PowerBead tubes. The rhizoplane compartment was derived from the root surface, which was removed by sonication for 5 min (Edwards et al., 2015). Seventy-two sugarcane roots were collected altogether from six blocks. Each soil sample consists of soil collected from 12 sugarcane roots which were excavated from two blocks through random selection in six blocks. Each composite soil sample was homogenized and stored at −80°C for less than 24 h before DNA extraction. After DNA extraction, the soil samples were air-dried and passed through a 2-mm sieve before measuring the TC, TN, and TP contents. Each soil sample was set up as three replicates with 0.5 g of each sample. The TC (Batjes, 1996) and TP (Sommers and Nelson, 1972) contents were determined as described previously, and the TN content was determined using the Kjeldahl method (Bremner and Tabatabai, 1972).

DNA Extraction and Sequencing

The total DNA was extracted from 1 g of each soil sample of three biological replicates, which was replicated three times using the E.Z.N.A soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, United States) following the manufacturer's instructions. The concentration and purity of the total DNA were measured using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, United States). Primers F338 (5′-ACT CCT ACG GGA GGC AGC A-3′) and R806 (5′-GGA CTA CHV GGG TWT CTA AT-3′) targeting the V4 region of the 16S rRNA gene were used for polymerase chain reaction (PCR) (Peiffer et al., 2013). This primer set provides a comprehensive coverage with the highest taxonomical accuracy for bacterial sequences. The reverse primer also contained a 6-bp error-correcting barcode unique to each sample. PCR comprised 25 μl of 2 × Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 μl of each primer (10 mM), and 3 μl DNA template (20 ng/μl) in a total volume of 50 μl. The reaction conditions were: initial denaturation for 5 min at 94°C; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; and a final elongation at 72°C for 10 min. The quantitative PCR was carried out using BioRad S1000 (Bio-Rad Laboratory, CA, United States). Each PCR product was subjected to sequencing by Magigene Technology (Guangzhou, China) using the Illumina HiSeq 2500 platform. FLASH software was used to merge pairs of reads from the original DNA fragments (Magoč and Salzberg, 2011). Further sequence analysis was performed using USEARCH v5.2.32 and was clustered using Unoise3. The quantitative insights into microbial ecology (QIIME) pipeline software was used to select 16S rRNA operational taxonomic units from the combined reads (Edgar, 2010). The 16S rRNA gene sequences obtained in this study have been deposited in the NCBI sequence read archive (SRA) database with accession number SRP238824.

Statistical and Bioinformatics Analysis

Alpha diversity was estimated using the Chao1 richness index and Shannon diversity index. Correlations between the alpha diversity and environmental factors were determined using the "corrplot" package (Wei et al., 2017) in the R v3.6.3. environment. Beta diversity, using the principal coordinates analysis (PCoA), was estimated using the Bray–Curtis distance matrix. Furthermore, we used the Mantel test to study the relationship between beta diversity and environmental factors. The Mantel test, PCoA, and distance-based redundancy analysis (dbRDA) were performed using "vegan" packages in R v3.6.3 (Oksanen et al., 2013). The relative abundance of bacterial communities was evaluated in "alluvial" and "ggplot" packages in R v3.6.3 (Wickham and Wickham, 2007), which showed the changing tendency of bacterial populations in each compartment, and the Simper function was used to make pairwise comparisons of population composition. Functional Annotation of Prokaryotic Taxa (FAPROTAX), a database that estimates the metabolism or other ecologically related functions of prokaryotes by extrapolating their functions, was used to predict the functions of rhizosphere bacterial communities under TG and WT intercropping patterns (Louca et al., 2016).

Networks were constructed for root-associated area and bulk soil communities based on operational taxonomic unit (OTU) relative abundances, resulting in two networks. Covariations were measured across nine biological replicates to create each network. Only OTUs detected in five out of nine replicate samples were used for network construction. Random matrix theory (RMT) was used to automatically identify the appropriate similarity threshold (St) prior to network construction; St defines the minimal strength of the connections between each pair of nodes (Deng et al., 2012). Global network properties were characterized according to Zhou et al. (2013). All analyses were performed using the molecular ecological network analyses (MENA) pipeline[1] and networks were graphed using Cytoscape 2.8.2 (Shannon et al., 2003). We characterized the modularity for each network created in this study. A module is a group of nodes (i.e., OTUs) that are highly connected within the group with few connections outside the group (Newman, 2006). In this study, modules were detected using the greedy modularity optimization method (Deng et al., 2012). Modularity (M) is an index measuring the extent to which a network is divided into modules, and we used $M > 0.4$ as the threshold to define modular structures. The connectivity of each node was determined based on its within-module connectivity (Z_i) and among-module connectivity (P_i), which were then used to classify the nodes based on the topological roles they play in the network (Guimera

[1]http://ieg2.ou.edu/MENA/

and Nunes Amaral, 2005). Node topologies are organized into four categories: module hubs (highly connected nodes within modules, $Z_i > 2.5$), network hubs (highly connected nodes within an entire network, $Z_i > 2.5$ and $P_i > 0.62$), connectors (nodes that connect modules, $P_i > 0.62$), and peripherals (nodes connected in modules with few outside connections, $Z_i < 2.5$ and $P_i < 0.62$) (Deng et al., 2012).

RESULTS

Soil Chemical Properties, Bacterial Alpha, and Beta Diversity

The TN content and TC/TN ration were significantly different between the two breeds. Furthermore, significant differences existed in the TC, TN, TC/TN, and TN/TP among the different soil layers. Considering the two influential factors together, there were extremely significant differences in TN and TP (**Table 1**). The alpha diversity indices, represented by the Chao1 richness and Shannon diversity indices, for the soil bacterial communities were significantly different between the TG and WT plants. However, no significant difference was observed between the rhizoplane and rhizosphere bacterial alpha diversity according to either index for the TG groups, whereas a difference was observed for the WT samples. Moreover, both indices demonstrated a certain degree of increase in the bacterial diversity of TG rhizocompartments compared with those of the WT (**Figures 1A,B**). Pearson's correlation analysis indicated that the Shannon diversity index of the WT plants was negatively correlated with the C/N ratio ($P < 0.05$), whereas that of the TG

TABLE 1 | Soil chemical properties and the ratio between them according to different compartments.

Line	Root compartment	TC (g·kg^{-1})	TN (g·kg^{-1})	TP (mg·kg^{1})	TC/TN	TN/TP	TC/TP
WT	Rhizoplane	14.03 ± 2.62ab	1.21 ± 0.20a	4.86 ± 1.59a	11.51 ± 0.58b	0.28 ± 0.15b	3.26 ± 1.73a
	Rhizosphere	11.43 ± 1.88ab	0.88 ± 0.02a	10.82 ± 0.66c	12.95 ± 2.37bc	0.08 ± 0.00a	1.07 ± 0.24a
	Bulk soil	11.88 ± 1.95ab	0.74 ± 0.02a	3.89 ± 0.69a	15.95 ± 2.29c	0.20 ± 0.04ab	3.18 ± 1.04a
TG	Rhizoplane	15.75 ± 1.80b	2.97 ± 0.56b	10.42 ± 0.15bc	5.35 ± 0.42a	0.29 ± 0.06b	1.51 ± 0.19a
	Rhizosphere	13.52 ± 0.58ab	1.21 ± 0.08a	5.77 ± 0.65ab	11.22 ± 0.25b	0.21 ± 0.04ab	2.37 ± 0.37a
	Bulk soil	9.73 ± 0.91a	0.67 ± 0.05a	10.45 ± 3.75bc	14.48 ± 1.42bc	0.07 ± 0.02a	1.04 ± 0.44a
Line		0.518	<0.001***	0.014*	<0.001***	0.938	0.057
Root compartment		0.006**	<0.001***	0.542	<0.001***	0.005**	0.432
Line × Root compartment		0.110	<0.001***	<0.001***	0.032*	0.026*	0.009**

*a TC, total carbon; TN, total nitrogen; TP, total phosphate. b Different letters indicate significant differences (ANOVA, P <0.05, Turkey's HSD post-hoc analysis) among root compartment. c *0.01 < P value < 0.05; **P value < 0.01; ***P value < 0.001.*

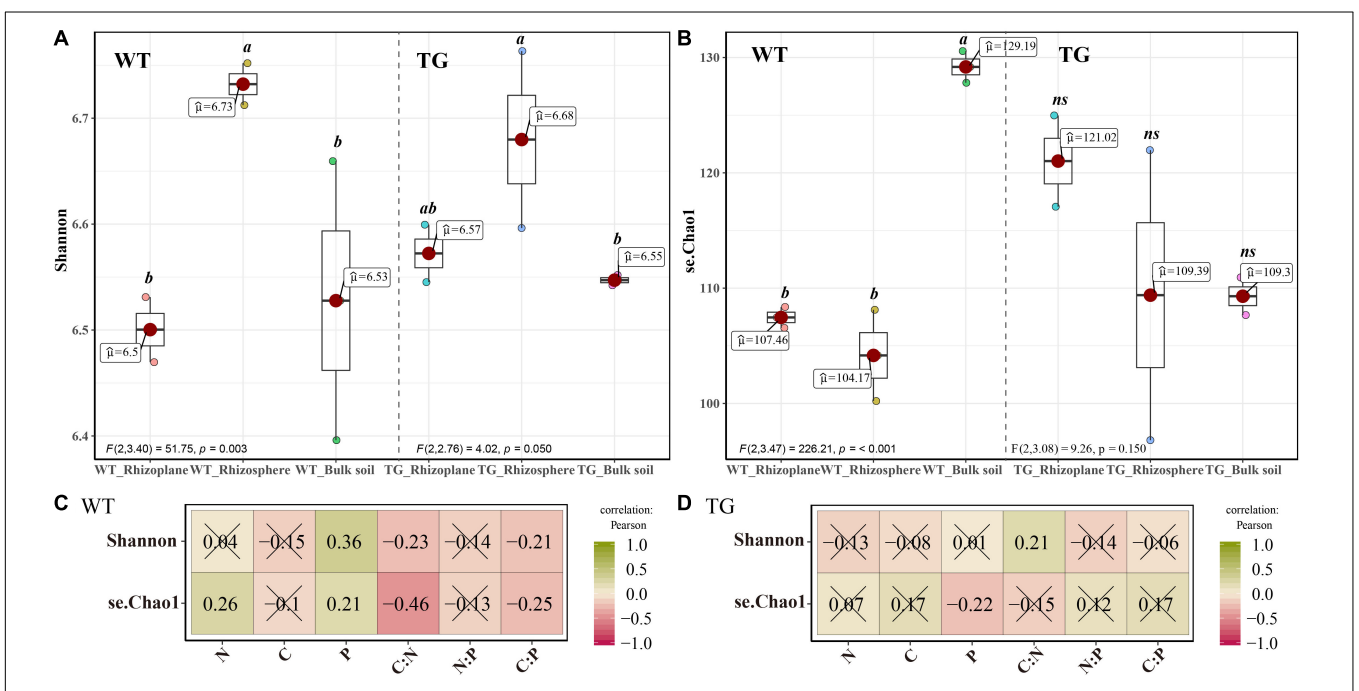

FIGURE 1 | (A,B) Bacterial alpha-diversity measurements of represented by Chao 1 Richness and Shannon Diversity in each area and cultivar. **(C,D)** The correlation between bacterial alpha-diversity and environmental factors using Pearson analysis.

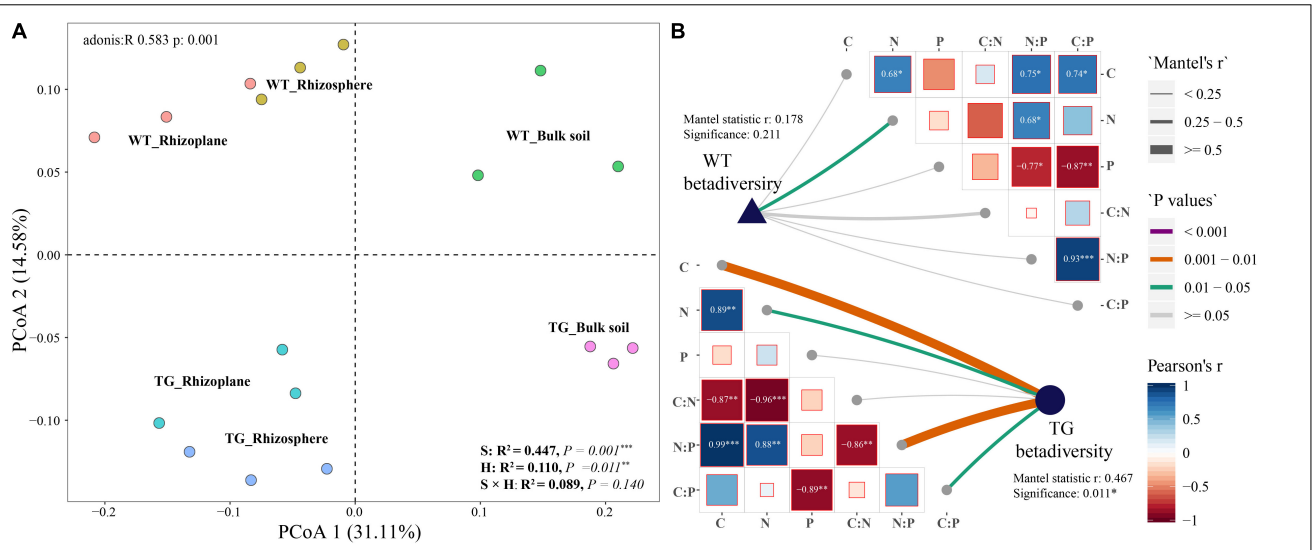

FIGURE 2 | (A) Principal coordinate analyses (PCoAs) using Bray distance metric indicates that the largest separation between bacterial communities is spatial distribution of three areas (PCoA.1) and the second largest source of variation is cultivar (PCoA.2). **(B)** Correlation between environmental factors and correlation between bacterial beta-diversity and environmental factors in two sugarcane cultivars using Mantel test.

plants was positively correlated with the C/N ratio ($P < 0.05$). Furthermore, the Chao1 index of the WT group was positively correlated with the TP content ($P < 0.05$) and negatively correlated with the TC content ($P < 0.05$), whereas that of the TG group showed the opposite relationships (**Figures 1C,D**).

The PCoA of the Bray distance was performed to investigate and visualize the patterns of separation among the three zones of the two sugarcanes. An obvious overlap was observed in the rhizosphere and rhizoplane areas in TG. In contrast, no apparent intersection was detected between the rhizoplane and rhizosphere of WT plants. In addition, the distance between the bulk soil bacteria community and the rhizosphere of TG plants was further than that of the WT (**Figure 2A**). The Mantel test further showed that the correlation between environmental factors in TG plants was greater than that in WT. The bacterial beta diversity of TG plants was positively correlated with the TC and TN contents, and the N/P and C/P ratios, respectively, and the correlations between TG bacterial beta diversity and the TC content and N/P ratio were relatively stronger than the others. However, the beta diversity of WT bacterial communities was only positively correlated with the TN content (**Figure 2B**).

Influence of the Genetic Modification on the Bacterial Community Composition

The relative abundance of bacterial communities based on phylum clearly differed between the TG and WT groups. An obvious fluctuation was observed between the relative abundances of the rhizoplane and rhizosphere bacterial communities in WT, whereas those of the TG plants tended to be similar. The relative abundance of Actinobacteria, Bacteroidetes, and Betaproteobacteria varied between breeds, and the relative abundance of Alphaproteobacteria, Actinobacteria, Bacteroidetes, Betaproteobacteria, Chloroflexi, and Verrucomicrobia were different among the layers. The relative abundance of Actinobacteria was different not only between breeds but also among layers (**Figure 3A**). The different compartments (71.5%) and sugarcane cultivars (21.2%) were observed to explain the variation in the bacterial composition in dbRDA (**Figure 3B**). The relationships between the main bacterial populations from the three zones and environmental factors were analyzed by dbRDA, showing that the most strongly affected populations were those of Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Deltaproteobacteria, Gammaproteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, and Verrucomicrobia. Among them, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Verrucomicrobia were the predominant groups of the rhizoplane and rhizosphere areas, whereas Chloroflexi, Acidobacteria, and Actinobacteria were mainly present in the bulk soil zone. Among the environmental factors, the C/N ratio ($R^2 = 0.451$, $P = 0.013$), TC content ($R^2 = 0.329$, $P = 0.048$), and TN content ($R^2 = 0.247$, $P = 0.125$) had the greatest influence on the bacterial community. In particular, the TC content was associated with Alphaproteobacteria, TN content was associated with Betaproteobacteria, Deltaproteobacteria, and Verrucomicrobia, and the C/N ratio was most strongly associated with the bulk soil area, mainly dominated by populations of Actinobacteria and Acidobacteria (arrows in **Figure 3B**).

Network Analyses of Bacterial Communities Among the Three Zones

The DESeq2 differential abundance analysis showed that 59% of the OTUs of TG plants were enriched in the rhizocompartments and 41% were detected in the bulk soil area, representing a statistically significant difference from

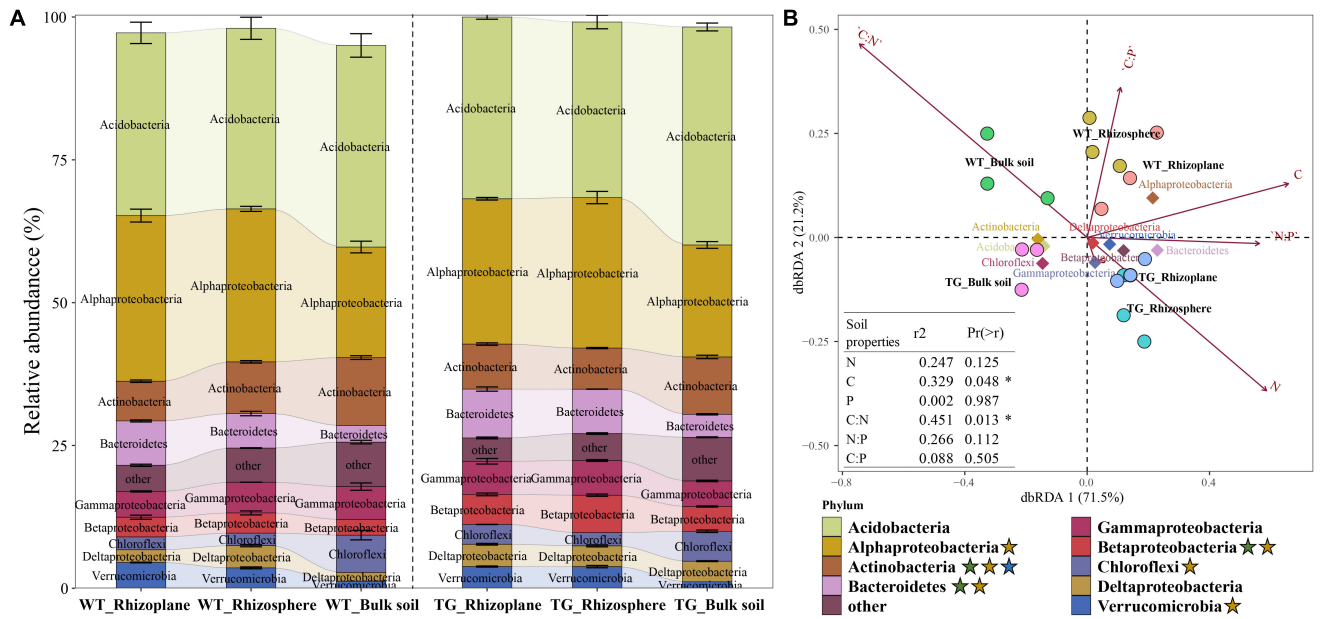

FIGURE 3 | (A) Bacterial relative abundance with Phylum of each zone in two cultivars. The green star represents that the relative abundance of the bacteria is different between breeds; the yellow star represents that the relative abundance of the bacteria is different among different soil layers; and the blue star represents that the relative abundance of the bacteria is different both between breeds and among different soil layers. **(B)** Distance-based Redundancy analysis of different zones, abundant classes, and 6 environmental factors (arrows) indicates the dominant communities and influential environmental factors.

the relatively random distribution of OTUs detected in the WT areas. Moreover, there was a strong positive correlation between the OTUs at the root-associated enrichment area of TG plants (**Figures 4A,C**). The MENA pipeline analysis divided all the identified OTUs into seven modules, and the distribution of these modules clearly differed between the TG and WT plants. The OTUs in the five modules of the root-associated area of TG plants showed a strong correlation with environmental factors, with negligible differences between the OTUs in these five modules. In contrast, the structure of the OTUs in the five modules representing colonization in the root-associated area of TG was significantly different from those in the other two modules in the bulk soil area (**Figures 4B,D**). The Module-EigenGene analysis showed that the eigengenes within the TG submodules that clustered into two groups were significantly correlated. Furthermore, one of the groups comprising five submodules exhibited significantly positive correlations with the TC and TN contents and the N/P ratio (**Figures 4B,D**). Network module separation and modularity calculation showed that the majority of the OTUs were peripherals, with most of their links remaining within their own modules. A total of two nodes were identified as connectors in the TG plants, and these OTUs were derived from Soilbacteriales and Rhizobiales, which were both enriched in the roots. In addition, two other nodes were identified as module hubs, which were mainly derived from Rhizobiales, similar to the connectors, and Actinomycetales (**Figure 5A**). The Map Prokaryotic clades tool was used to associate the OTUs to established metabolic or other ecologically relevant functions for predicting the strengthened ecological functions of the bacterial communities in TG plants. This analysis showed that communities related to chemoheterotrophy and nitrogen fixation were significantly stronger in the TG plant environment than in WT environments (**Figure 5B**).

DISCUSSION

Through this field experiment, we demonstrated that drought-resistant TG sugarcane shapes the root-associated bacterial community assembly, which may in turn contribute to the ability of the host plant to respond appropriately to abiotic environmental stress. Thus, our results confirm that the diversity and composition of the bacterial communities of TG plants considerably differed from those of WT, with respect to both root-associated area and bulk soil, indicating that the genetic modification altered the plant-associated bacterial communities. The host plant genotype explained a significant portion of the variation in the diversity and composition of the bacterial communities. In addition, variation in the TG bacterial communities was more strongly correlated with soil environmental factors than that of the WT, indicating that some compounds in the root exudates have specific effects on the bacterial communities of TG plants. Finally, the rhizoplane and rhizosphere compartments of TG plants were more closely associated, whereas the dissimilarity in the bacterial communities between the rhizosphere and bulk soil was greater than that of the WT. Here, we discuss how these three main results can provide new insights into the factors

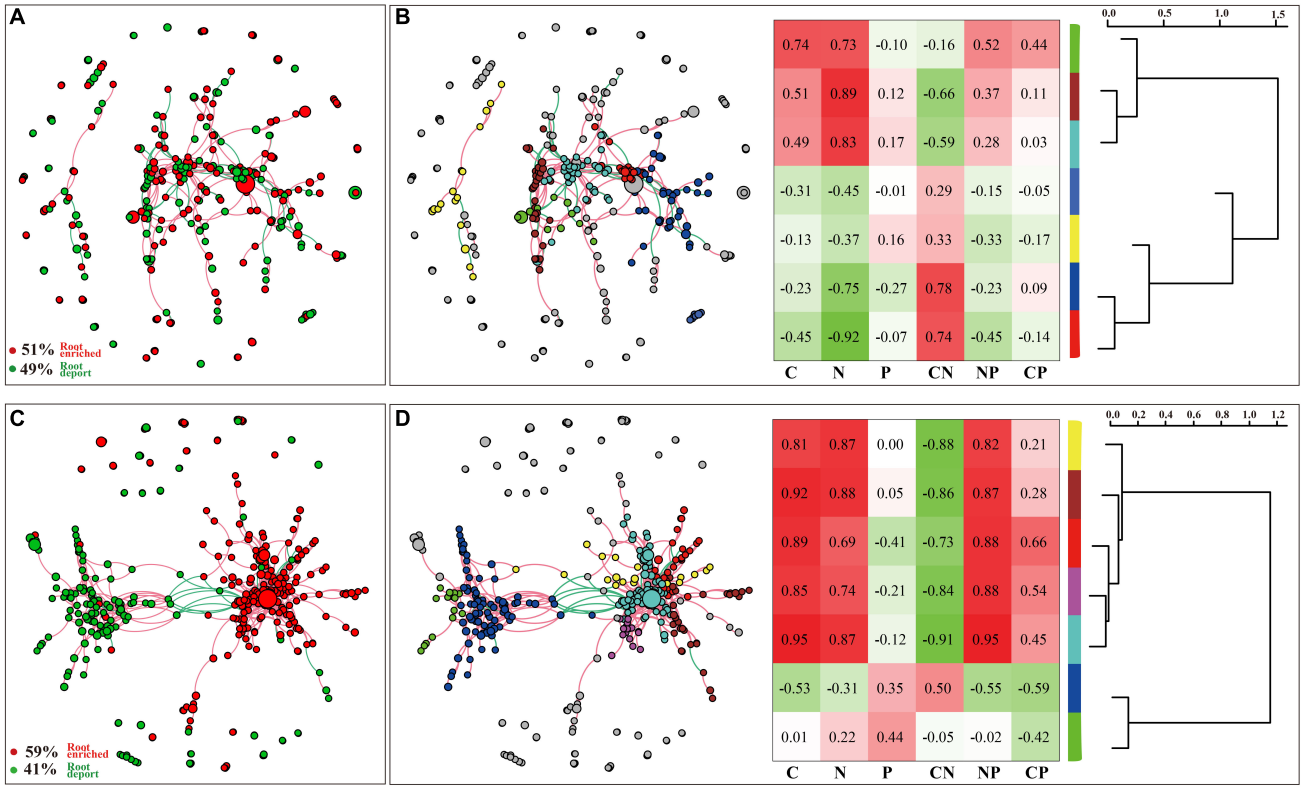

FIGURE 4 | (A,C) DESeq2 differential abundance indicates the enrichment and deportation of OTUs within the overall area of three zones in two WT and TG, respectively. Each node represents an individual OTU, and the red edge is drawn between OTUs if they are positively correlated, while the green edge is drawn between OTUs if they are negatively correlated. **(B,D)** OTUs are divided into seven modules using the molecular ecological network analyses (MENAP), indicating the ecological network relationship between OTUs. Module-Eigen Gene analyses indicates the Module correlation with environmental factors and the Module-Eigen Gene hierarchy structure. The heatmap shows the correlation between modules and environmental factors, and the hierarchy clustering located on the right shows the Pearson correlation among module eigengenes.

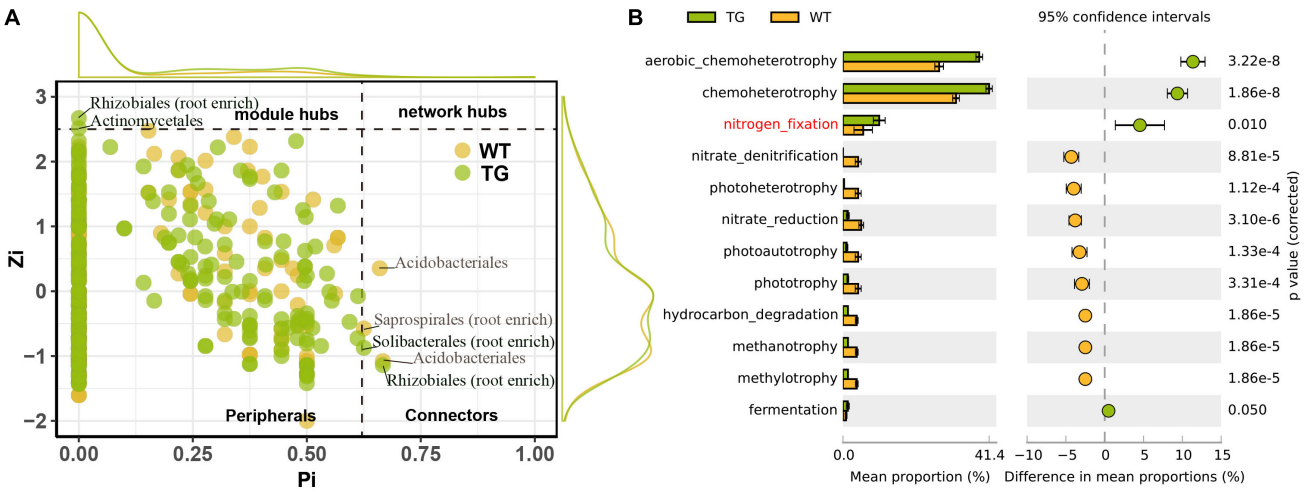

FIGURE 5 | (A) Network module separation and modularity calculation analysis shows dominantly functioning bacterial communities. Each dot represents an OUT in two sugarcanes. The x-Zi represents within-module connectivity and the y-Pi represents among-module connectivity. **(B)** Map prokaryotic clades to established metabolic or other ecologically relevant functions based on DESeq2 shows the ecological functions of root bacterial communities in TG and WT, indicating the enhanced ecological functions of TG root bacterial communities compared with WT.

that shape root-associated bacterial communities and their ecological relevance.

Variation in Bacterial Community Diversity in TG Sugarcane

In the present study, we investigated the influence that the plant genotype exerts on bacterial community diversity in three layers (the rhizoplane, rhizosphere, and bulk soil), which is extremely correlated with the environmental factors (TC, TN, and TP contents). We found obvious variations in the root bacterial community diversity between the TG and its parental non-TG variety WT (**Figures 1**, **2**), which have been reported by several studies in other plants (Brusetti et al., 2005; Fang et al., 2005; Li et al., 2018). In the rhizosphere, alterations in the structure, abundance, and diversity of the bacterial communities show great differences in transgenic rice and corn, which are consistent with our results (Brusetti et al., 2005; Fang et al., 2005; Li et al., 2018). However, some studies have reported conflicting results. In drought-tolerant crops, such as transgenic corn expressing the *Hahb-4* gene and transgenic rice expressing the *CaMSRB2* gene, only minor effects on the root-associated bacterial community were observed (Sohn et al., 2016; Ibarra et al., 2020). These conflicting results are likely due to the different genes that were modified in the plants. Hamonts et al. (2018) demonstrated that sugarcane-associated bacterial assemblage is primarily determined by plant compartment, followed by other factors such as the growing region and sugarcane variety. Indeed, plant genotype is responsible for some of the variations observed in root microbiomes, suggesting an active role of the host in the establishment of the communities (Colombo et al., 2017). Additionally, we also found a stronger correlation between the relative metrics of bacterial diversity and environmental factors in TG compared with those in WT, especially for the TC and TN contents (**Figures 1C,D**, **2B**), suggesting that the changes in the soil environment of the root microbiome influence the bacterial community diversity. A strong relationship between root exudates and microbial diversity has been previously proven (Eisenhauer et al., 2017). Furthermore, the root exudates from GM plants strongly influence the rhizosphere microbial communities (Dunfield and Germida, 2004), and the quantity and quality of the root exudates are determined by plant genotype (Badri and Vivanco, 2009). Several studies have shown that the expression of several drought-inducible genes in an ABA-independent pathway is regulated by the DREB transcription factors (Agarwal et al., 2006; Upadhyay et al., 2017). Moreover, overexpression of *Ea-DREB2* in sugarcane leads to a higher photosynthetic rate and chlorophyll content than those of WT sugarcane under drought stress (Augustine et al., 2015). Plants may release up to 20% of their photosynthesis products into the soil, providing a basis for the establishment of plant–microorganism interactions that will benefit plant growth by, for example, increasing the availability of mineral nutrients or the production of phytohormones (el Zahar Haichar et al., 2008). Besides, a previous study showed that C cycling enzyme potential activities increased with inorganic N availability, while those of N cycling enzymes increased with C availability (Bowles et al., 2014), indicating that the increases in TC and TN in the root-associated area of TG plants might be related to the soil enzyme activities. Overall, our analysis of bacterial diversity revealed that the plant genotype is one of the primary factors contributing to changes in root bacterial diversity due to changes in the physicochemical environment of the microorganisms.

Similar Bacterial Composition of the Rhizoplane and Rhizosphere in TG Sugarcane

In our study, we confirmed that the similar bacterial composition of the rhizoplane and rhizosphere in TG plants was related to the changes in environmental factors. The rhizoplane is the root surface where the host plants are in direct contact with the rhizosphere soil. Based on a study of the root-associated microbial community assembly, the microbial community associated with the roots was proposed to be assembled in two steps: the rhizosphere is first colonized by a subset of the bulk soil community, and then the rhizoplane and endosphere are colonized by a subset of the rhizosphere community (Sasse et al., 2018). The dynamics of microbiome acquisition in our study provide experimental support for this model, given that step 2 of the microbial community assembly is consistent with our data. That is, we observed an increase in the relative microbial abundance in the TG rhizoplane and a reduction in abundance in the rhizosphere, suggesting that some bacteria migrate from the rhizosphere to the rhizoplane (**Figure 3A**). It has been reported that several plant growth-promoting bacteria colonize in the rhizosphere of sugarcane under drought stress (Pereira et al., 2019). Besides, previous studies have suggested that the phylum Proteobacteria comprises several plant growth-promoting rhizobacteria (PGPR) (Bruto et al., 2014), which may facilitate plant growth by promoting the acquisition of nutritional resources such as N, P, and iron (Vurukonda et al., 2016). In our study, the dominant taxa in the root-related area were Proteobacteria, Verrucomicrobia, and Bacteroidetes (**Figure 3B**), especially the Proteobacteria including Betaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria, which observed apparent increases in the rhizoplane of TG compared with WT plants (**Figure 3A**), suggesting an increase in beneficial bacterial communities. In moisture-limited soils, the relative abundance of the phyla Proteobacteria, Verrucomicrobia, and Bacteroidetes was found to decrease (Naylor and Coleman-Derr, 2017). On the contrary, Proteobacteria and Bacteroidetes were enriched in the rainy season (Barnard et al., 2013). These reports indicate the intimate relationship between the phyla mentioned above and the drought-resistant capacity of plants and also suggest the contribution made by those phyla to the enhanced drought-resistant ability of the TG plants in our study. Additionally, TC and TN were detected as the most important contributors to the variations in the bacterial communities (**Figure 3B**), indicating that bacterial community

distribution changes with changes in environmental factors. The rhizosphere is the soil area that is most strongly influenced by the exudates released by the roots. Thus, an assembly of the rhizosphere microbiome is also influenced by the root exudates to a certain degree, which can help select beneficial soil microbial communities (Backer et al., 2018; Williams and de Vries, 2020). Therefore, the recruitment of TG rhizoplane bacteria might represent the beneficial bacterial selection from the root exudates, especially the recruitment of Proteobacteria. Indeed, exudation has been shown to play an active role in bacterial proliferation in the rhizosphere soil (Baudoin et al., 2003).

Intimate Relationship Between the Bacterial Communities of the Rhizocompartments in TG Sugarcane

In the present study, the enhanced drought-resistant ability of TG sugarcane is closely related to the intimate relationship between the bacterial communities of the rhizocompartments in TG plants due to both the function of plant root exudation and the beneficial bacterial communities colonized in the root-related area. The rhizoplane and rhizosphere communities are extremely close, and thus these zones are commonly regarded as a continuum (Johri et al., 2003). We found a closer relationship along this continuum in the TG plants than in the WT plants (**Figures 4C,D**), indicating that the roots of TG plants have a more dynamic activity to uptake more nutrients from the rhizosphere soil. As mentioned above, PGPR along with nitrogen-fixing bacteria are rhizosphere organisms with well-established beneficial effects on plant growth and health (Mendes et al., 2013). In our study, Rhizobiales (Alphaproteobacteria), which belongs to PGPR (Bresson et al., 2013), was identified as a highly enriched member of the core functional bacterial community in all the three zones of TG plants, and the nitrogen-fixing and chemoheterotrophic functions of bacteria in the TG plants were stronger than those of the WT (**Figures 5A,B**), both of that indicating the enhanced nutrition-absorbing ability of the TG plant bacterial community. Mineral nutrients (inorganic carbon, inorganic nitrogen, and immobile phosphate) can be dissolved by the release of some compounds of the root exudates (e.g., organic acids and amino acids) that are used by rhizosphere-dwelling microbes (Song et al., 2012; Canarini et al., 2019). It has been reported that members of Alphaproteobacteria can efficiently use carbon from metabolites generated by primary assimilators in the sugarcane rhizosphere (Da Costa et al., 2018), which is consistent with our results that the Alphaproteobacteria population increased with the enhancement of TC content in the root-associated area. In addition, changes in microbial communities can act as a feedback to plant growth (Williams and de Vries, 2020). Some specific soil microbes have been confirmed to have the ability to modify the metabolite composition of the whole plant (Fernandez et al., 2012). Plant-associated microorganisms also constitute a strong sink for plant carbon, thereby increasing the concentration gradients of metabolites and affecting root exudation (Canarini et al., 2019). Our study revealed that the levels of nutrients (TC, TN, and TP) in the TG rhizosphere increased to feed more bacteria, especially beneficial communities, residing around the roots. Plant strategies for nutrient foraging may be strongly affected by the root-associated microbial population, especially the dominant beneficial communities (Pii et al., 2015). Therefore, the changing soil environment around the root may not only be the result of root exudation but also of the activity of certain beneficial bacterial populations colonized in the rhizosphere. Such an intensified and beneficial root–microbiome interaction is expected to facilitate the growth and development of the plant and further enhance the plant's resistance to abiotic stresses.

CONCLUSION

We investigated the effects of TG sugarcane harboring the drought-resistant gene *Ea-DREB2B* on the bacterial communities of the root-associated layers (rhizoplane and rhizosphere) and bulk soil. Our results support the influence of alterations in plant genotypes by genetic modifications on plant growth and health due to the feedback from changes induced in the surrounding environment. Accordingly, the diversity and composition of the bacterial community were altered by the genetic modification in sugarcane. Most importantly, we identified a stronger and more similar relationship between the rhizoplane and rhizosphere bacterial communities and a more distant relationship between the rhizosphere and bulk soil bacterial communities in TG than in WT plants, due to a change in the soil environment caused by the alteration in root exudation. The enhancement of specific ecological functions (nitrogen fixing and chemoheterotrophy) of the TG bacterial communities further indicated their stronger beneficial effects for the plant. Overall, our study provides evidence that sugarcane root-related bacterial communities can be altered by modification in the *Ea-DREB2B* gene, which influences the ABA-mediated pathway to enhance the photosynthetic rate in plants. As DREBs are important genes for crop improvement, by enhancing the resistance of plants, we focused on the effects of TG sugarcane on the bacterial communities that interact with plants. The results will help in understanding the mechanisms of drought resistance induced by DREBs. Furthermore, our study provides information about the effects of GM plants on soil bacterial communities. However, root-associated bacterial communities are influenced by numerous factors (e.g., genotype, temperature, soil texture, and soil enzymes activities), and therefore, a comprehensive evaluation of the effects of transgenic plants on bacterial communities should be conducted taking into consideration other potentially influential factors in the future.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI SRP238824.

AUTHOR CONTRIBUTIONS

XZ and ZW contributed to design of the experiments, data analysis, and manuscript writing. YJ contributed to experimentation. QL, HY, and MZ contributed to data interpretation and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00704/full#supplementary-material

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Bacillus Responses to Plant-Associated Fungal and Bacterial Communities

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Some members of root-associated *Bacillus* species have been developed as biocontrol agents due to their contribution to plant protection by directly interfering with the growth of pathogens or by stimulating systemic resistance in their host. As rhizosphere-dwelling bacteria, these bacilli are surrounded and constantly interacting with other microbes via different types of communications. With this review, we provide an updated vision of the molecular and phenotypic responses of *Bacillus* upon sensing other rhizosphere microorganisms and/or their metabolites. We illustrate how *Bacillus* spp. may react by modulating the production of secondary metabolites, such as cyclic lipopeptides or polyketides. On the other hand, some developmental processes, such as biofilm formation, motility, and sporulation may also be modified upon interaction, reflecting the adaptation of *Bacillus* multicellular communities to microbial competitors for preserving their ecological persistence. This review also points out the limited data available and a global lack of knowledge indicating that more research is needed in order to, not only better understand the ecology of bacilli in their natural soil niche, but also to better assess and improve their promising biocontrol potential.

Keywords: *Bacillus*, rhizosphere, bioactive secondary metabolites, microbial interaction, biocontrol, molecular cross-talk, phenotype modulation

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INTRODUCTION

Some *Bacillus* species of the *B. subtilis* complex are plant-associated and important members of the microbiome (Mendes et al., 2013; Müller et al., 2016; Fierer, 2017). During the last decades, their potential use as biocontrol agents with protective activity toward economically important plant pathogens has been highlighted thereby representing a promising alternative to chemical pesticides (Expósito et al., 2017; Fan et al., 2017; Finkel et al., 2017; Fira et al., 2018; Köhl et al., 2019). The efficacy of such bacilli in plant protection, as well as their constant presence in the strongly competitive rhizosphere niche, are due to their high potential to synthesize a wide range of volatile organic compounds (VOCs) and soluble bioactive secondary metabolites (BSMs). High structural diversity is observed in the patterns of VOCs formed by *Bacillus* (Caulier et al., 2019; Kai, 2020) but also in BSMs which can be either ribosomally synthesized and post-translationally modified like bacteriocins and lantibiotics or enzymatically formed via multi-modular mega-enzymes as in the case of polyketides (PKs), di-peptides or cyclic lipopeptides (CLPs) (Harwood et al., 2018; Kaspar et al., 2019; Rabbee et al., 2019). A prime role of some soluble BSMs and volatiles in plant protection is related to their strong antimicrobial activity leading to direct antagonism against plant

pathogens (Raaijmakers and Mazzola, 2012; Borriss, 2015; Chowdhury et al., 2015a; Fan et al., 2018; Caulier et al., 2019; Rabbee et al., 2019; Kai, 2020). A second important biocontrol-related trait of those compounds is their ability to trigger an immune reaction in the host plants which leads to systemic resistance (Induced SR) rendering the plant less susceptible to pathogen infection (Pieterse et al., 2014; Chowdhury et al., 2015a; Fan et al., 2018; Caulier et al., 2019; Rabbee et al., 2019). An additional role of BSMs is also linked to an efficient plant root colonization ability of *Bacillus* which indirectly protects the plant by decreasing the space and nutrient availability for pathogens (Raaijmakers et al., 2010; Borriss, 2015; Nayak et al., 2020). Some BSMs also contribute to colonization since they are involved in the developmental processes of *Bacillus* social motility and biofilm formation (Raaijmakers and Mazzola, 2012; Borriss, 2015; Pandin et al., 2017).

As rhizosphere-dwelling bacteria, these plant-associated bacilli are influenced by various environmental factors, such as temperature, pH, moisture, light, and nutrient composition dictated by plant exudation (Santoyo et al., 2017). In this competitive niche, *Bacillus* species are also surrounded by and constantly interacting with a myriad of other (micro)organisms (Mendes et al., 2013; Traxler and Kolter, 2015; Fierer, 2017; Schmidt et al., 2019). In this review, we illustrate the diversity of BSMs produced by different *Bacillus* species and how this metabolome and phenotypic traits dictating ecological fitness can be impacted upon interaction with other fungal and bacterial microorganisms. The outcomes of volatile-based microbial interactions, in general, have been recently reviewed (Schmidt et al., 2015; Tyc et al., 2017). However, when dealing with interactions involving bacilli, information is scarce concerning possible changes in VOCs production upon cross-talk or perception of volatiles produced by other microorganisms (Chen et al., 2015; Tahir et al., 2017; Martínez-Cámara et al., 2019). Thus, we focus hereafter on interactions based on cross-talks mediated by the perception of soluble metabolites.

DIVERSITY AND BIOACTIVITIES OF *BACILLUS* BSMs

In the comparative genomic era, numerous adjustments have been done in the last years to clarify the phylogeny of the *B. subtilis* complex, which includes, among others, species, such as *B. velezensis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. subtilis* subspecies *subtilis*, *B. licheniformis*, *B. pumilus*, and *B. siamensis* with potential as biocontrol agents (Expósito et al., 2017; Fira et al., 2018; Maksimov et al., 2020), and which led to some confusion in species names but also to misassignments (Dunlap et al., 2016; Fan et al., 2017; Harwood et al., 2018; Du, 2019; Torres Manno et al., 2019). Many isolates, such as strains FZB42, QST713, or SQR9 formerly assigned to the *B. subtilis* or *B. amyloliquefaciens* species have been re-classified as *B. velezensis* representing the model species for plant-associated bacilli (Dunlap et al., 2016; Fan et al., 2017). A large part of the genome of these species is devoted to the production of antimicrobial compounds with up to 12% annotated as involved in the synthesis of bioactive secondary metabolites (Molinatto et al., 2016; Fan et al., 2017; Pandin et al., 2018).

Non-ribosomal metabolites are synthesized either by polyketide synthases (PKS) or non-ribosomal peptide synthase (NRPS), both acting as assembly lines catalyzing different steps for the incorporation of amino acid residues (Dutta et al., 2014; Winn et al., 2016; Bozhüyük et al., 2019). The three main families of *Bacillus* CLPs are surfactins, fengycins, and iturins (**Figure 1**). According to this limited number of families identified so far, the structural diversity of *Bacillus* CLPs may appear quite limited compared to other bacterial genera, such as *Pseudomonas*, for which many more different groups have been discovered (Geudens and Martins, 2018; Götze and Stallforth, 2020). However, reduced specificity of adenylation domains allows substitutions at specific places in the peptide chain and the NRPS machinery can bind different fatty acids with various chain lengths in the initiation step leading to co-production of various homologs within the three families as illustrated in **Figure 1** (Kraas et al., 2010; Bozhüyük et al., 2019). Interestingly, some CLP peptidic variants are synthesized through species-specific clusters, like pumilacidin and lichenysin which are only produced respectively by *B. pumilus* and *B. licheniformis* (**Figure 1**).

The three different types of CLPs retain specific but complementary functions considering biocontrol efficiency and, more generally, ecological fitness of the producing strains. By contributing to motility and biofilm formation, surfactins are involved in colonization of plant tissues which indirectly allow *Bacillus* to outcompete phytopathogens for space and nutrients. Surfactins are also involved in the molecular cross-talk with the host and it is well-characterized as an elicitor of plant immunity leading to ISR (Ongena and Jacques, 2008; Henry et al., 2011; García-Gutiérrez et al., 2013; Cawoy et al., 2015; Chowdhury et al., 2015a). Direct antibiotic activity of surfactins at biologically relevant concentrations toward soil-dwelling or plant-associated microbes has been only occasionally reported (Qi et al., 2010; Liu et al., 2014). By contrast, fengycins and iturins are best characterized for their antifungal activities against a wide range of plant pathogens (Caulier et al., 2019; Rabbee et al., 2019). This is mainly due to their ability to perturb fungal cell membrane integrity resulting in cytoplasm leakage and finally hyphae death and inhibition of spore germination (Chitarra et al., 2003; Romero et al., 2007; Deleu et al., 2008; Etchegaray et al., 2008; Gong et al., 2015; Gao et al., 2017; Zhang and Sun, 2018). The three CLPs retain some selectivity but may also act synergistically to inhibit fungal growth (Liu et al., 2014). The lipid composition of the plasma membrane could explain differences in the sensitivity of fungal targets to one or more CLPs (Wise et al., 2014; Fiedler and Heerklotz, 2015).

Besides lipopeptides, most species of the *B. subtilis* group also produce other non-ribosomal oligopeptide derivatives, such as bacilysin, chlorotetaine, bacitracins, and rhizocticins which are known to be efficient as antibacterial compounds targeting cell wall biosynthesis (Zhao and Kuipers, 2016). The siderophore bacillibactin is highly conserved in the *B. subtilis* group (**Figure 1**)

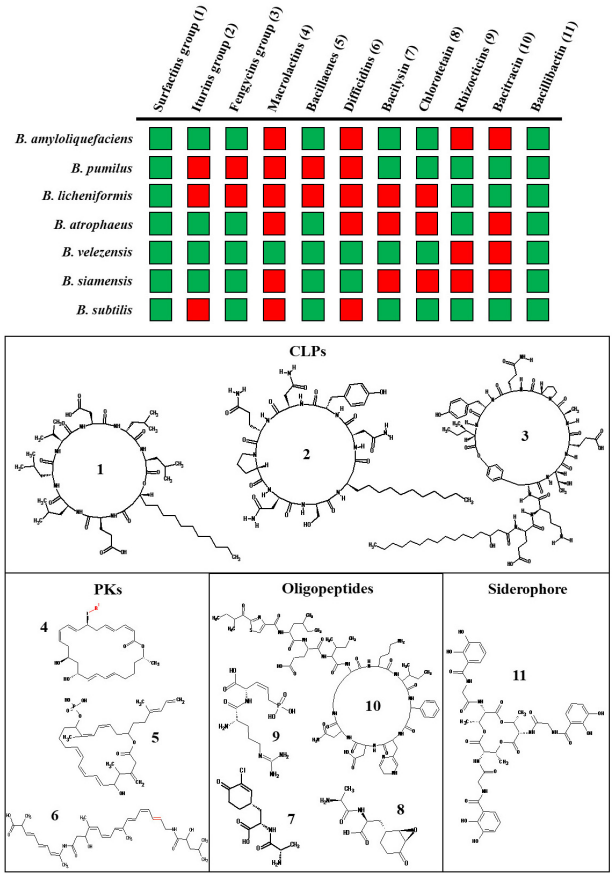

FIGURE 1 | Main non-ribosomal BSMs produced by the various species in the *B. subtilis* complex. The BSMs production is indicated for the following species *B. subtilis, B. siamensis, B. velezensis, B. atrophaeus, B. amyloliquefaciens, B. pumilus*, and *B. licheniformis* by a green square whereas red square indicates an absence of production of the BSMs in this species (Zhao and Kuipers, 2016; Fan et al., 2017; Harwood et al., 2018; Du, 2019; Torres Manno et al., 2019). The surfactins, iturins, and fengycins groups include lichenycin (1;AA1:L-Gln) and pumilacidin (1;AA4: L-Leu, AA7 = I-Ile); mycosubtilin (2;AA6: D-Ser, AA7 = L-Thr) and bacillomycin (2; AA6: D-Ser, AA7 = L-Asn); maltacin (4;AA1: L-Ser), agrastatin (4;AA10: L-Val) and plipastatin (4;AA9: D-Tyr), respectively. The structure of the representative metabolite is indicated by a number and represented below. The possible variations in the PKs structure are highlighted in red. For the macrolactin family, the main variants are R = H; CO-CH_2-COOH; CO-CH_2-CH_2-COOH or 6-*O*-succinyl-β-glucose (for review see Piel, 2010).

and is induced in response to iron limitation in the environment. It allows *Bacillus* to efficiently acquire Fe^{3+} and other metals (Miethke et al., 2006, 2008; Li et al., 2014) thereby depriving phytopathogens of this essential element (Miethke et al., 2006; Niehus et al., 2017).

Polyketide biosynthesis is performed by successive condensation of small carboxylic acids mediated by core domains of the corresponding enzyme machinery but some PKs are synthesized via hybrid NRPS/PKS systems leading to the integration of amino acid residues (Piel, 2010; Olishevska et al., 2019). The three main PKs produced by *Bacillus* are difficidins, macrolactins, and bacillaenes, the latter being more widespread across species (**Figure 1**). The main PKs role is related to their antibacterial activity via the ability to inhibit protein biosynthesis in numerous phytopathogenic bacteria but certain antifungal activity has been reported for bacillaenes and macrolactins (Caulier et al., 2019; Olishevska et al., 2019).

Ribosomally synthetized BSMs encompass bacteriocins and lantibiotics including plantazolicin, subtilin, ericin, mersacidin, amylolysin, and amylocyclicin that are specifically produced by some species or strains (Brötz et al., 1998; van Kuijk et al., 2012; Arguelles Arias et al., 2013; Scholz et al., 2014; Torres Manno et al., 2019). These BSMs are responsible for growth inhibition of Gram-positive bacteria by acting via different modes of action (Abriouel et al., 2011; Acedo et al., 2018).

PERCEPTION OF FUNGI TRIGGERS THE PRODUCTION OF APPROPRIATE BSMs

Several works have illustrated the impact of phytopathogenic fungi on BSMs production by soil bacilli. Some *B. amyloliquefaciens*, *B. velezensis*, and *B. subtilis* strains respond to the presence of antagonistic fungi by stimulating the production of the antifungal CLPs fengycins and/or iturins (**Table 1**). Not only the production of specific CLPs varies in a species-dependent manner but it is also highly dependent on the interacting fungal species. For example, much higher production of iturins and fengycins by *B. subtilis* 98S was observed in confrontation with *Pythium aphanidermatum* and *Fusarium oxysporum* but not with *Botrytis cinerea* (Cawoy et al., 2015). Further, upon interaction with fungi, some *B. velezensis* strains (SQR9, FZB42, and S499) overproduced either iturins or fencycins (Li et al., 2014; Chowdhury et al., 2015b; Kulimushi et al., 2017). For instance, Li et al. (2014) showed that when confronted with *Sclerotinia sclerotiorum, B. velezensis* SQR9 overproduces bacillomycin D (iturin family), but not fengycins. An overproduction of bacillomycin along with a reduced production of fengycins was also reported by Chowdhury et al. (2015b) upon *B. velezensis* FZB42 interaction with *Rhizoctonia solani* in the rhizosphere of lettuce plants. Differentially, Kulimushi et al. (2017), showed that strains S499 and FZB42 improved production of fengycin but not iturins upon interaction with *Rhizomucor variabilis*. Most of these studies also indicated that fengycins and iturins are the main BSMs responsible for antifungal activities (**Table 1**). Thus, *Bacillus* cells could specifically sense the presence of fungal competitors and accordingly overproduce appropriate antifungal BSMs to outcompete the interacting fungi. Moreover, besides modulating the production of fengycins and iturins, some strains of *B. velezensis* (SQR9, FZB42, and QST713) and *B. subtilis* (B9-5) may overproduce surfactins when sensing phytopathogenic fungi (Li et al., 2014; Chowdhury et al., 2015b; DeFilippi et al., 2018; Pandin et al., 2019). In support to this hypothesis, surfactin production of *B. velezensis* FZB42 was highly induced in the presence of fungal pathogen *R. solani* in the lettuce rhizosphere where it was found as the main produced compound (Chowdhury et al., 2015b). A similar response was recorded when *B. velezensis* SQR9 was confronted with

S. sclerotiorum and *Phytophthora parasitica* (Li et al., 2014) or when *B. subtilis* B9-5 interacted in liquid medium with *Rhizopus stolonifer* (DeFilippi et al., 2018). In contrast to fengycins and iturins, surfactins are not strong direct antifungal metabolites in biologically relevant concentrations (Raaijmakers and Mazzola, 2012). Thus, it stays unclear why *Bacillus* responded by surfactin overproduction to the presence of antagonistic fungi. A possible explanation could be rooted in its global role promoting the rhizosphere and thereby, contributing to competition for nutrients and space with the interacting fungi (Ongena and Jacques, 2008; Rabbee et al., 2019).

Even though the siderophore bacillibactin is produced by all members of the *B. subtilis* species complex (**Figure 1**), its possible overproduction upon microbial interactions has been poorly investigated. Interestingly, the work of Li et al. (2014) showed that *B. velezensis* SQR9 overproduces bacillibactin when grown in presence of a range of fungi including *V. dahliae*, *S. sclerotiorum*, *F. oxysporum*, *R. solani*, *F. solani*, and *P. parasitica*. This may be interpreted as a response of the bacterium to some iron-limitation in the medium caused by the fungi via the release of their own chelatants.

In *B. subtilis*, the expression of many BSMs biosynthesis genes is transcriptionally fine-tuned by compound-specific regulation but also by global regulators governing the transition to crucial developmental processes like motility, biofilm formation and sporulation (Inaoka et al., 2009; López et al., 2009; Vargas-Bautista et al., 2014). Fungal triggers may affect both types of regulatory systems involved in BSMs production. For instance, upon sensing *F. verticillioides,* the global stress-related regulator SigB is activated in *B. subtilis* NCIB3610 which in return enhances surfactin production (Bartolini et al., 2019). In interaction with *F. culmorum* under biofilm-conducive conditions, *B. subtilis* Bs12 down-regulates the expression of the *sin*R gene known as a repressor of biofilm formation which also negatively regulates surfactin production (Kearns et al., 2005; Khezri et al., 2016; Zhi et al., 2017). These observations strongly suggest that specific soluble signals, emitted by fungal pathogens, could be perceived by bacilli which in turn modulate BSMs synthesis. As observed by Bartolini et al. (2019), cells of the *Bacillus* colony, physically close to the fungal culture, responded to signals by over-expressing genes coding for transcription factors involved in CLPs synthesis regulation. In contrast, colony cells positioned on the opposite side of the fungi did not react to the fungus (Bartolini et al., 2019). This phenomenon indicates that the specific fungal metabolite diffuses on a short distance and has an influence on closely located *Bacillus* cells. Currently, no fungal compounds have been identified as triggers of BSM stimulation in *Bacillus*. Nonetheless, few commonly produced metabolites by *Fusarium* species were suggested to modify *Bacillus* behavior. It was shown that two cyclic depsipeptides (enniatins B1 and enniatins A1) and a pyrone (lateropyrone) had an antagonistic effect on *B. subtilis* growth (Ola et al., 2013). Fusaric acid also modified antibacterial activity of *B. mojavensis* but it was not related to a decrease in the production of specific BSMs (Bacon et al., 2004, 2006; Bani et al., 2014). These metabolites could also play a triggering role at sub-inhibitory concentration and could have an inducible effect on the range of *Bacillus* responses as has been shown for other signal metabolites (Bleich et al., 2015; Liu et al., 2018).

BACILLUS PHENOTYPE IS MODULATED UPON PERCEPTION OF BACTERIAL COMPETITORS

Some BSMs may also act as molecular determinants driving outcomes of interactions between *B. subtilis* and bacterial competitors as illustrated for the bacillaene polyketide displaying an essential protective role for survival in competition with *Streptomyces* soil isolates (Straight et al., 2007; Barger et al., 2012). However, there are few direct evidences for enhanced expression of BSMs upon interbacteria interactions. The only convincing examples involve the interaction of plant-associated bacilli with plant pathogens, such as *Ralstonia solanacearum* (Almoneafy et al., 2014) and *Pseudomonas fuscovaginae* (Kakar et al., 2014). In these two studies, improved expression of surfactin, bacilysin, and iturin biosynthesis genes were observed when *Bacillus* and pathogens were grown together in dual-cultures. Nevertheless, no clear indication about the enhanced production of the aforementioned BSMs based on their quantification nor improved antibacterial activities of *Bacillus* was presented as a result of this interaction.

Interestingly, at the phenotypical level, the development of soil bacilli is differentially altered upon sensing other bacteria from the same natural environment. Some of these phenotypical changes can be associated or due to a modulated production of specific BSMs. First, exogenous antibiotics or signals may stimulate biofilm formation which depends, to some extent, on surfactin production (López et al., 2009) and which may be viewed as a defensive response against exogenous toxic compounds and/or infiltration by competitors (Flemming et al., 2016; Townsley and Shank, 2017; Molina-Santiago et al., 2019). For instance, *B. subtilis* increased its relative subpopulation of biofilm matrix-producing cells in response to small molecules secreted by other bacterial species (López et al., 2009; Shank et al., 2011). The same phenomenon was illustrated for thiazolyl peptides emitted by closely related species, such as *B. cereus* and putatively formed by other soil microbes, such as *Streptomyces* isolates (Bleich et al., 2015). However, no change in surfactin production associated with the stimulation of biofilm was reported in these studies.

Besides biofilm formation, other mechanisms drive bacteria to initiate protective responses upon the detection of competitors. The flagellum-independent sliding motility is considered as an adaptive mechanism that allows bacterial cells to physically relocate in the context of a competitive interaction (Wadhams and Armitage, 2004; Jones et al., 2017; McCully et al., 2019). Upon sensing *S. venezuelae*, the *B. subtilis* ability to slide was increased (Liu et al., 2018). It depends in part on the production of surfactin (Grau et al., 2015; van Gestel et al., 2015) but a potential boost in lipopeptide synthesis upon the perception of the *Streptomyces* challenger was not demonstrated. Chloramphenicol and derivatives produced by *S. venezuelae* were identified as

TABLE 1 | Change in expression and bioactivity of BSMs produced by members of *B. subtilis* group, upon interaction with fungal species.

BSMs	Change in expression	Involvement in antifungal activity	*Bacillus* species (strains)	Fungal species	References
Fengycins	0	Yes	*B. subtilis* (98S)	*B. cinerea*	Cawoy et al., 2015
	+	Yes	*B. subtilis* (98S)	*F. oxysporum*	Cawoy et al., 2015
	+	No	*B. subtilis* (98S)	*P. aphanidermatum*	Cawoy et al., 2015
	+	Yes	*B. velezensis* (S499)	*R. variabilis*	Kulimushi et al., 2017
	+	Yes	*B. velezensis* (FZB42)	*R. variabilis*	Kulimushi et al., 2017
	0	Yes	*B. velezensis* (QST713)	*R. variabilis*	Kulimushi et al., 2017
	+	Yes	*B. velezensis* (SQR9)	*Verticillium dahliae*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*F. oxysporum*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*Phytophthora parasitica* var. *nicotianae*	Li et al., 2014
	-	Mediating the plant defense expression	*B. velezensis* (FZB42)	*R. solani*	Chowdhury et al., 2015b
	+	ND	*B. subtilis* (B9-5)	*R. stolonifer*	DeFilippi et al., 2018
	+	ND	*B. subtilis* (B9-5)	*Fusarium sambucinum*	DeFilippi et al., 2018
	+	ND	*B. subtilis* (B9-5)	*V. dahliae*	DeFilippi et al., 2018
	+	ND	*B. velezensis* (QST713)	*Trichoderma aggressivum f. europaeum*	Pandin et al., 2019
Iturins	0	Yes	*B. subtilis* (98S)	*B. cinerea*	Cawoy et al., 2015
	+	Yes	*B. subtilis* (98S)	*F. oxysporum*	Cawoy et al., 2015
	+	No	*B. subtilis* (98S)	*P. aphanidermatum*	Cawoy et al., 2015
	+	No	*B. velezensis* (SQR9)	*V. dahliae*	Li et al., 2014
	+	No	*B. velezensis* (SQR9)	*S. sclerotiorum*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*F. oxysporum*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*P. parasitica*	Li et al., 2014
	+	Mediating the plant defense expression	*B. velezensis* (FZB42)	*R. solani*	Chowdhury et al., 2015b
Surfactins	+	Yes	*B. velezensis* (SQR9)	*S. sclerotiorum*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*R. solani*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*Fusarium solani*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*P. parasitica*	Li et al., 2014
	+	Mediating the plant defense expression	*B. velezensis* (FZB42)	*R. solani*	Chowdhury et al., 2015b
	+	ND	*B. subtilis* (B9-5)	*R. solani*	DeFilippi et al., 2018
	+	ND	*B. subtilis* (B9-5)	*F. sambucinum*	DeFilippi et al., 2018
	+	ND	*B. subtilis* (B9-5)	*V. dahliae*	DeFilippi et al., 2018
	+	ND	*B. velezensis* (QST713)	*T. aggressivum f. europaeum*	Pandin et al., 2019
Bacillibactin	+	Yes	*B. velezensis* (SQR9)	*V. dahliae*	Li et al., 2014
	+	No	*B. velezensis* (SQR9)	*S. sclerotiorum*	Li et al., 2014
	+	No	*B. velezensis* (SQR9)	*F. oxysporum*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*R. solani*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*F. solani*	Li et al., 2014
	+	Yes	*B. velezensis* (SQR9)	*P. parasitica*	Li et al., 2014

"0" indicates no changes, "+" enhanced and, "–"decreased BSMs production by Bacillus upon interaction with fungi. "Yes" indicates fungitoxic activity, "No" no antifungal activity, "ND" indicates that BSMs with antifungal activity are not detected.

molecular triggers acting at subinhibitory concentrations for inducing *Bacillus* motility (Liu et al., 2018).

Multiple bacteria promote sporulation in *B. subtilis* which represents another example of alteration of the physiological development of this species. In a context of distant interactions, exogenous siderophores accelerate the differentiation of *Bacillus* cells into spores. It was notably shown for enterobactin from *E. coli* and for ferrioxamine E produced by *Streptomycetes* (Grandchamp et al., 2017). In iron-limited environments, *B. subtilis* cells would thus respond by taking up those "piratable" siderophores and start sporulating. This is not a general response to xenosiderophores since for instance, pyochelin from *Pseudomonas* does not affect *Bacillus* sporulation (Molina-Santiago et al., 2019). Nevertheless, the ability of siderophores

to alter cellular differentiation in *B. subtilis* suggests that those molecules are likely to mediate complex microbial interactions in iron-depleted conditions, as often met in a soil environment. However, induction of *B. subtilis* sporulation by other bacteria may also occur in a cell-to-cell contact situation. Upon interaction with *P. chlororaphis*, its type VI secretion system acted as a trigger for sporulation, independently from its established role as cargo for delivering toxic effectors into the target *Bacillus* cells (García-Bayona and Comstock, 2018; Molina-Santiago et al., 2019).

That said, interspecies interactions may also result in inhibition rather than in stimulation of key developmental processes determining the fate of *Bacillus* multicellular communities. As an example, 2,4-diacetylphloroglucinol, a broad-spectrum antibiotic synthesized by fluorescent *Pseudomonas*, alters colony morphology, inhibits biofilm formation and sporulation in *B. subtilis* populations grown adjacent to *P. protegens* colonies (Powers et al., 2015). This antibiotic seems to act as an interspecific signaling molecule that inhibits bacterial differentiation at subinhibitory concentrations (Powers et al., 2015).

CONCLUSION

Here we provide an overview of the phenotypic and molecular responses of plant-beneficial soil bacilli upon sensing signals from other microorganisms that can be encountered in the rhizosphere niche. It is clear that BSMs production by *Bacillus* can be modulated upon interactions with other microbes and that key BSM-driven developmental processes may undergo unsuspected changes. It somehow illustrates the flexibility of these bacteria in re-directing their secondary metabolome to adapt environmental fitness upon sensing the presence of neighboring microorganisms. Nevertheless, the molecular mechanisms integrating the perception of exogenous triggers with a regulatory response leading to enhanced production of BSMs still remain unclear.

A significant boost in BSMs production by soil bacilli has been reported in most cases as an outcome from interactions with plant pathogenic fungi. This is of value in the context of biocontrol of fungal pathogens since direct antagonism is considered as the most powerful mode of action for suppression of plant diseases (Fravel, 2005; Frey-Klett et al., 2011; Köhl et al., 2019). By contrast, direct evidence for an impact of interbacteria interactions on the expression of the secondary metabolome in *Bacillus* is still globally missing. Nevertheless, interaction-mediated variations in colony morphology, motility, biofilm formation, or sporulation illustrate how soil bacilli can protect themselves from antimicrobials emitted by bacterial competitors. Such an impact on those key developmental processes should thus be coupled with significant modulation in the production of specific BSMs underpinning these phenotypes. Depending on the concentration, these BSMs would then act as antimicrobials in interference competition or as signals in cooperative interspecies communication processes not necessarily affecting the growth of the partners (Bleich et al., 2015; Liu et al., 2018). However, this has yet to be thoroughly demonstrated and future examination of developmental controls for BSMs biosynthesis will likely bring light upon the key principles driving environmental fitness of soil bacilli as intrinsically influenced by interspecies competition.

From an ecological viewpoint, further investigations would also help to better understand why soil amendment with selected bacilli, even at high doses, do not durably impact the composition of the rhizosphere microbiome despite their huge arsenal in antimicrobial weapons (Correa et al., 2009; Chowdhury et al., 2013; Kröber et al., 2014; Qiao et al., 2017) and by contrast with some other bacteria and fungi (Buddrus-Schiemann et al., 2010; Chowdhury et al., 2013; Erlacher et al., 2014; Thomas and Sekhar, 2016; Wu et al., 2016). Those bacilli may thus provide protection to their host plant toward microbial pathogen ingress but would avoid detrimental effect on its naturally selected beneficial microbiome which is of prime interest for future application as biocontrol agents.

AUTHOR CONTRIBUTIONS

SA, TM, and MO conceived the idea, designed the outlines of the review, and wrote the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Bacterial Communities of the Canola Rhizosphere: Network Analysis Reveals a Core Bacterium Shaping Microbial Interactions

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The rhizosphere hosts a complex web of prokaryotes interacting with one another that may modulate crucial functions related to plant growth and health. Identifying the key factors structuring the prokaryotic community of the plant rhizosphere is a necessary step toward the enhancement of plant production and crop yield with beneficial associative microorganisms. We used a long-term field experiment conducted at three locations in the Canadian prairies to verify that: (1) the level of cropping system diversity influences the α- and β-diversity of the prokaryotic community of canola (*Brassica napus*) rhizosphere; (2) the canola rhizosphere community has a stable prokaryotic core; and (3) some highly connected taxa of this community fit the description of hub-taxa. We sampled the rhizosphere of canola grown in monoculture, in a 2-phase rotation (canola-wheat), in a 3-phase rotation (pea-barley-canola), and in a highly diversified 6-phase rotation, five and eight years after cropping system establishment. We detected only one core bacterial Amplicon Sequence Variant (ASV) in the prokaryotic component of the microbiota of canola rhizosphere, a hub taxon identified as cf. *Pseudarthrobacter* sp. This ASV was also the only hub taxon found in the networks of interactions present in both years and at all three sites. We highlight a cohort of bacteria and archaea that were always connected with the core taxon in the network analyses.

Keywords: bacteria, archaea, microbial ecology, agroecosystem, crop rotations, *Brassica napus*

INTRODUCTION

A plant in its natural environment coexists with myriads of archaea, bacteria, fungi, as well as with other unicellular eukaryotic microorganisms that constitute its microbiota. The rhizosphere is a hotspot of microbial interactions between species that have various ecological functions. These microbial communities are particularly important for plant health as they influence its development

and its productivity (Barriuso et al., 2008; Bulgarelli et al., 2013; Bakker et al., 2014). Throughout their life, plant roots exude compounds creating the rhizosphere environment (Bais et al., 2006). Spatial and temporal variation in rhizodeposition allows plants to shape their rhizosphere microbial communities to their benefit (Tkacz et al., 2015; Pii et al., 2016; Eisenhauer et al., 2017).

Plant rhizosphere can host mutualistic microbes such as mycorrhiza or plant growth promoting bacteria (PGPB) that facilitate nutrient uptake, mitigate abiotic stress, and prevent root infection by pathogens (Barriuso et al., 2008; Farina et al., 2012; Fincheira and Quiroz, 2018). Plant-microbe and microbe-microbe interactions are diverse. Plants live in symbiotic and commensal relationships with numerous organisms, but they must also face pathogenic attacks (Hajishengallis et al., 2012). Rhizosphere organisms may influence each other, thus forming a complex web of interactions. For example, we know that mycorrhizal fungi have their own bacterial microbiota (Bianciotto et al., 2003; Iffis et al., 2014, 2017). These bacteria can be endophytic or form biofilm at the surface of the hyphae and can facilitate symbiosis formation in plants (Fitter and Garbaye, 1994; Iffis et al., 2014; Taktek et al., 2017).

Since the last decade, new generation sequencing (NGS) improved our access to microbial genetic information leading to significant advances in microbial ecology. This technological improvement lead to new ways of analyzing plant microbial communities (Duffy et al., 2007; Bulgarelli et al., 2013; Mendes et al., 2015). Now, we can identify with confidence the factors shaping the microbial communities of the rhizosphere (Kuramae et al., 2011; Agler et al., 2016). The microbiome of the rhizosphere is extremely large and diverse. To summarize this complexity, we can divide it into pools of microbes based on their functions or occurrence (Ridout and Newcombe, 2016). In a given community, microbial taxa are likely to be favored by their host plant throughout its existence (Rout, 2014). These taxa are expected to be always part of the plant microbiota at a defined time *t*, regardless of environmental conditions. According to Vandenkoornhuyse et al. (2015), the taxa always present in association with the plant forms the core microbiome and have preferential interaction with their host. The definition of a pool of microorganisms always present at t time in the plant microbiota is appropriate for most ecological studies concerning the plant microbiota as they mostly rely on a single sampling time. However, it was necessary to consider temporal variation in our definition of the core microbiota, and this is what we did in this study.

The interactions between microbes in the plant rhizosphere remains largely obscure. Next Generation Sequencing techniques can provide information on the abundance of the taxa interacting in a microbiome, but cannot reveal the biochemistry of interacting microbes in the ecosystem. That is why computational approaches aiming at identifying the nature of the links between the variations in the abundance of microbial taxa were developed as a complement to NGS (Ings et al., 2009; Deng et al., 2012; van der Heijden and Hartmann, 2016). Network analysis allows us to identify microbial taxa that are functionally linked to others within the microbiome. Highly connected microorganisms may have a greater impact on plants and ecosystem functioning than others, because they theoretically interact with many partners and antagonists; these highly interacting species are named hub taxa (Agler et al., 2016). Interactions occurring in microbial communities are known to be complex and difficult to retrieve with usual statistical methods (Kurtz et al., 2015). However, the information provided by NGS can be processed through network analysis to identify cohorts represented by hub taxa. Simplifying the study of complex microbiome, Taktek et al. (2017) showed taxa that recruit organisms beneficial to the host plant, but hub taxa could also be pathogens. Some hub taxa in the human microbiome can articulate infection by consortia of pathogens (Hajishengallis et al., 2012). As pathogens can affect the plant microbiome, pathogenic hub taxa may occur in the rhizosphere. The hub taxa are a useful concept and help to understand the ecology of the root and rhizosphere ecosystems, which could lead to the development of applications in crop plant root systems.

Canola was shown to possess a specific bacterial component of the core microbiota conserved across the Canadian prairie (Lay et al., 2018). Floc'h et al. (2020) reported the temporal stability of the fungal component of the core microbiota in canola rhizosphere, despite considerable changes in the plant rhizosphere microbiota across years. In the present study, we aimed to test if the bacterial component of the canola microbiota has a similar pattern of temporal variation. We investigated the temporal stability of the bacterial component of the core canola rhizosphere microbiota in order to ascertain whether a persistent bacterial component exists. Another aim was to determine if the canola rhizosphere harbors bacterial hub taxa, and to visualize the variation between years in the structure of interactions among the bacteria living in the canola rhizosphere microbiota. We sought to identify a universal bacterial component of the core microbiota in the rhizosphere of a plant species, specifically canola grown over the years under a range of climatic conditions and biological environments. We thus used a gradient of crop diversification levels to create variation in the biological environment of rhizosphere soil and examine over two years what in the bacterial component of the canola microbiota is invariable: the core microbiota. Canola is a crop of economical importance for Canada. It is also a good model plant to study the rhizosphere microbiome as canola produce antimicrobial isocyanates (Zheng et al., 2014) leading to simpler microbial communities in its rhizosphere (Rumberger and Marschner, 2003).

MATERIALS AND METHODS

Three sites located in three pedoclimatic zones of the canola-producing area of western Canada were used. Two sites were in Alberta, specifically in Lacombe (lat. 52.5°N, long. 113.7°W) and Lethbridge (lat. 49.7°N, long. 112.8°W), and the third site was in Scott, Saskatchewan (lat. 52.4°N, long 108.8°W). The soil in Lethbridge is a Brown Chernozem with a silty loam texture, while the Dark Brown Chernozems have a loamy texture at the Scott site and a clay loam texture at Lacombe.

Plots of a larger long-term crop rotation experiment initiated in 2008 were used for this study. Site description, experimental design and sampling methods are described in details in Floc'h

et al. (2020). This study had a complete randomized block design replicated at three geographic sites, each with 4 blocks and 4 crop rotation treatments, and we collected samples from the Roundup Ready (RR) canola phase of the crop rotations on 2 years, in 2013 and 2016. The four treatments were four levels of cropping system diversification: (1) monoculture of RR canola, (2) wheat-RR canola, (3) pea-barley-RR canola, and (4) lentil-wheat-Liberty Link canola-pea-barley-RR canola (**Table 1**). Crops were grown according to best management practices. Information on crop management is described in Harker et al. (2015).

Rhizosphere samples were collected during the fourth week of July in 2013 and 2016, which corresponds to the end of canola flowering period. Three to four plants randomly selected within each plot were uprooted with a shovel. The shoots were removed and roots were placed in plastic bags and brought to the laboratory on ice in a cooler. About 5 g of rhizosphere soil per plot was collected by gently brushing the roots. The samples were kept at 4°C before being shipped on ice to Lethbridge, Alberta, where they were preserved at -80°C until DNA extraction.

DNA Extraction and Amplification

DNA extraction was conducted as described in Floc'h et al. (2020). We constructed amplicon libraries for bacterial 16S rRNA gene sequences by using target-specific PCR primers attached to Illumina overhang sequences for NextEra library preparation. The primer pairs were GTGCCAGCMGCCGCGGTAA (515F-Illu) and GGACTACHVGGGTWTCTAAT (806R-Illu). This primer set was selected because it is used by the Earth Microbiome Project.[1] Two PCR reactions were performed to prepare the amplicon library. In the first PCR reaction, the V4 hypervariable region of prokaryotic 16S RNA genes was amplified using primers previously described (515F and 806R). The PCR reaction was performed in a 25-μL reaction mixture containing 1 μL of template DNA, 1 × PCR-buffer (Qiagen, Germantown, MD, United States), 1.8 mM $MgCl_2$, 1.25 μL of 5% dimethylsulfoxide (DMSO), 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, United States), and 0.6 μM of each primer. The 5′ ends of the forward and reverse primers were tagged with CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT), respectively, which were used as anchors for the PCR reaction. The conditions to amplify the prokaryotic 16S rRNA fragments consisted of an initial denaturation at 94°C for 2 min, 33 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s, followed by a final elongation at 72°C for 7 min.

The second PCR reaction was used to add barcodes to each sample and the Illumina sequencing adapters. This PCR reaction was performed in a 20-μl reaction mixture, containing 1 × PCR-buffer (Qiagen, Germantown, MD, United States), 1.8 mM $MgCl_2$, 1 μl of 5% DMSO, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Roche, Branford, CT, United States), 2 μM of NextEra XT index primers (Illumina Inc., San Diego, CA, United States), and 1 μL of 1/150 dilution of the first PCR products. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, 15 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min followed by a final elongation at 72°C for 3 min. After the second amplification, PCR products were quantified using Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Life Technologies, Canada) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (D-Mark, Canada). The amplicon library was purified using calibrated AMPure XP beads (Agencourt, United States), and the average size and quantity of each library were assessed on the LabChip GX (Perkin Elmer, United States) instrument. The library was then sequenced on Illumina MiSeq using the paired-end 250 protocol at Génome Québec Innovation Centre at McGill University (Montreal, Canada).

ASV Determination and Bioinformatic Pipeline

The bioinformatic pipeline used for the processing of our 16S rRNA gene sequences from 2013 and 2016 was DADA2 v1.8 (Callahan et al., 2016). We first used Cutadapt 1.13 to remove the primer part of the 16S rRNA gene sequences. Then, we excluded the sequences with less than 200 bp as the base quality of the sequences tended to diminish below that threshold in our data with the command "filterAndTrim" with a "maxEE" score of 2, "trunQ" score of 2 and "minLen" argument set to 50. Then, we calculated the error rate using the machine learning algorithm implemented in DADA2 with the command "learnErrors." As the error rate was satisfying according to developer's recommendations, we merged the forward and reverse sequences using the command "mergePairs." Afterward, the Amplicon Sequence Variant (ASV) table was calculated and the chimeras eliminated using the command "makeSequenceTable," resulting in a sequence length ranging from 250 to 253 nucleotides. ASVs were then identified using the naïve Bayesian classifier method on the databases SILVA and RDP, and the identity of ASVs of interest was verified manually using BLAST on the NCBI nt database. With the taxonomic resolution of the 16S RNA gene, it is generally not possible to identify a bacterium at the species level. Thus, the identifications at species level presented here must be consider with caution despite they perfectly match (100% similarity and coverage) the reference sequences of NCBI.

[1] http://www.earthmicrobiome.org/emp-standard-protocols/16s/

TABLE 1 | Selected treatments from a long-term experiment established in 2008 at three different sites in the Canadian prairies (Harker et al., 2015).

	Cropping systems	
Diversification level	**2008–2013**	**2008–2016**
Monoculture	RR-RR-RR-RR-RR-RR[1]	RR-RR-RR-RR-RR-RR-RR-RR-RR
Low	W-RR-W-RR-W-RR	RR-W-RR-W-RR-W-RR-W-RR
Medium	P-B-RR-P-B-RR	P-B-RR-P-B-RR-P-B-RR
High	Len-W-LL[2]-P-B-RR	Len-W-LL-P-B-Len-W-P-RR

The rotation phases examined in this study in 2013 and 2016 are underlined. [1]RR, canola 71-45, a Roundup Ready cultivar resistant to glyphosate. [2]LL, canola InVigor 5440 Liberty Link, cultivar resistant to glufosinate.

The MiSeq sequencing data generated as part of this work are publicly available on Zenodo.[2]

Data Processing and Statistical Analyses

We first wanted to assess the variation occurring in canola rhizosphere caused by the crop diversification systems. The dataset was standardized by randomly subsampling the read data from each sample to the lowest number of reads (13 241) encountered for a sample, using the function "rrarefy" of the vegan package v.2.4.6 in R v. 3.4.3, before calculating Chao1 (Chao, 1984), Shannon and Simpson's α-diversity indices using the same package.

The significance of crop diversification effect on α-diversity indices was tested by analysis of variance (ANOVA) one year at a time, combining sites and blocks in one random effect with 12 blocks (four blocks per each of the three sites), and comparisons between treatment means were made with Tukey's *post-hoc* tests using the R package agricolae v1.3.1 (Mendiburu, 2015). The effect of crop diversification on bacterial community structure was assessed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), considering 12 blocks (four blocks per each of the three sites), using the function "adonis" of the vegan package v 2.4.6 (Oksanen et al., 2019) in R v3.4.3, and the entire (non-subsampled) set of relative abundance data. The blocked multi-response permutation procedure (BMRPP) was used for pairwise comparison of community structure under the different crop diversification treatments. using Šidák correction for pairwise comparison in the R package "RVAideMemoire" v0.9 (Hervé, 2015).

After determining the impact of crop diversification on canola rhizosphere, we aimed at identifying its universal bacterial component of the core microbiota and hub taxa. We defined the core microbiota as the set of organisms that are present in the microbiota at all sites and plots at t and t+1. To assess the interactions among bacterial taxa in the microbiota, we created a co-occurrence network using the package SPIEC-EASI v 1.0.6 in R 3.4.3 (Kurtz et al., 2015). The analysis was conducted over all bacterial rhizosphere communities of each year. The input data consisted in the matrix of the raw abundance of ASVs of one year of sampling. We first filtered the dataset to remove the ASVs with a frequency less than 20%. The SPIEC-EASI run was done with the algorithm "mb" with the lambda min ratio set at 10^{-2} and 50 repetitions. We then imported the networks in Cytoscape 3.7.1 (Smoot et al., 2011) for plotting and used the "organic" layout to draw the network. Edges where defined as co-occurrences or mutual exclusion regarding the positives or negatives values of inverse covariance linking the nodes. Betweenness centrality, defined as the fraction of the shortest path between all other nodes in the network containing the given node, and degree score, highlight central nodes and provide information about network architecture. A score of betweenness centrality and degree of connectivity greater than the score of 95% of the network taxa could suggest participation in multipartite interactions in the community and allow us to flag the highly connected taxa as hub-taxa. Hub-taxa were defined as the nodes possessing a score of betweenness centrality > 0.40 and a degree score > 10.

Spearman's correlations between abundance of hub-taxa and of their cohorts with canola yield were computed on R 3.4.3.

[2]https://zenodo.org/record/3626047#.XisHASZOmV4

RESULTS

Taxonomic Affiliation of the Bacterial Component of the Canola Rhizosphere Microbiota

Our bioinformatic pipeline retrieved 2 175 992 reads from the 96 samples, that were assigned to 10 385 ASVs. Read number per sample ranged from 10 938 to 60 896. The ASVs belong mostly to four bacterial phyla that did not vary substantially in abundance in the two years of study: *Proteobacteria* (25%), *Actinobacteria* (22.5%), *Acidobacteria* (16%), and *Chloroflexi* (13%) (**Figure 1**). Rarefaction curves indicated that read abundances were close to saturation for all the samples (**Supplementary Figure S2**).

Effect of Treatments on Communities

Crop diversification had no significant influence on α-diversity indices (**Table 2**) or on the structure of bacterial communities of canola rhizosphere in 2013 (**Table 3**). On the other hand, crop diversification significantly affected the community structure of canola rhizosphere in 2016 by PERMANOVA ($P = 0.047$), where the rhizosphere communities of canola in monoculture and in the highly diversified system were structurally different according to the BMRPP test (**Table 3**). PCoA analyses showed a clear segregation of prokaryotic communities by site, but did not show clear patterns between diversification levels either in 2013 and 2016 (**Supplementary Figures S3, S4**). Since the prokaryotic communities segregated per site, additional PERMANOVA were conducted to assess whether, within each site, the crop diversification level has an effect on the microbiota structure. Results did not show any differential effect of crop diversification per site and year (**Table 3**). Indicator species analysis revealed ASVs significantly associated with crop diversifications. In 2013, the highest level of crop diversification had the highest number of indicator species (15), whereas monoculture had nine and the diversification treatment with wheat and canola had only one (**Table 4**). No indicator species was found in association with the medium crop diversification level in 2013. In 2016, monoculture showed the highest number of indicator species with 26 ASVs, the low crop diversification had four and the medium diversification had one. No indicator species were found in association with the highest level of crop diversification in 2016. ASV108 (cf. *Thermomicrobiales* sp.) was and indicator species of the monoculture in 2013 and 2016; it is also the only indicator species to be found in both years of sampling.

Core Bacterial Component of the Canola Rhizosphere Microbiota

Only one bacterial ASV remained present across all the samples in every crop rotation and both years: ASV1. ASV1 was identified as cf. *Pseudarthrobacter* sp. according to SILVA and RDP

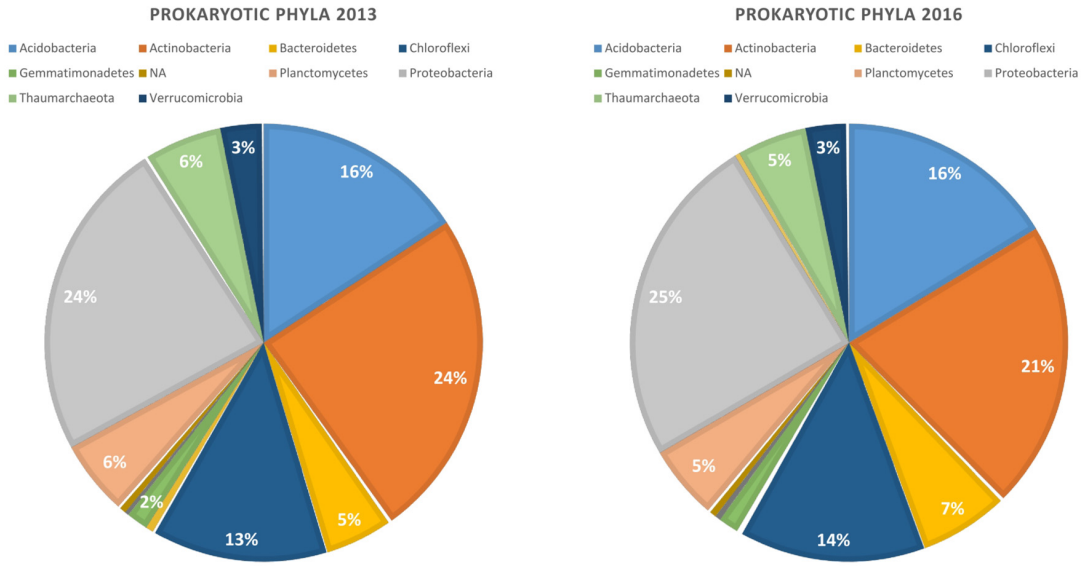

FIGURE 1 | Relative abundance of dominant bacterial phyla in the rhizosphere of canola in 2013 and 2016.

TABLE 2 | Mean values of bacterial α-diversity indices in the rhizosphere of canola under different crop diversification levels, in 2013 and 2016.

	2013				2016			
Index[1]	**Monoculture[2]**	**Low**	**Medium**	**High**	**Monoculture**	**Low**	**Medium**	**High**
Shannon	5,256	5,297	5,318	5,219	5,397	5,321	5,183	5,227
Simpson	0,990	0,989	0,990	0,988	0,990	0,989	0,985	0,986
Chao1	347,961	385,078	394,167	365,999	441,829	429,286	396,087	428,412
Richness	346,467	382,942	396,768	364,275	436,833	423,442	390,867	420,333

[1]No significant differences in diversity index values were detected between the crop rotations by Tukey test α = 0.05. [2]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR.

databases and was the most abundant bacterial ASV in the canola rhizosphere in both years of the study. Its relative abundance ranged from 3.4% of the bacterial community in 2013 to 2.6% in 2016 and was not influenced by cropping system diversification.

Network Analysis of the Bacterial Component of the Microbiota

A network composed of 47 ASVs and 56 edges was found in 2013 (**Figure 2**). This network was modular and included 13 mutual exclusions and 43 co-occurrences between bacterial taxa. A module was organized around ASV12 (cf. *Acidobacteria* sp.) which shared 5 co-occurrences and 2 mutual exclusions. Another module was organized around ASV1 (cf. *Pseudarthrobacter* sp.) which shared 9 co-occurrences and 3 mutual exclusions with other bacterial taxa. In 2016, the interaction network between bacteria was more complex than in 2013, with 51 ASVs and 83 edges (**Figure 3**). The network showed no modularity but was organized on ASV1 which shared 10 co-occurrences and 3 mutual exclusions with the other members of the network. Taxonomical affiliation of the ASVs of the networks in 2013 and 2016 can be found in **Supplementary Tables S1, S2**.

There was one ASV identified as hub-taxa in 2013 and in 2016, ASV1 (cf. *Pseudarthrobacter* sp.), that was also the only relatively abundant member of the bacterial component of the canola rhizosphere microbiota. In 2013, ASV1 had a score of betweenness centrality of 0.44 and a degree score of 11, and in 2016 a score of betweenness centrality of 0.44 and a degree score of 13. No other ASV of the networks had values of betweenness centrality and degree score above the threshold of 95% as it was the case for ASV1. We were able to identify a cohort of bacterial taxa that were connected with ASV1 in 2013 and in 2016 (**Table 5**). The types of interaction between ASV1 and its cohort members were consistent and stable through years. In particular, ASV1 was always positively linked with ASV2 (cf. *Yersinia* sp.), ASV4 (cf. *Stenotrophomonas* sp.), ASV11 (cf. *Stenotrophomonas* sp.), ASV25 (cf. *Candidatus Nitrosocosmicus* sp.) and ASV71 (cf. *Paenarthrobacter* sp.), and negatively linked with ASV3 (cf. *Nitrosophaeraceae* sp.) and ASV6 (cf. *Chloroflexi* KD4-96).

Correlation Between ASV1 and Its Cohort Members, and Canola Yield

Spearman's correlations were used to assess the relationship between ASV1 and its cohort members and canola yield in each

TABLE 3 | Effects of crop diversification on the structure of the bacterial community in the canola rhizosphere, in 2013 and 2016, according to PERMANOVA (α = 0.05, *n* = 12), and significant differences between the structure of bacterial communities per crop diversification level according to Blocked Multi-Response Permutation Procedures (BMRPP) with Šidák correction for two-way comparisons (α = 0.035, *n* = 12).

	PERMANOVA			
	2013		2016	
Source	**DF[1]**	***P*-value**	**DF**	***P*-value**
Overall model				
Crop diversification	3	0,202	3	0,047*
Residuals	44		44	
Lacombe				
Crop diversification	3	0,88	3	0,262
Residuals	12		12	
Lethbridge				
Crop diversification	3	0,131	3	0,292
Residuals	12		12	
Scott				
Crop diversification	3	0,319	3	0,479
Residuals	12		12	
MRPP				
Monoculture[2]		a[3]		a
Low		A		ab
Medium		A		ab
High		A		b

[1]DF : Degree of Freedom. [2]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR. [3]Within each column, crop rotations associated with the same letters are not significantly different.

years (**Table 5**). ASV1 and most of its cohort members were not related to canola yield in 2013, only ASV6 showed a moderate negative correlation (R = -0.40, P = 0.0149) with canola grain yield, according to Ratner (2009). However, in 2016, ASV1 was positively correlated with canola yield (R = 0.46, P = 0.001), as it was the case for ASV3 (R = 0.23, P = 0.05) and ASV71 (R = 0.45, P = 0.0012). ASV6 remained negatively correlated with canola yield (R = -0.41, P = 0.003).

DISCUSSION

We validated that a core bacterial component of the canola rhizosphere microbiota cannot only be stable across pedoclimatic zones but also through years. This core bacterial component was formed of only one taxon, ASV1 identified as cf. *Pseudarthrobacter* sp., which was also identified as a hub taxon and had a cohort of seven bacterial taxa with stable relationships across the two years of the study.

ASV1, cf. *Pseudarthrobacter* sp.

ASV1 was the only bacterial member that fit the definition of a core microbiota member that was detected in the canola rhizosphere and it was the most abundant ASV in both years of sampling. With our current sequencing technology (Illumina MIseq), it is likely that prokaryotes can go unseen if their abundance is low in a sample. ASV1 was the only bacterial core member identified, but it is probable that other less abundant prokaryotic members of this core microbiota were undetected. Furthermore, 16S rRNA gene sequences obtained with Illumina MiSeq technology do not have enough taxonomic resolution to distinguish between closely related species and uncertainty exists: ASV1 matches with 100% identity with at least 100 *Arthrobacter* and *Pseudarthrobacter* sequences in NCBI database. *Arthrobacter* is a genus of gram-positive bacteria from the *Micrococcaceae* family that was subdivided in several other genera like *Pseudarthrobacter* (Busse, 2016). This genus includes mainly soil bacterial species (Busse, 2016). *Arthrobacter* is also a genus with many species known as PGPB (Chan and Katznelson, 1961; Manzanera et al., 2015; Ullah and Bano, 2015; Aviles-Garcia et al., 2016; Fincheira and Quiroz, 2018) colonizing the roots and rhizosphere of a large spectrum of agricultural crops, such as rice or tomato. Lay et al. (2018) reported a member of canola rhizosphere core microbiota identified as *Arthrobacter* that shared 100% identity with ASV1 in similar sites of the Canadian Prairies in 2014. They also reported that their *Arthrobacter* was positively correlated with canola yield as it was the case here with ASV1 in 2016. Furthermore, an *Arthrobacter* sp. was previously shown to increase canola yield and acts as PGPB (Kloepper, 1988). This genus was reported as a highly competitive and fast growing bacteria in canola rhizosphere (Tkacz et al., 2015). Lay et al. (2018) also reported the presence of *Arthrobacter* sp. in wheat and pea rhizospheres in rotation with canola, but in smaller proportions than in canola rhizosphere. That omnipresence and abundance of ASV1 (cf. *Pseudarthrobacter* sp.) in all our plots suggest a selection by canola and highlight this taxon as a good PGPB candidate.

Variations in Bacterial Microbiota

Bacterial communities are known to be sensitive to changes in abiotic factors such as pH and humidity, or nutrient availability (Norman and Barrett, 2016; Wan et al., 2020). As plants actively control their rhizosphere microbiota through root exudates (Bais et al., 2006; Eisenhauer et al., 2017), we expected important differences in the bacterial communities of our crop diversification treatments. This was not the case. In 2013, no effect of crop rotation on bacterial community structure was detected and in 2016, the only significant difference was between the two extreme treatments, i.e., canola monoculture and the highest level of crop diversification, and the difference was marginally significant (P = 0.047). Indicator species analysis showed those two crop diversification treatments as the ones that had the highest number of indicator species. It is possible that the number of indicator species (26) of the monoculture in 2016 with a dominance of *Chloroflexi* (**Table 4**) could be the source of the difference in community structure, with the highest level of crop diversification with the BMRPP, even if no significant differences was found in 2013 between those two crop diversification treatments. Long lasting effect of agricultural management such as crop rotation were reported in the literature (Buckley and Schmidt, 2001). In the Brazilian Amazon for example, crop management

TABLE 4 | Indicator species analysis of the prokaryotic ASV residing in the rhizosphere of canola in response to cropping diversification treatment in 2013 and 2016.

	2013			2016		
Crop diversification[1]	**Indicator species ASV**	**Closest identity**	***P* value**	**Indicator species ASV**	**Closest identity**	***P* value**
Monoculture	ASV315	*Thermomicrobiales*	0,003**	ASV399	*Acidobacteria* sp.	0,002**
	ASV833	*Paracoccus* sp.	0,001**	ASV202	*Thermomicrobiales*	0,003**
	ASV380	*Chloroflexi* sp.	0,003**	ASV309	*Actinobacteria* sp.	0,002**
	ASV409	*Haliangium* sp.	0,010*	ASV576	*Thermomicrobiales*	0,007**
	ASV16	*Intrasporangiaceae*	0,026*	ASV276	*Chloroflexi* sp.	0,002**
	ASV108[2]	*Thermomicrobiales*	0,031*	ASV848	*Micromonosporaceae*	0,009**
	ASV280	*Chthoniobacter* sp.	0,042*	ASV119	*Rhizobiaceae*	0,013*
	ASV251	*Chthoniobacter* sp.	0,042*	ASV547	*Chloroflexi* sp.	0,003**
	ASV838	*Rhodanobacteraceae*	0,049*	ASV680	*Tepidisphaera* sp.	0,009**
				ASV334	*Rhizobiaceae*	0,015*
				ASV809	*Planctomycetes*	0,020*
				ASV315	*Thermomicrobiales*	0,022*
				ASV321	*Thermomicrobiales*	0,034*
				ASV460	*Chloroflexi* sp.	0,032*
				ASV60	*Gaiella* sp.	0,037*
				ASV142	*Chthoniobacter* sp.	0,030*
				ASV181	*Tepidisphaerales*	0,034*
				ASV629	*Solirubrobacter* sp.	0,025*
				ASV552	*Pseudonocardia* sp.	0,036*
				ASV137	*Rubinisphaeraceae*	0,045*
				ASV613	*Chloroflexi* sp.	0,043*
				ASV108	*Thermomicrobiales*	0,039*
				ASV463	*Parafilimonas* sp.	0,041*
				ASV227	*Acidobacteria*	0,045*
				ASV183	*Chitinophagaceae*	0,047*
				ASV1287	*Pirellula* sp.	0,047*
Low	ASV529	*Rubrobacter* sp.	0,015*	ASV501	*Pyrinomonadaceae*	0,001**
				ASV577	*Streptosporangium* sp.	0,008**
				ASV377	*Lysobacter* sp.	0,030*
				ASV697	*Frankiales*	0,036*
Medium				ASV1624	*Acidobacteria*	0,033*
High	ASV182	*Pseudomonas* sp.	0,002**			
	ASV214	*Gaiellales*	0,009**			
	ASV34	*Gaiella* sp.	0,012*			
	ASV283	*Haloactinopolyspora* sp.	0,014*			
	ASV498	*Rhizobiales*	0,014*			
	ASV624	*Bacteria*	0,017*			
	ASV93	*Nitrososphaeraceae*	0,016*			
	ASV751	*Acidobacteria*	0,003**			
	ASV59	*Holophagae* sp.	0,027*			
	ASV24	*Burkholderiaceae*	0,027*			
	ASV53	*Nitrososphaeraceae*	0,025*			
	ASV248	*Iamia* sp.	0,029*			
	ASV127	*Acidobacteria*	0,046*			
	ASV262	*Sphingomonas* sp.	0,040*			
	ASV302	*Acidobacteria*	0,048*			

*Indicator values (IndVal) were tested for significance by Monte Carlo permutation tests (α = 0.05, 999 permutations). [1]Monoculture, canola monoculture; Low, wheat-canola rotation; Medium, pea-barley-canola rotation; High, lentil-wheat-LL-pea-barley-RR. An empty row indicates the absence of indicator species. [2]ASV in bold are indicator species found in 2013 and 2016 in the same crop diversification. Level of significance, *p < 0.05, **p < 0.01.*

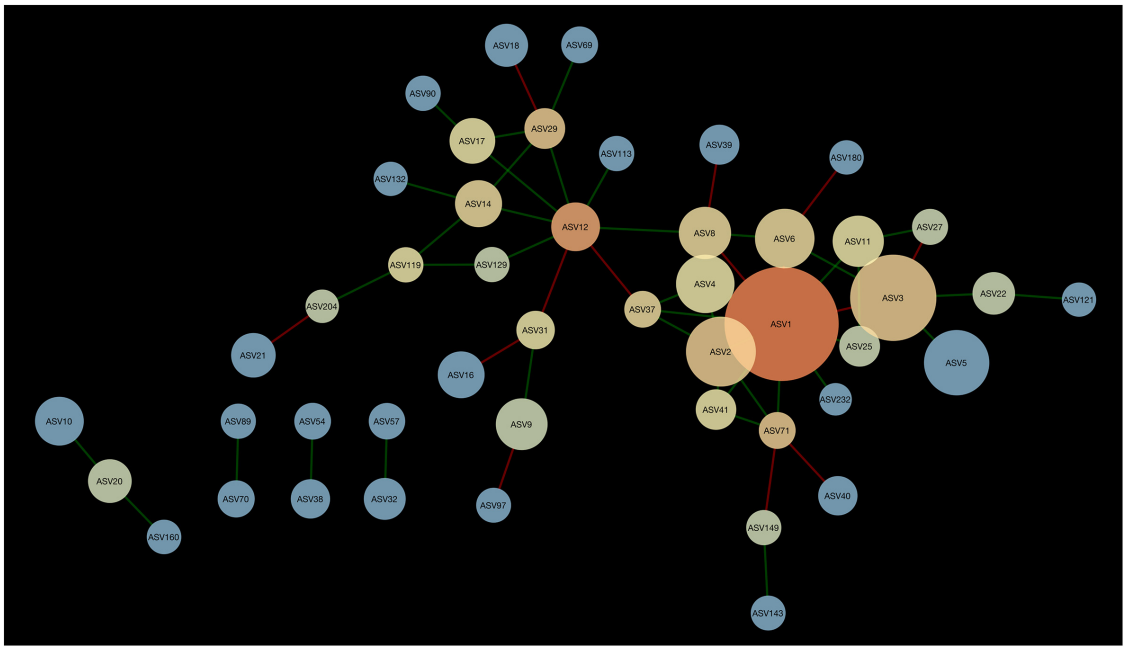

FIGURE 2 | Network of interactions between bacteria forming the microbiome of canola rhizosphere in 2013. Dot size is proportional to the relative abundance of ASV, and shades indicate the degree of betweenness centrality: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

seems to have a significant impact on microbial community structure (Jesus et al., 2009). For temperate environments, our results are consistent with Jesus et al. (2016) who did not find any influence of crop rotation on soil microbial communities in Michigan.

In our study, we examined the bacterial community in the canola rhizosphere, a component of the microbiota that is principally influenced by canola root exudates (Rumberger and Marschner, 2003), mitigating the effects of other crops in the rotation systems. We do not know if the crop diversification levels influenced the bulk soil bacterial communities. However, our results showed that canola recruited similar bacterial communities between all crop diversification levels in 2013. Even if most of the microbes in the rhizosphere are probably selected by the plant from its surrounding soil, it is also possible that a part of the canola rhizosphere microbiota can be inherited maternally with the seed microbiome as it is known to be the case for a wide range of plants (Shade et al., 2017). That could explain the similarities of canola rhizosphere community structure in systems with different levels of diversification. It is also possible that the bacterial communities in our diversified system were not host-specific, but colonize the roots of all crop species used in rotation, as it was reported by Lay et al. (2018). They found that the bacterial microbiota of canola rhizosphere was more similar to the one found in pea than the one found in wheat rhizosphere. But here, we did not find significant difference in community structure between the low, medium and high crop diversification in 2013 and only a slightly significant difference in 2016, suggesting that rotation crops have a limited influence on the bacterial communities of canola rhizosphere. Thus, we can consider the influence of abiotic variation on bacterial community in our study. A previous study showed that soil type and the frequency of rainfall have stronger effects on the microbial community of canola rhizosphere than crop rotations (Schlatter et al., 2019). Floc'h et al. (2020) also found a large variation in fungal rhizosphere community structure that was linked with difference in water availability in canola rhizosphere. In the present study, the experimental plots and sampling times were the same as those used in Floc'h et al. (2020). But the difference in precipitation between years (**Supplementary Figure S2**) did not affect the stability of the bacterial community structure observed in 2013 and 2016, contrarily to what was found for the fungal community in Floc'h et al. (2020). This stability is noteworthy. Bacterial interactions in canola rhizosphere microbiota also showed stability through years, here.

Interactions in the Bacterial Component of the Microbiota

Using the same rhizosphere soil samples, Floc'h et al. (2020) reported drastic changes between years in the dynamics of fungal interactions in the microbiota of canola rhizosphere. In the present work, if the complexity of the interaction network changed between the two years of sampling, the pool of bacteria forming its nucleus remained the same. The hotspot of interaction was always articulated around ASV1 (*Pseudarthrobacter* sp.). ASV1 was the only core bacterial member of the microbiota of canola rhizosphere and the only hub taxa detected with network analysis for both years of the present study. The fungal hub taxa in canola rhizosphere were subject to

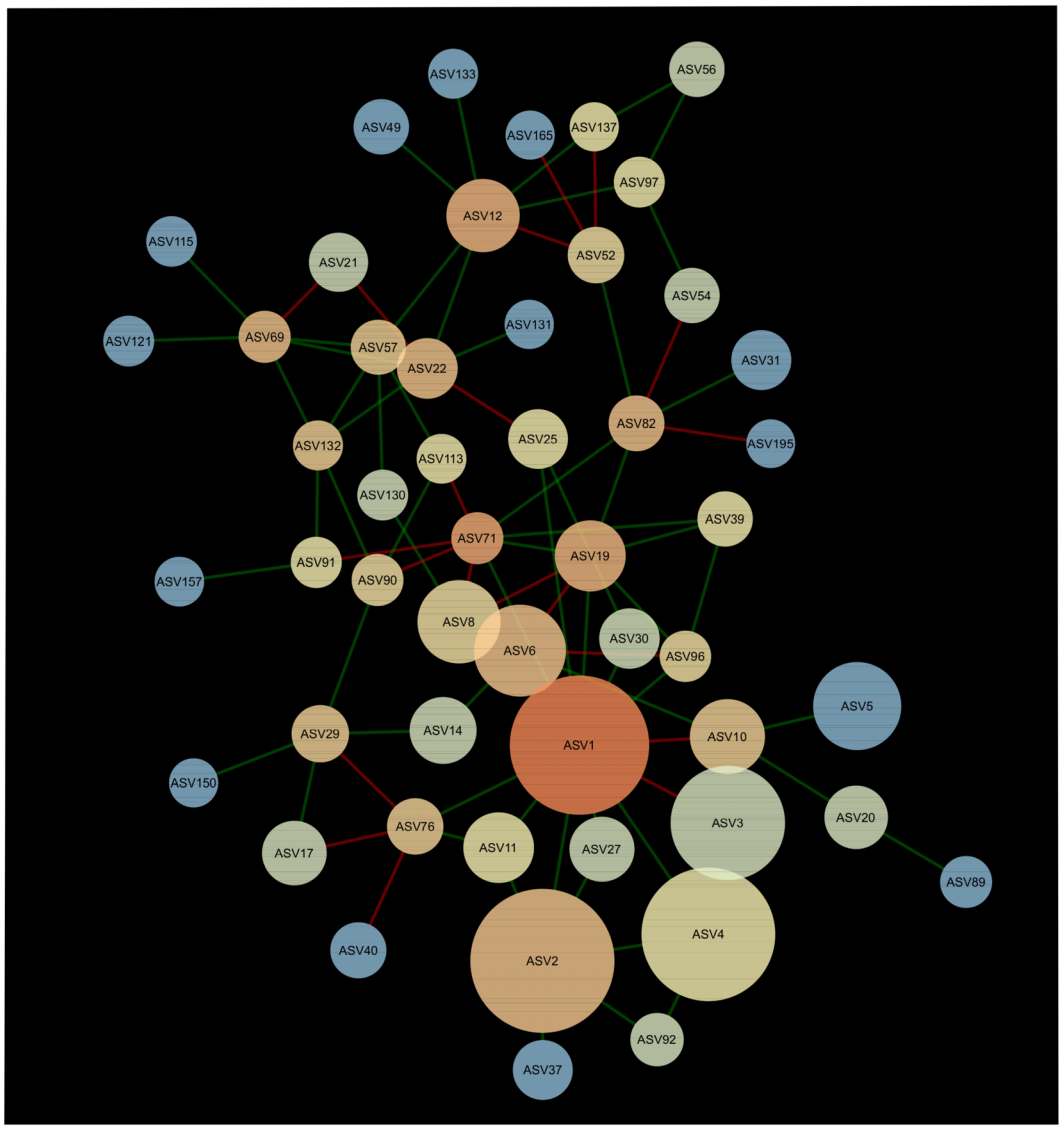

FIGURE 3 | Network of interactions between the bacteria forming the microbiome of canola rhizosphere in 2016. Dot size is proportional to relative abundance of ASV, and shades indicate the degree of betweenness centrality: ASVs with warm colors are more connected with the other members of the network than the cold colored ones. Green edges indicate positive relationships and red edges, negative relationships.

change between the years of the study, but it was not the case for bacterial hub taxa.

For both year of sampling, ASV1 was interacting with seven other taxa: ASV2 (cf. *Yersinia* sp.), ASV3 (cf. *Nitrososphaeraceae* sp.), ASV4 (cf. *Stenotrophomonas* sp.), ASV6 (cf. *Chloroflexi* KD4-96), ASV11 (cf. *Stenotrophomonas* sp.), ASV25 (cf. *Candidatus Nitrosocosmicus* sp.) and ASV71 (cf. *Paenarthrobacter* sp). The persistence of these interactions across time suggests a close interaction of ASV1 with these other members of the community. The fact that ASV6 was negatively linked with ASV1 and negatively correlated with canola yield raises interest. This phylum is associated with several agricultural plants like potato (Ýnceoğlu et al., 2011), lettuce (Cardinale et al., 2015) or maize (Peiffer et al., 2013) and was found in a large spectrum of soil ecosystems including forest, grassland, and tundra ecosystems (Fierer et al., 2012). *Chloroflexi* appears as characteristic of the rhizosphere of canola monoculture: 3 of 9 ASVs in 2013 and 9 of 26 ASVs were identified as indicator species in 2016 (**Table 4**). Monoculture of canola was found to have lower yield values across time and favour accumulation of microbial pathogenic taxa in soil (Hummel et al., 2009; Harker et al., 2015). *Chloroflexi* have been reported in the canola rhizosphere previously, but there was no mention of *Chloroflexi* species being pathogenic to canola (Gkarmiri et al., 2017). Correlations do no indicate that there is a causal relationship between the abundance of the different bacterial ASVs and canola yield. Correlations may point to bacteria that benefit from higher canola growth, or to a condition favorable to both

TABLE 5 | Spearman's correlation between the hub taxa ASV1 and its cohort members with canola yield (*N* = 48) in 2013 and 2016.

ASV[1]	Identity	Hub taxa[2]	2013		2016	
			% relative abundance	Spearman r	% relative abundance	Spearman r
ASV1	*Pseudarthrobacter* sp.	Y	3,430	ns	2,692	0,462*
ASV2	*Yersinia* sp.	N	1,599	ns	2,829	ns
ASV3	*Nitrosphaeraceae*	N	2,275	ns	1,981	0,286*
ASV4	*Stenotrophomonas* sp.	N	1,129	ns	2,533	ns
ASV6	*Chloroflexi KD4-96*	N	1,172	−0,400***[2]	1,341	−0,412***
ASV11	*Stenotrophomonas* sp.	N	0,831	ns	0,734	Ns
ASV25	*Candidatus Nitrosocosmicus* sp.	N	0,397	ns	0,422	Ns
ASV71	*Paenarthrobacter* sp.	N	0,234	ns	0,203	0,45***

*[1]ASV: Amplicon Sequence Variant. [2]Taxa with high connectivity in network analysis in 2013 and 2016 (see section "Materials and Methods" for details). Level of significance, *p < 0.05, **p < 0.01, and ***p < 0.001.*

canola and these bacteria, rather than an effect of the bacteria on plant productivity. However, the correlation values can be used as an index for identifying potential bacterial ASV of interest for the enhancement of canola production, since the bacteria directly beneficial to canola would be among those showing positive correlation with yield. It is possible that ASV6 could be commensal of canola fungal pathogens or of other microbes that are favored by monoculture (Floc'h et al., 2020), or pathogenic itself. Tests of pathogenicity should be made, or cross-kingdom network interactions studies conducted to verify the occurrence of ASV6 with pathogenic microbes.

In the cohort of taxa associated with ASV1, two other taxa were positively correlated with canola yield in 2016: ASV3 and ASV71. ASV71 was identified as *Arthrobacter*, so it is phylogenetically closely related to ASV1, and could be a potential PGPB with ASV1 (Manzanera et al., 2015; Ullah and Bano, 2015; Pereira et al., 2019). ASV3 is an archaea identified as a member of the *Nitrososphaeraceae* family that was poorly correlated with canola yield. Little information about this family is available. The presence of *Nitrososphaeraceae* was previously reported by Gkarmiri et al. (2017), and Lay et al. (2018) found core microbiota members of canola rhizosphere that were genetically close to *Nitrocosmicus* spp. Another study mentioned *Nitrososphaeraceae* as a microbial taxa retrieved from spacecraft surfaces (La Duc et al., 2012). This family appears to be widely distributed in the environment. As hub taxa can have very strong influence on the whole microbiota and on plant performance, ASV1 and its cohort members could be important. These bacteria should be isolated and tested under controlled conditions in structured experiments to examine their potential PGPB activity or pathogenic behavior on canola.

CONCLUSION

In this work, we have shown that the bacterial component of the core microbiota of canola rhizosphere is stable across years despite dissimilarity in precipitations. We identified the single core bacterial ASV in the microbiota of canola rhizosphere as cf. *Pseudarthrobacter* sp. In both years of the study, this single bacterial core microbiota member was a hub taxon in stable association with a cohort of bacteria. *Chloroflexi* were somewhat typical of canola monoculture, but the influence of crop diversification level on bacterial community structure, was only marginal, showing that the bacterial component of the microbiota of canola rhizosphere is more stable than its fungal component. This study provides information about bacterial and archaeal species in canola rhizosphere that could be important for future enhancement of canola production through microbiota manipulation or development of new cohorts for bio-inoculants.

DATA AVAILABILITY STATEMENT

The MiSeq sequencing data generated as part of this work are publicly available on Zenodo (https://zenodo.org/record/3626047#.XisHASZOmV4).

AUTHOR CONTRIBUTIONS

J-BF and CH designed and performed the experiment. MS-A, CH, and MH supervised the project. NL, KH, CH, and MS-A provided the material and analytic tools. J-BF analyzed the data. J-BF, MS-A, and CH wrote the manuscript. All authors revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01587/full#supplementary-material

FIGURE S1 | Rarefaction curves for each rhizosphere soil sample, showing the relationship between the number of ASVs and the abundance of 16S rRNA gene sequences reads, in the 2013 and 2016 dataset.

FIGURE S2 | Cumulative precipitation proportion at each site in 2013 and 2016.

FIGURE S3 | PCOA analysis of prokaryotic communities of canola rhizosphere using Hellinger distances in 2013.

FIGURE S4 | PCOA analysis of prokaryotic communities of canola rhizosphere using Hellinger distances in 2016.

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Impact of Quorum Sensing Molecules on Plant Growth and Immune System

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Bacterial quorum-sensing (QS) molecules are one of the primary means allowing communication between bacterial cells or populations. Plants also evolved to perceive and respond to those molecules. *N*-acyl homoserine lactones (AHL) are QS molecules, of which impact has been extensively studied in different plants. Most studies, however, assessed the interactions in a bilateral manner, a nature of interactions, which occurs rarely, if at all, in nature. Here, we investigated how *Arabidopsis thaliana* responds to the presence of different single AHL molecules and their combinations. We assumed that this reflects the situation in the rhizosphere more accurately than the presence of a single AHL molecule. In order to assess those effects, we monitored the plant growth and defense responses as well as resistance to the plant pathogen *Pseudomonas syringae* pathovar *tomato* (*Pst*). Our results indicate that the complex interactions between multiple AHL and plants may have surprisingly similar outcomes. Individually, some of the AHL molecules positively influenced plant growth, while others induced the already known AHL-priming for induced resistance. Their combinations had a relatively low impact on the growth but seemed to induce resistance mechanisms. Very striking was the fact that all triple, the quadruple as well as the double combination(s) with long-chained AHL molecules increased the resistance to *Pst*. These findings indicate that induced resistance against plant pathogens could be one of the major outcomes of an AHL perception. Taken together, we present here the first study on how plants respond to the complexity of bacterial quorum sensing.

Keywords: AHL, quorum sensing, priming, growth promotion, resistance induction

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INTRODUCTION

Interactions between plant and the associated bacteria are based on an exchange and perception of diverse molecules that are both plant- and bacteria-originated. In the last decade, bacterial quorum sensing molecules were shown to play a crucial role in the communication between the associated bacterial community and the host plant.

Quorum sensing (QS) was discovered as a means of communication within bacterial populations; it is a process based on the synthesis and detection of autoinducer or QS molecules. This phenomenon enables bacteria to monitor the cell density and to coordinate collective changes

in behavior. Gram-negative bacteria generally rely on the synthesis of autoinducers like *N*-acyl homoserine lactones (AHL) or cyclodipeptides. Perception of QS molecules in bacteria activates or deactivates transcription of numerous QS-regulated genes including virulence factors, biofilm formation, chemotaxis, and many more (Bellezza et al., 2014). AHL is one of the major and most extensively studied class of QS molecules. Molecules from this group are comprised of two moieties: a homoserine lactone ring and an acyl side-chain, ranging from 4 to 18 carbons. The acyl chain may vary in the length or in the substitution of the hydrogen at the C-3 position with a hydroxyl or a ketone group (Whitehead et al., 2001; Marketon et al., 2002; Von Bodman et al., 2003). The precise recognition of the AHL by its cognate receptor depends on the lactone ring; the amide group and the fatty acid chain length that together determine the specificity of the cell-to-cell recognition and interaction (Churchill and Chen, 2011).

Since bacteria very often interact with other organisms, it is maybe not surprising that QS molecules may modulate the behavior of other bacterial species and even higher organisms (Williams, 2007). Even though it is still not clearly understood how plants perceive these signaling molecules, the impact of AHL was reported on many occasions including changes in the expression of various genes, proteomes and root development (Mathesius et al., 2003; Ortiz-Castro et al., 2008; Von Rad et al., 2008; Schikora et al., 2011; Schenk et al., 2012). The first evidence of the impact of bacterial AHL on plants was presented by Mathesius et al. (2003). In this study, proteomic analysis showed significant differences in the abundance of more than 150 proteins involved mainly in physiological activities of *Medicago truncatula* including flavonoid synthesis, hormone metabolism and oxidative stress in the roots when treated with two different AHL (oxo-C12-HSL and oxo-C16-HSL). Similarly, in response to the AHL, oxo-C8-HSL, *Arabidopsis* seedlings showed differences in the accumulation of proteins that were involved in carbon metabolism, protein biosynthesis and plant resistance (Miao et al., 2012; Ding et al., 2016).

Multiple studies suggested that plant responses to a particular AHL molecule are very specific and depend on the length of the acyl moiety. AHL with a short acyl chain length of 4 to 6 carbons have been shown to increase primary root elongation and growth rate. These effects were mainly attributed to changes in auxin level (Von Rad et al., 2008; Bai et al., 2012; Liu et al., 2012; Schenk et al., 2012; Zhao et al., 2016). Treatment with oxo-C6-HSL resulted in the expression of genes predominantly associated with auxin and cytokinin signaling pathways (Von Rad et al., 2008; Zhao et al., 2016). Auxin was also shown to be involved in the formation of AHL-induced adventitious roots in *Vigna radiata* (Bai et al., 2012). In a similar study, treatment with oxo-C10-HSL induced formation of adventitious roots in mung bean via an H_2O_2- and NO-dependent cyclic GMP-signaling (Bai et al., 2012). Nonetheless, the formation of lateral roots in *Arabidopsis* due to pretreatment of oxo-C10-HSL seems independent of auxin concentration (Ortiz-Castro et al., 2008). Palmer et al. (2014) argued that AHL-dependent growth induction is associated with alteration in transpiration intensity.

Apart from the AHL-induced plant growth and changes in root architecture, AHL may elicit changes in defense mechanisms. AHL with a long acyl chain length of 12 to 16 carbon atoms were shown to induce AHL-priming for enhanced resistance in *M. truncatula, Arabidopsis thaliana,* and *Hordeum vulgare* (Schikora et al., 2011; Schenk et al., 2012, 2014; Zarkani et al., 2013; Shrestha et al., 2019). The systemic effect of enhanced resistance in AHL-primed plants is notably associated with salicylic acid signaling. In addition, AHL-priming with oxo-C14-HSL induced oxylipin accumulation in distal tissues of *Arabidopsis* that promoted stomatal closure and accumulation of callose and phenolic compounds like e.g., lignin in cell walls, resulting in enhanced resistance toward bacterial and fungal pathogens (Schenk and Schikora, 2014). Very interesting is the fact that different plants may respond differently to the same AHL molecule. For example, oxo-C14-HSL synthesized by *Ensifer meliloti* stimulated root nodulation in *M. truncatula* (Veliz-Vallejos et al., 2014) whereas in *Arabidopsis* and barley, it enhanced resistance against *Pseudomonas syringae* and *Blumeria graminis*, respectively (Schikora et al., 2011; Schenk et al., 2012, 2014; Zarkani et al., 2013; Shrestha et al., 2019).

Despite the numerous reports on the linear interactions between specific AHL molecules and the plant, studies on interaction between the host plant and complex AHL combination are missing. Therefore, in this study we investigated how plant responds to complex AHL combinations, in which not a single but multiple AHL molecules are present. We assumed that this reflects the situation in the rhizosphere more accurately than the presence of a single AHL molecule. We monitored the plant growth and defense responses as well as resistance to the plant pathogen *P. syringae* pathovar *tomato* (*Pst*). Our results indicate that the complex interactions may have surprisingly similar outcomes. Whereas the presence of single AHL molecules induces changes in the growth and AHL-priming, known already from previous reports, the combinations had relatively low impact on the growth but seemed to induce resistance mechanisms. We present here the first data on how a plant respond to the complexity of bacterial quorum sensing.

MATERIALS AND METHODS

Plant Growth

Wild-type *A. thaliana Col*-0 seeds were surface sterilized by washing with sterilization mix of 12% bleach: deionized water: 100% EtOH (1:3:4) for 10 min and further rinsed twice with 100% EtOH for 1 min. The seeds were dried and placed on 1/2-strength MS (Murashige and Skoog) agar plates under sterile conditions. For *P. syringae* pathogenicity assay, root growth and biomass assays, 1-week old seedlings were transferred to square Petri dishes containing 50 ml of 1/2-strength MS medium with different *N*-acyl homoserine lactones (AHL) (Sigma-Aldrich) or their possible combinations, or acetone as a control, and were allowed to grow vertically (see below). Plants were grown at controlled conditions: day/night 8/16 h photoperiod and 22°C, light intensity of 150 μmol/m^{2}s and 60% humidity, in a growth chamber for 3 weeks. For gene expression analysis, 2-week old seedlings were transferred to six-well plates

with 3 ml 1/2-strength MS medium per well 1 day prior to pretreatment with AHL.

Pretreatment With AHL

Arabidopsis Col-0 seedlings grown on 1/2-strength MS medium under sterile conditions were transferred to square Petri dishes or six-well plates containing 1/2-strength MS medium with different AHL (Schikora et al., 2011): *N*-(3-oxohexanoyl)-*L*-homoserine lactone (oxo-C6-HSL), *N*-(3-oxooctanoyl)-*L*-homoserine lactone (oxo-C8-HSL), *N*-(3-oxododecanoyl)-*L*-homoserine lactone (oxo-C12-HSL) and *N*-(3-oxotetradecanoyl)-*L*-homoserine lactone (oxo-C14-HSL) (Sigma-Aldrich) and their possible combinations at a final concentration of 6 μM. Oxo-C6-HSL, oxo-C8-HSL, oxo-C12-HSL and oxo-C14-HSL were dissolved prior in acetone to acquire a stock solution of 60 mM. The seedlings on square Petri dishes were grown for 3 weeks whereas the seedlings in six-well plates were grown for additional 3 days.

Root Elongation and Biomass Assay

In order to reveal the differences in growth parameters of *Arabidopsis* seedlings due to different AHL-treatments, 1-week old seedlings were transferred to Petri dishes containing 1/2-strength MS medium with different AHL, either individually or in combinations or acetone as a control. The seedlings were allowed to grow vertically in controlled conditions for three additional weeks. For the root elongation analysis, the length of each root was measured manually. For biomass analysis, three to five plants from each plate were pooled together and weighed.

Gene Expression Analysis

In order to induce AHL-priming, *Col*-0 *Arabidopsis* seedlings grown on 1/2-strength MS medium under sterile conditions were transferred to six-well plates with 3 ml 1/2-strength MS medium 24 h prior to pretreatment with AHL at a final concentration of 6 μM. The plants were grown for additional 3 days in the same conditions: day/night 8/16 h photoperiod and 22°C, light intensity of 150 $\mu mol/m^2s$ and 60% humidity. All experiments were performed with the solvent control, acetone. To reveal the differences in defense responses between AHL-primed and naïve plants, 3 days after the pretreatment, seedlings were treated with 100 nM flg22. *Arabidopsis* seedlings pretreated with different combinations of AHL were harvested at 0 and 2 h post flg22 treatment. Whole seedlings were homogenized and total RNA was extracted using peqGOLD TriFast (VWR) following manufacturer's recommendations. RNA concentration and quality were determined using the Nanodrop Bioanalyzer. One μg of total RNA was DNAse digested using the PerfeCTa DNAse I (Quanta Biosciences) and subsequently cDNA synthesis was carried out using the qScript cDNA Synthesis kit (Quanta Biosciences) according to the manufacturer's recommendations. Quantitative RT-PCR (qPCR) was performed using primers listed in **Supplementary Table S1**. All expression levels were normalized to the expression of *AtUBQ*. The experiments were performed in a minimum of six independent replications.

Challenge With *Pseudomonas syringae*

Pseudomonas syringae pv. *tomato* DC3000 *(Pst)* was grown on King's B medium containing selective antibiotics for 2 days at 28°C. The bacteria were washed and resuspended in 10 mM $MgCl_2$ and $O.D_{600}$ of the bacterial culture was adjusted to 0.01. Plants growing on different combinations of AHLs were sprayed homogenously with *Pst* at $O.D_{600} = 0.01$, corresponding to 10^7 colony forming units (CFU)/ml. Twelve and 96 h post inoculation with *Pst*, three plants were pooled, weighed and then homogenized in 10 mM $MgCl_2$, serial dilution was plated in duplicates on King's B agar plates containing selective antibiotics to assess the CFU number. The experiments were performed in six independent replications in two independent experiments.

Statistical Analysis

Root growth assays were performed in a minimum of 75 biological replicates from five independent experiments. Biomass assays were performed in at least 20 biological replicates from five independent experiments. Quantitative PCR assays were performed in six biological replicates from three biologically independent experiments. *Pst* pathogenicity assay was performed in six biological replicates from two independent experiments. p-values < 0.05 in the Student's t-test were considered as indicative for a significant difference. Graphs were made using Prism 7 (GraphPad Software, La Jolla, CA, United States).

RESULTS

Bacterial Quorum Sensing Molecules Affect the Plant Growth

In order to assess how the presence of multiple *N*-acyl homoserine lactones (AHL) in the rhizosphere influences the growth of plants, we exposed *A. thaliana Col*-0 seedlings to four AHL molecules (A: oxo-C6-HSL; B: oxo-C8-HSL; D: oxo-C12-HSL and E: oxo-C14-HSL) individually, and in all possible combinations (**Supplementary Figure S1A**). *Arabidopsis* seeds were germinated on sterile 1/2-strength MS medium for 1 week and transferred to fresh 1/2-strength MS media supplemented with single AHL or their combinations for an additional 3 weeks (**Supplementary Figure S1B**). Assessment of the root length revealed that while the treatment of plants with combinations AD, BD, BE, ABDE or the single D (oxo-C12-HSL) had no impact on roots length, if compared to the control (**Figure 1A**). Contrarily, treatments with combinations ABD, ABE, AB or the single A (oxo-C6-HSL) and E (oxo-C14-HSL) molecules, if compared to the control, resulted in longer roots (**Figure 1A**). The impact of other single or combinations of AHL was less elusive since the treatment resulted in an intermediate root length (**Figure 1A**).

In addition to root length, we also measured the plant weight. The acquired results revealed that treatment with combinations AD, BD, ABDE, and BE had no impact on plant weight, while treatments with combinations AB, ABD, ABE and single A and E molecules resulted in plant with the highest biomasses

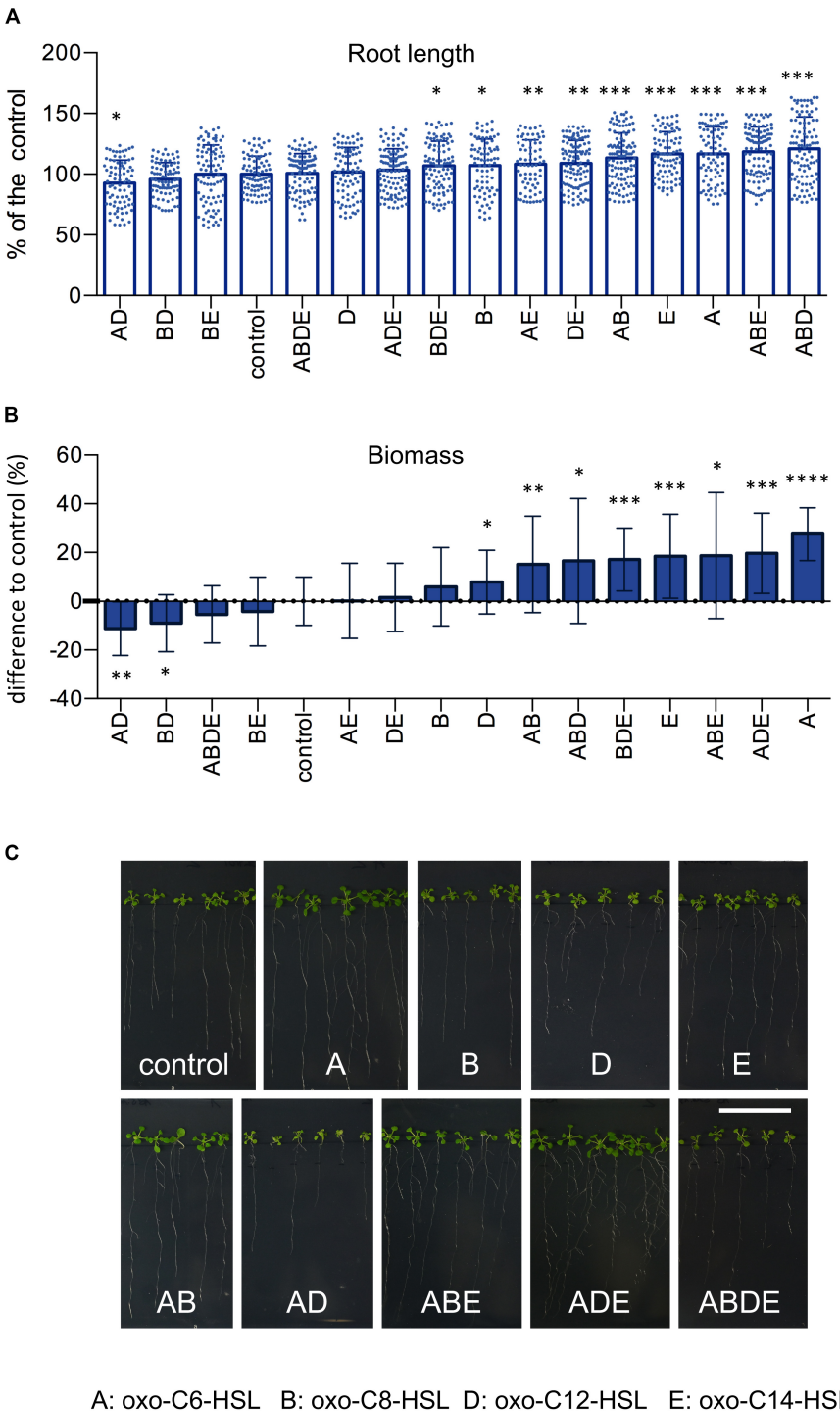

FIGURE 1 | AHL and their combinations have an impact on plant growth. *Arabidopsis thaliana Col*-0 plants were grown under the treatment of 6 μm AHL alone or their combinations for 3 weeks and the root length **(A)** and plant biomass **(B)** were assessed. The control, acetone-treated plants, was set to 100%. * indicates $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and **** $p < 0.00005$ in Student's *t*-test, minim. $n = 20$. Representative photographs presenting the phenotype of *A. thaliana Col*-0 after the exposition to AHL, are presented in **(C)**, the bar represents 3 cm.

(**Figure 1B**). In addition, we also observed a negative correlation between root length and weight. For instance, treatment with the single molecule D (oxo-C12-HSL) resulted in shorter roots, however, higher plant biomass. The same was true for the treatment with combinations ADE and BDE, which resulted in shorter roots but higher plant biomass (**Figure 1B**).

Taking together, we observed that some AHL molecules or their combinations were able to significantly enhance root growth or plant biomass, while others, for example combinations AD and BD, negatively influenced plant growth.

Pretreatment With oxo-C14-HSL Induces AHL-Priming

In the next step, we wondered if the treatment with the different AHL or their combinations would impact immune responses of *Arabidopsis*. To answer this question, we first monitored the expression of four defense-related genes: *WRKY22*, *WRKY29*, the *Glutathione S-Transferase 6* (*GST6*) and the *Heat Shock Protein 70* (*Hsp70*), in plants pretreated with the AHL solvent control (acetone) or with 6 μM oxo-C14-HSL (E). This AHL is known to induce AHL-priming for enhanced resistance. We assessed the gene expression before (0 h) and 2 h after a challenge with 100 nM flg22 in oxo-C14-HSL pretreated plants (**Figure 2**). Our results aligned with the findings reported previously for AHL-priming (Schikora et al., 2011; Schenk et al., 2014), we observed higher induction of *WRKY22*, *WRKY29,* and *GST6* expression in AHL-pretreated plants, if compared to the control plants after the challenge with flg22 (**Figure 2**).

Pretreatment With AHL Affects the Responsiveness to flg22

Following those results, *Arabidopsis* plants were pretreated with all four tested AHL molecules individually as well as in all possible combinations, similar to experiments described above. The expression levels of the four defense-related genes: transcription factors *WRKY22* and *WRKY29*, the *GST6* and *Hsp70*, were assessed 2 h after the challenge with 100 nM flg22. The induction ratio for each gene (difference in gene expression between the 0 h and the 2 h time points) in control plants was set to 100%. The highest induction rates were observed after pretreatment with either the single molecule E (oxo-C14-HSL) or the combinations DE (in case of *WRKY22* and *GST6*) and AD in case of *WRKY29*. On the other hand, the lowest induction rates were observed after pretreatment with the single molecule A (oxo-C6-HSL) in case of *WRKY22* and *Hsp70* and after pretreatment with molecule B (oxo-C8-HSL) in case of *Hsp70* (**Figure 3**). Very striking in our results was a particular tendency, it appears namely that pretreatment with triple AHL combinations as well as combinations with long-chained AHL (D and E) resulted in higher induction rate of particular genes, than pretreatments with single short-chained AHL (A or B) or their combinations. This was especially apparent for the expression levels of *WRKY22* (**Figure 3**) and *GST6* (**Figure 3**).

Comparison Between Growth and Defense Parameters Reveals That Different Interactions May Result in a Similar Outcome

The seemingly diverse impact of the pretreatment with AHL molecules or their combinations prompted us to compare the outcome of such interactions in more detail. To this end, we set the root length, biomass as well as the induction of gene expression in the control plants (pretreatment with acetone) to 100% and calculated the results accordingly. Several patterns emerged from such comparison, apparently, pretreatment with the short-chained AHL, oxo-C6-HSL (A) induced the root length and biomass, while pretreatment with the long-chained AHL oxo-C14-HSL (E) enhanced expression of several defense-related genes, as observed after an additional challenge with flg22 (**Figure 4**; Schenk et al., 2012). In addition, except for the combination of the shortest AHL (AB), all other double combinations seem to enhance the induction of gene expression after flg22 treatment, while having no or only very low impact on the plant growth. Similarly, pretreatments with all triple and the quadruple combinations resulted in AHL-priming for enhanced gene induction as indicated by a higher induction of the expression of the four tested genes, when compared to the induction rate of the control pretreatment after flg22 challenge (**Figure 4**).

Taken together, the direct comparison indicated that although in bilateral interactions, it is the type of the AHL molecule that decides on the outcome of the interaction, in complex interactions, it is rather the number of different AHL molecules. Pretreatment with multiple AHL molecules induced AHL-priming for enhanced resistance, the only exception was the pretreatment with the combination of short-chained AHL (AB), in this case the AHL-priming was not observed.

Enhanced Resistance Seems to Be a General Response to Complex AHL Combinations, With Some Exceptions

In order to test our hypothesis that the impact of AHL molecules on plant immunity is linked to their complexity rather than specific molecules, we pretreated *Arabidopsis* plants with two double combinations (one included short-chained AHL molecules AB and the other long-chained AHL molecules DE) and four triple combinations as well as the quadruple mix of all tested AHL molecules. Plants were grown for additional 3 weeks and challenged with *P. syringae* pathovar *tomato* (*Pst*). Bacterial proliferation was assessed 96 h after the spray-inoculation. Since tri-partied systems are prone to variability, in addition to biological triplicates used in the experiment, the entire assay was performed independently and the results are presented in **Figures 5A,B**, respectively. Previous studies showed that pretreatment with single short-chained AHL had no impact on the resistance toward *Pst*, and pretreatment with a long-chained AHL induced an enhanced resistance to *Pst* via the AHL-priming (Schenk et al., 2012). Here, the proliferation of *Pst* revealed that our hypothesis was only partially correct. Indeed, pretreatment with all triple and the quadruple combinations induced enhanced resistance against *Pst*, if compared to the pretreatment with acetone (**Figure 5**). However, while comparing the bacterial proliferation on plants pretreated with the double AHL combinations and control, it was apparent that only the

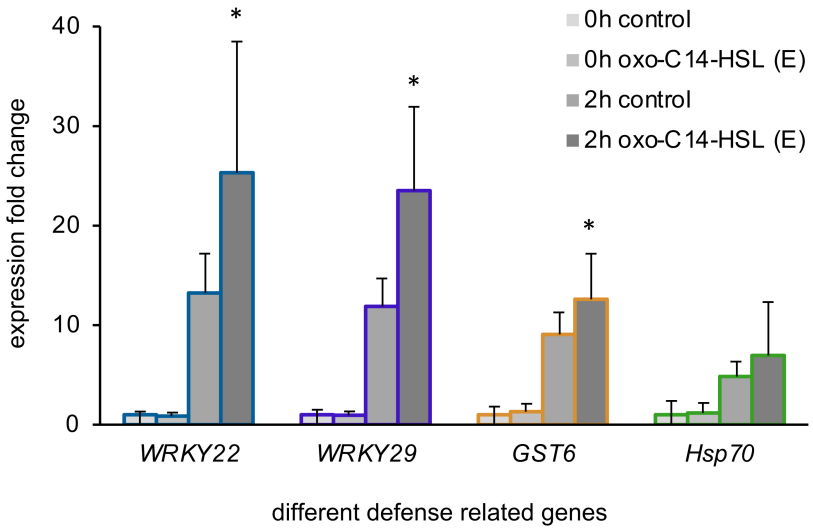

FIGURE 2 | Long-chained AHL, oxo-C14-HSL, induced AHL-priming. Expression levels of four defense-related genes: the transcription factors *WRKY22* and *WRKY29*, the *Glutathione S-Transferase 6 (GST6)* and the *Heat Shock Protein 70 (Hsp70)* assessed in plants pretreated for 3 days with either the acetone solvent (control) or 6 μM oxo-C14-HSL (E), prior (0 h) and 2 h (2 h) after the challenge with 100 nM flg22. The mRNA level in control plants (0 h control) was set to 1, the expression was normalized to the expression levels of housekeeping gene *At5g25760* (Ubiquitin ligase). * indicates $p < 0.05$ in Student's *t*-test.

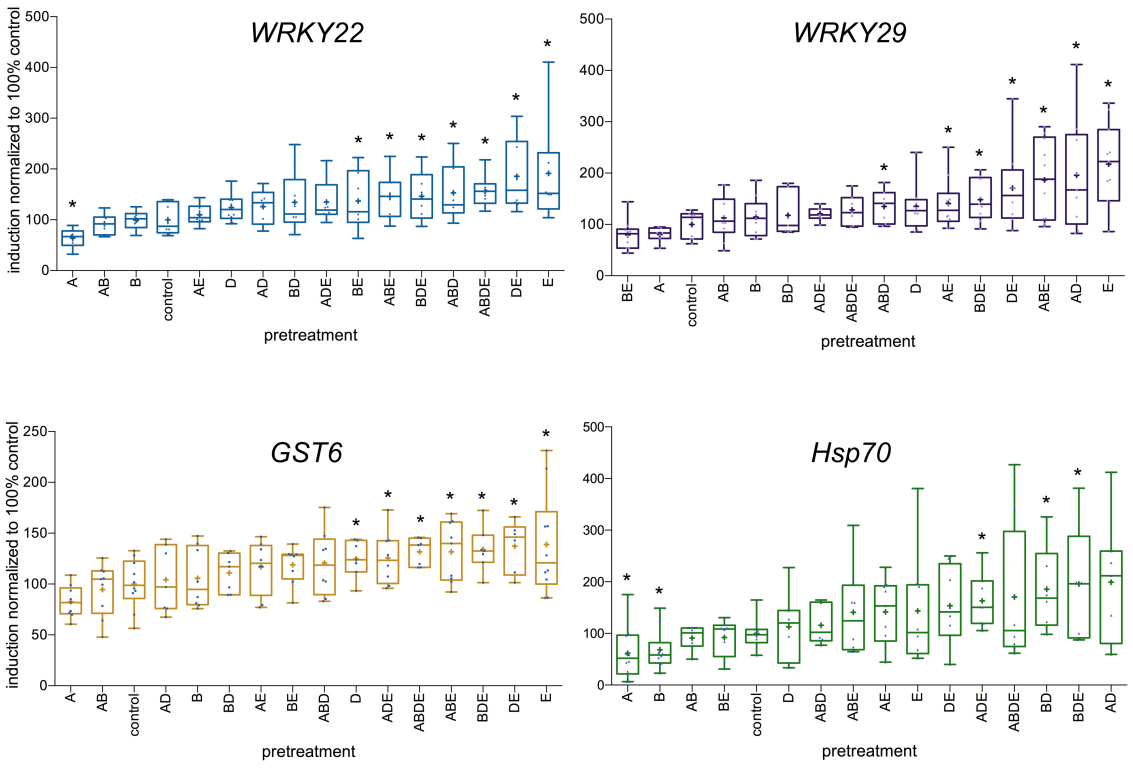

FIGURE 3 | Differences in the induction of defense-related genes upon AHL pretreatment. Single AHL: *N*-(3-oxohexanoyl)-*L*-homoserine lactone (oxo-C6-HSL) (A), *N*-(3-oxooctanoyl)-*L*-homoserine lactone (oxo-C8-HSL) (B), *N*-(3-oxododecanoyl)-*L*-homoserine lactone (oxo-C12-HSL) (D) and *N*-(3-oxotetradecanoyl)-*L*-homoserine lactone (oxo-C14-HSL) (E), as well as their combinations, were used as pretreatment for 3 days prior to the challenge with 100 nM flg22. The induction of the expression of the transcription factors *WRKY22* and *WRKY29*, the *Glutathione-S-Transferase 6 (GST6)* and the *Heat Shock Protein 70 (Hsp70)* was assessed 2 h after the flg22 challenge. The induction in control plants (acetone pretreatment) was set to 100%. The level of expression was normalized to the expression of the housekeeping gene *At5g25760* (Ubiquitin ligase). * indicates $p < 0.05$ in Student's *t*-test.

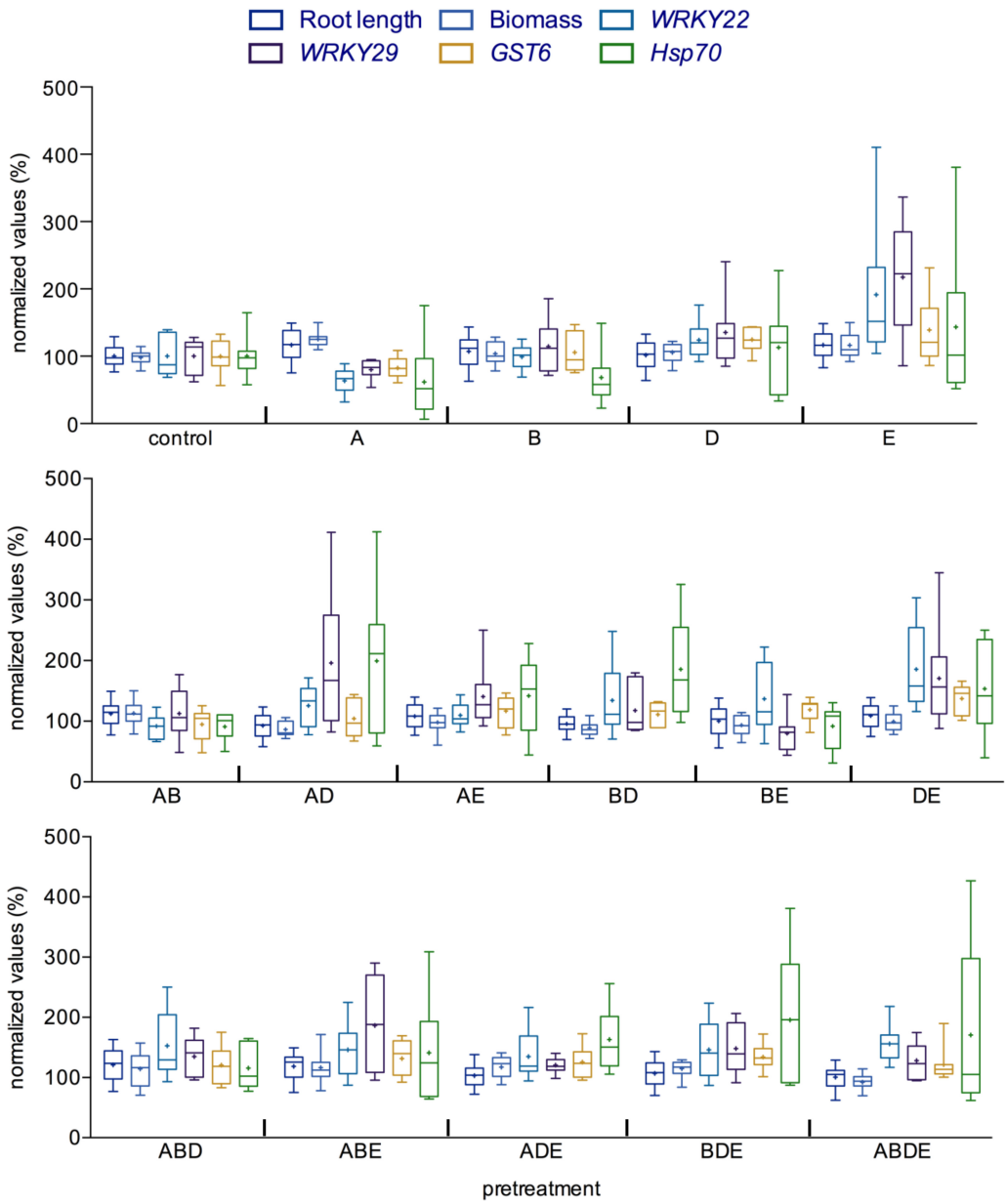

FIGURE 4 | Comparison between growth parameters and immune response indicates significant impact of complex AHL combinations. All parameters measured during this study were compared with each other for each AHL treatment/pretreatment. The values of the control plants were set to 100%. Root length and biomass are presented as a direct ratio of the control values; the gene expression analysis is presented as a percentage of the induction in the gene expression 2 h after challenge with flg22. *WRKY22* and *WRKY29* encode for transcription factors, the *GST6* for the *Glutathione S-Transferase 6* and *Hsp70* for *Heat Shock Protein 70*. A: *N*-(3-oxohexanoyl)-*L*-homoserine lactone (oxo-C6-HSL); B: *N*-(3-oxooctanoyl)-*L*-homoserine lactone (oxo-C8-HSL); D: *N*-(3-oxododecanoyl)-*L*-homoserine lactone (oxo-C12-HSL); and E: *N*-(3-oxotetradecanoyl)-*L*-homoserine lactone (oxo-C14-HSL). Multiple letters indicate the used combination.

pretreatment with long-chained AHL (DE) induced enhanced resistance. The pretreatment with the short-chained AHL (AB) resulted in bacterial proliferation similar to control plants.

In summary, pretreatment with the complex triple or more AHL combinations induced enhanced resistance regardless of its composition, whereas a combination with only two short-chained AHL molecules was not able to induce AHL-priming for enhanced resistance.

DISCUSSION

The association between plant roots and bacteria possesses the potential to shape both, the host plant as well as the associated community. While the soil serves as the reservoir of a plethora of microbial species, it is the plant which models the microbial community in its rhizosphere and on the rhizoplane. Our knowledge regarding bacteria, which associate

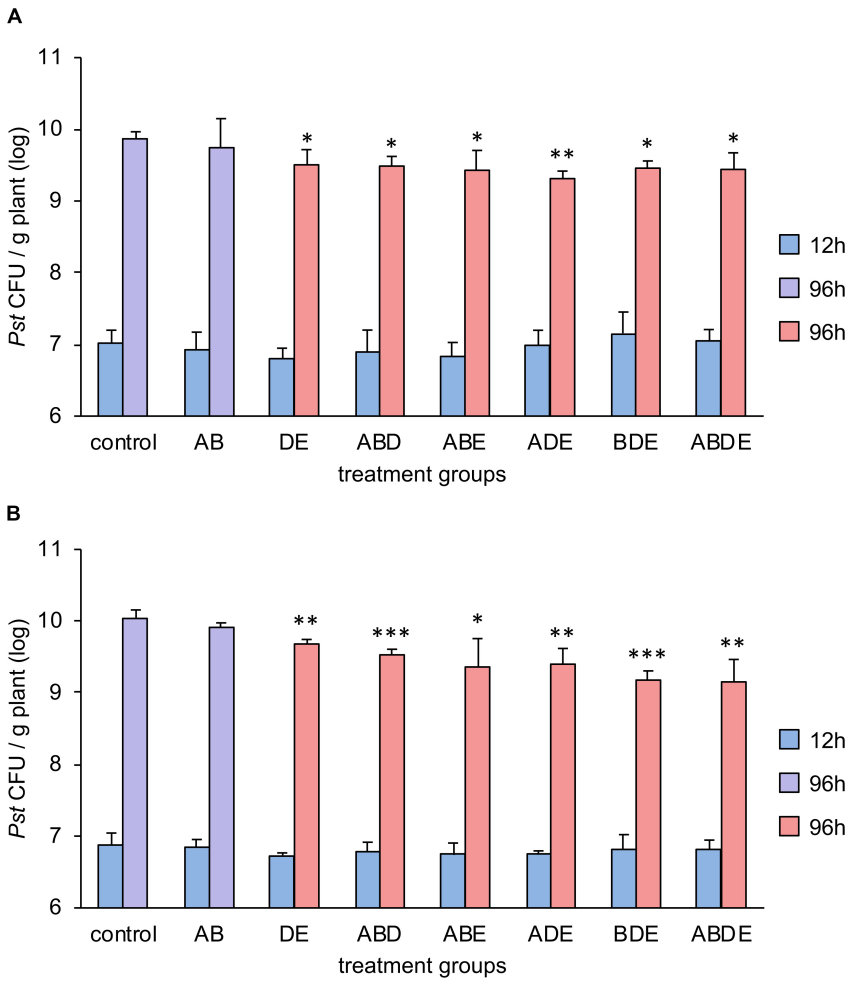

FIGURE 5 | Multiple AHL combinations induce AHL-priming for enhanced resistance against *Pst*. *Arabidopsis thaliana Col*-0 plants were transferred to 1/2-strength MS medium supplemented with combinations of AHL as indicated, 1 week after germination. After additional 3 weeks, plants were challenged with *Pseudomonas syringae* pathovar *tomato (Pst)*. Bacteria were grown on King's B medium, washed in 10 mM $MgCl_2$ prior use and the optical density was adjusted to 0.01 (10^7 CFU/ml). Bacterial colony forming unit (CFU) number was assessed 12 h and 96 h after the spray-inoculation. A: *N*-(3-oxohexanoyl)-*L*-homoserine lactone (oxo-C6-HSL); B: *N*-(3-oxooctanoyl)-*L*-homoserine lactone (oxo-C8-HSL); D: *N*-(3-oxododecanoyl)-*L*-homoserine lactone (oxo-C12-HSL); and E: *N*-(3-oxotetradecanoyl)-*L*-homoserine lactone (oxo-C14-HSL). Multiple letters indicate the combination used for the pretreatment. Different colors represent the significance groups at $p < 0.05$ in Student's *t*-test. In addition, * indicates $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$ in Student's *t*-test. **(A,B)** Display two independent experiments.

with plant increased significantly during the last decade, and lead to the definition of a core microbial community associated with plants, notably *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012). The diversity within the community as well as the influence of external factors (e.g., soil type) differs in different root-related compartments. Nevertheless, root exudates have the biggest influence on the structure of the rhizosphere community. Their composition was postulated as the driving force structuring microbial communities on many occasions (Zhalnina et al., 2018). The microbial community in turn may influence the physiology of the host plant. The presence of so-called plant growth promoting bacteria is an excellent example, another are soil-borne pathogens. In fact, diversified root microbiome may be one of the key players in plant health, preventing diseases (Berg et al., 2017; Durán et al., 2018).

In this study, we assessed how plants respond to the complex communication between bacteria, which takes place in such communities. As a model, we chose the well-known response of *Arabidopsis* to bacterial quorum sensing molecules from the *N*-acyl homoserine lactones (AHL) group. These molecules are usually used by Gram-negative bacteria in their intra- and inter-population communication and have well-documented impact on *Arabidopsis* and other plants (Hartmann et al., 2014; Schikora et al., 2016). Several reports have shown that AHL may promote root length or induce AHL-priming for enhanced resistance (Mathesius et al., 2003; Ortiz-Castro et al., 2008; Hartmann et al., 2014; Schikora et al., 2016; Zhao et al., 2020).

Here, we broadened our knowledge on the interactions between AHL molecules and the plant from linear interactions with single molecules to the outcome of complex interactions between multiple AHL molecules and the host plant. The multitude of AHL-producing bacteria that have been already identified on plant roots (Berg et al., 2002; Balasundararajan and Dananjeyan, 2019) implies that a host plant may indeed encounter more than a single AHL molecule, a situation which would necessitate a coordinated response.

One of the responses to the presence of AHL molecules was a modification in root morphology. The intermediate C10-HSL seemed to have the strongest effect on *Arabidopsis* and induced shortening of roots as well as increased formations of lateral roots and root hairs (Ortiz-Castro et al., 2008). Similarly, other AHL molecules like the short-chained C6-HSL and C8-HSL also increased root length (Von Rad et al., 2008; Schenk et al., 2012). Such modifications were observed also in other plants; for instance, in wheat and barley, where the presence of AHL increased root length and plant biomass (Rankl et al., 2016; Moshynets et al., 2019), or in mung bean, where AHL induced the formation of adventitious roots (Bai et al., 2012). The morphological response to multiple AHL molecules seems to be different from the response to a single AHL. Although we observed the previously reported enhanced root elongation after treatment with single short-chained AHL, their combinations (AD and BD) inhibited both, root length and plant biomass. On the contrary, the presence of all four tested AHL molecules didn't change those parameters. Whether this outcome is a result of contrary physiological reactions, for example contra-balancing hormone levels, is still an open question. It is also probable that the downstream signaling cascade initiated by a particular AHL molecule might interfere with the signaling cascade initiated by another AHL molecule.

AHL-priming for induced resistance is another phenomenon, widely discussed as a consequence of AHL presence. It gained much attention recently as a possible target for breeding approaches and alternative plant protection strategy (Hernandez-Reyes et al., 2014; Shrestha et al., 2019; Wehner et al., 2019). AHL-producing bacteria and also AHL molecules were shown to induce AHL-priming and therefore protect plants from diseases (Bauer and Mathesius, 2004; Hartmann et al., 2014; Schikora et al., 2016). Very effective is the long-chained oxo-C14-HSL (Schenk et al., 2014), however, other AHL molecules also enhanced resistance against pathogens (Mathesius et al., 2003; Schuhegger et al., 2006; Liu et al., 2020). In addition to the response to single AHL molecules, our results suggest that the induction of AHL-priming could be the general response to multiple AHL molecules. Indeed, we observed here that the presence of all combinations, except the double short-chain AB combination, induces not only the expression of several defense-related genes but also the resistance against the foliar pathogens *P. syringae*. Whether the response is based on the presence of long-chained AHL molecules or whether the responses to for example oxo-C14-HSL, a known AHL-priming inducer, overwrites other responses requires further studies. It is also important to note that in recent report (Liu et al., 2020), AHL-induced enhanced resistance was observed after treatment with the short-chained AHL, oxo-C8-HSL. This indicates, that different experimental conditions may play a role. Resistance to pathogens is a crucial feature desired in crop plants and therefore, results obtained in this study could open new opportunities for modern strategies in plant protection. It is namely envisageable to use diverse AHL-producing strains as members of the applied inoculum.

Yet another discovery is very interesting in this context, the resistance to abiotic stresses (high salt concentrations) was enhanced after the treatment with oxo-C6-HSL (Barriuso et al., 2008; Zhao et al., 2020). Even though the exact mechanism of AHL-induced salt tolerance is not yet known, the possibility to modulate the plant tolerance to abiotic stresses like salt or drought using AHL is very interesting.

CONCLUSION

It is important to note that the response of the model plant *A. thaliana* to the presence of different AHL molecules reflects only one level of the interactions in the rhizosphere. Nevertheless, our results indicate that the complex interactions of multiple AHL on plants may have surprisingly similar outcomes. Their combinations had a relatively low impact on the growth but seemed to induce resistance mechanisms. Our findings indicate that induced resistance against plant pathogens could be one of the major outcomes of AHL perception. Such findings are indeed very interesting since they open new possibilities for plant protection approaches and improve our knowledge on how complex bacterial communities may influence the host plant.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ASh and ASc designed the study and wrote the manuscript. ASh, MG, IO, and JK performed the experiments. ASh analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01545/full#supplementary-material

FIGURE S1 | Experimental approach. **(A)** The structures of *N*-acyl homoserine lactone (AHL) molecules used in this study. *N*-(3-oxohexanoyl)-*L*-homoserine lactone (oxo-C6-HSL) (A), *N*-(3-oxooctanoyl)-*L*-homoserine lactone (oxo-C8-HSL) (B), *N*-(3-oxododecanoyl)-*L*-homoserine lactone (oxo-C12-HSL) **(D)** and *N*-(3-oxotetradecanoyl)-*L*-homoserine lactone (oxo-C14-HSL) **(E)**. **(B)** Timelines of different experiments in this study. *Arabidopsis thaliana Col*-0 seeds were germinated on sterile 1/2-strength MS medium and grown for an additional week, before transplanting to fresh medium supplemented with single AHL or their combinations for: (i) additional 3 weeks for the measurement of growth parameters, or (ii) 3 weeks before the challenge with *Pseudomonas syringae* pathovar *tomato (Pst)*. Two-week old plants designated for expression analysis were transferred to liquid MS medium supplemented with single AHL or their combinations 3 days prior to the challenge with 100 nM flg22.

TABLE S1 | Primers used in this study. List of primers used for quantitative PCR in this study.

TABLE S2 | Measured values. Experimentally acquired values for root length, plant biomass and induction of gene expression. Data were used in **Figures 1–4** as the basis for normalization. Values for acetone-pretreated plants were set to 100%.

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Framework for Quantification of the Dynamics of Root Colonization by *Pseudomonas fluorescens* Isolate SBW25

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Colonization of the root surface, or rhizoplane, is one of the first steps for soil-borne bacteria to become established in the plant microbiome. However, the relative contributions of processes, such as bacterial attachment and proliferation is not well characterized, and this limits our ability to comprehend the complex dynamics of microbial communities in the rhizosphere. The work presented here addresses this knowledge gap. A model system was developed to acquire quantitative data on the colonization process of lettuce (*Lactuca sativa* L. cultivar. All Year Round) roots by *Pseudomonas fluorescens* isolate SBW25. A theoretical framework is proposed to calculate attachment rate and quantify the relative contribution of bacterial attachment to colonization. This allows the assessment of attachment rates on the root surface beyond the short time period during which it can be quantified experimentally. All techniques proposed are generic and similar analyses could be applied to study various combinations of plants and bacteria, or to assess competition between species. In the future this could allow for selection of microbial traits that improve early colonization and maintenance of targeted isolates in cropping systems, with potential applications for the development of biological fertilizers.

Keywords: rhizosphere, microbiome, bacterial dynamics, attachment, colonization, root surface, *Pseudomonas fluorescens*

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INTRODUCTION

The region of soil under direct influence of a plant root is termed the rhizosphere. The rhizosphere hosts a complex microbiome, distinct from both the bulk soil and other plant associated environments (Lundberg et al., 2012). Biological interactions taking place on the surface of the root shape microbial diversity in the rhizosphere. Successful colonization of the root surface, or rhizoplane, is often the first step toward entering the plant microbiome for soil-borne bacteria, including pathogens (Walker et al., 2004). High levels of microbial competition are observed at or near the surface of the root because bacteria seek plant-derived nutrients and space in which to establish themselves (Reinhold-Hurek et al., 2015). Colonization of the rhizoplane is an important step prior to internalization and translocation of bacteria within plant tissue (Berggren et al., 2005).

The root surface is the point at which many plant growth promoting, and pathogen suppressing, bacteria are established and act to influence the plant (Köhl et al., 2019; Shinde et al., 2019). The rhizoplane is also susceptible to colonization by human pathogens (Wright et al., 2017).

Understanding the process of root surface colonization is challenging. Soil is a heterogeneous environment and enables very diverse forms of biological activity (Hinsinger et al., 2009; Kuzyakov and Blagodatskaya, 2015). Plants and microorganisms secrete a broad range of chemical compounds which can impact bacterial growth and alter their physiology (Dennis et al., 2010; Yu et al., 2019). Plants are known to recruit certain bacteria while suppressing others through immune responses (Chowdhury et al., 2015; Feng et al., 2019). Differences in community structure are observed at different stages of root development, or depending on root anatomy (Humphris et al., 2005; Schmidt et al., 2018). Bacteria associated with roots are also impacted by temporal variations in root exudation (Kuzyakov and Blagodatskaya, 2015).

Colonization of the rhizoplane requires complex and often very specific mechanisms that develop in a progressive manner. First, bacteria must be able to detect the presence of a root. Subsequently, bacteria must be able to move toward the root and then establish themselves in a location from which plant-derived nutrients are accessible. The chemotactic response of soilborne bacteria to plant derived signaling molecules has been well documented (Pliego et al., 2008; Feng et al., 2019). Mobility of microbes in soils and toward roots has been demonstrated in a many soil-borne bacteria. Bacterial motility and physical soil properties determine the ability of bacteria to approach the root. For example, soil moisture has been found to be the main factor effecting the movement of *Pseudomonas fluorescens* toward wheat roots (Bashan, 1986). As a result of this movement, higher microbial diversity is observed in the soil directly surrounding the root relative to that of the bulk soil (Robbins et al., 2018). Bacterial numbers on the rhizoplane can increase in two ways; (i) recruitment from the surrounding medium resulting in attachment and/or (ii) proliferation of established bacteria on the root. Bacteria can form weak reversible bonds, then strong permanent attachments to the root surface (Rossez et al., 2014). Root growth leads to the dilution of bacterial density on the rhizoplane and, eventually, displacement of bacterial colonies from sites of heavy exudation (Dupuy and Silk, 2016). It is likely that bacterial mobility on the rhizoplane contributes to maintenance of colonization at sites of exudation, but this has not been well studied.

Bacteria vary significantly in their ability to move, attach, and proliferate in the rhizosphere. In soil, dynamic interactions take place between the root and microbes which can either compete or cooperate during colonization (Lareen et al., 2016). The rhizosphere microbiome structure emerges as a result of these interactions. Recent developments in genomics, sequencing and bioinformatics have revealed the taxonomic diversity and positioning of bacteria within the rhizosphere. The specificity of certain taxonomic groups to host plants and environmental conditions has also been investigated through microbiome analysis (Dawson et al., 2017; Lucaciu et al., 2019). Bioinformatics approaches are increasingly focusing on extracting information on community functional traits (Fitzpatrick et al., 2018). Unfortunately, top-down molecular approaches lack the ability to identify factors that contribute to the maintenance of bacteria in the rhizosphere. To date, however, these approaches have only had a limited impact on agriculture and our ability to manipulate the plant microbiome (Gopal and Gupta, 2016). This may be due to a lack of understanding of the mechanisms through which bacteria are recruited and maintained on the root surface.

To address this knowledge gap, the work presented here proposes a mathematical framework to dissect the factors contributing to maintenance and recruitment on the rhizoplane. This framework links the relative contribution of attachment and proliferation on the rhizoplane to the overall colonization rate of the root. A model system was developed to acquire quantitative data on the colonization process of lettuce roots by *Pseudomonas fluorescens* isolate SBW25, an isolate with well characterized interaction with plants (Jackson et al., 2005). Through a series of colonization experiments we determined the key parameters that need to be measured in order to characterize microbial colonization. A theoretical framework is proposed to calculate attachment rate and quantify the relative contribution of recruitment to colonization. We have developed the techniques here to be able to be applied to study other combinations of plant and bacterial isolates, alone or in competition, thus the work has broad impact and value.

MATERIALS AND METHODS

Plant Growth and Microcosm Set Up

Lettuce (*Lactuca sativa* L. cultivar. All Year Round) seeds were obtained from Sutton Seeds, United Kingdom. Prior to germination, seeds were surface sterilized by soaking in a solution of 2% w/v calcium hypochlorite (Sigma Aldrich 12116) for 15 min. They were subsequently washed in sterile distilled water. Seeds were then plated on 1.5% water agar. Plates were sealed and covered with foil. They were then incubated at 21°C for 3 days. Sterile microcosms were set up in 75 mm round bottom culture tubes (VWR 211-0046). 1.5% water agar (1 ml) was melted and pipetted into culture tubes. Tubes were set on their sides to allow agar to form a slope and a well in which microbial suspensions in liquid solution could interact with the root. Once agar had set, a small section was removed to form a platform (**Figures 1A,B**).

Each microcosm contained 1 ml of 0.5 × concentration Murashige and Skoog (MS) media without sucrose (Sigma Aldrich M5524). Light was prevented from reaching the roots by covering the bottom part of the tube with tape. Following germination, individual plants were transferred from plates to microcosms. They were placed on the water agar platform, with their root tip in the well (**Figure 1A**). Microcosms were then sealed, using a plastic lid, and placed in a growth-cabinet. Growth conditions were 21°C with 16 h of light at 60 μmol m^{-2} s^{-1}. Plants were grown for 3 days before further treatment.

To assess the efficiency of surface sterilization, 20 seeds were sterilized as described above, imprints were made by placing sterilized seeds on Lysogeny Broth (LB) (Sigma Aldrich

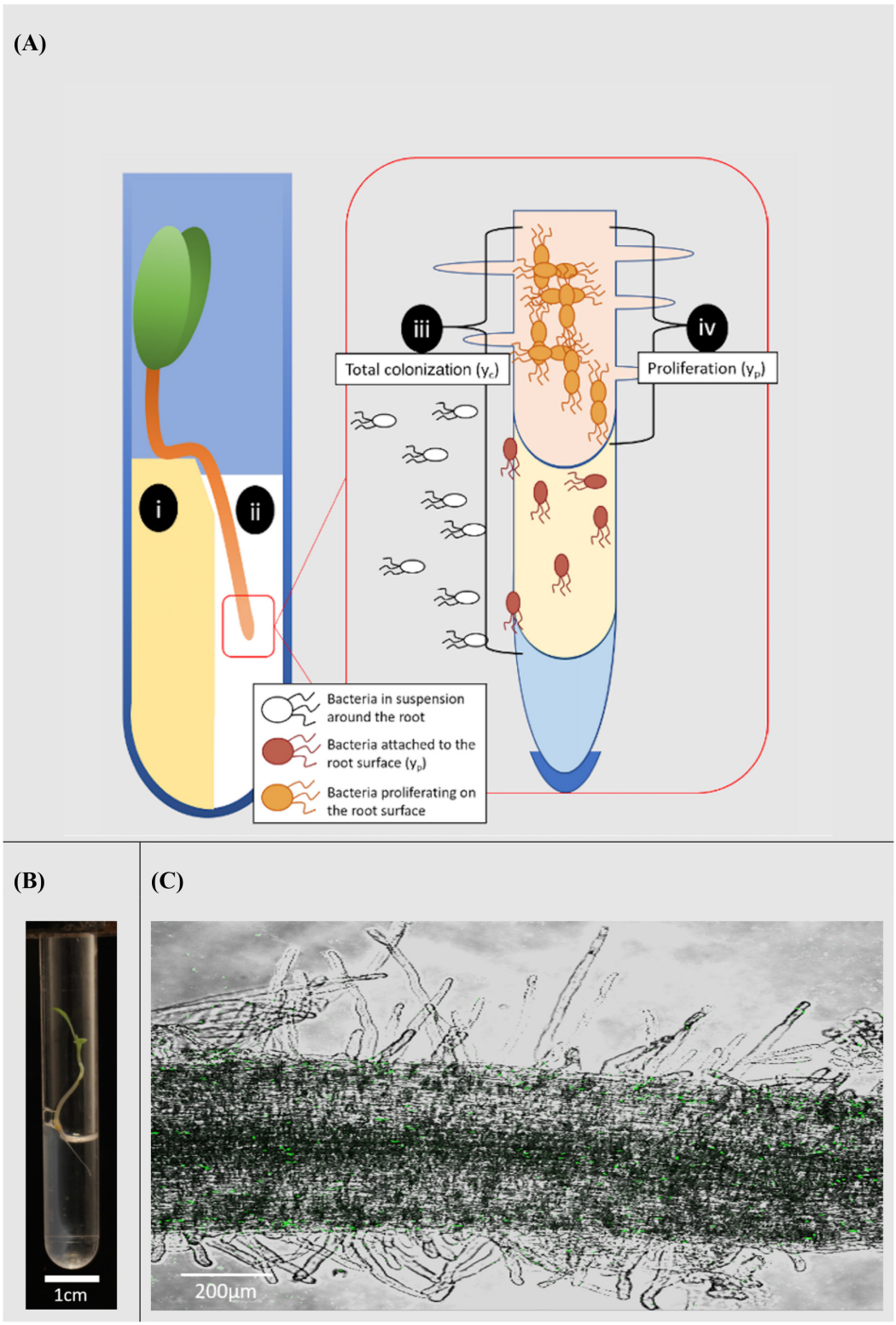

FIGURE 1 | Microcosm system for the study of rhizoplane colonization. **(A)** Diagram of the microcosm system; (i) plants are grown on a water agar slope. (ii) A bacterial suspension is introduced to a level no higher than the hypocotyl. The system allows bacterial movement along the root and quantification of the colonization process; (iii) total colonization (y_c) was the result of both attachment to and proliferation on the root surface. (iv) Proliferation on the root surface (y_p) was quantified in the absence of attachment. **(B)** Microcosm chamber containing a growing lettuce seedling. **(C)** A confocal image of *Pseudomonas fluorescens* SBW25 E1433 (shown in green) superimposed over a brightfield image of a lettuce root.

L9234) agar for ∼30 s, followed by incubation at either 18 or 27°C for 24 h.

Bacterial Isolates and Bacterial Transformation

Pseudomonas fluorescens isolate SBW25 (genome accession AM181176.4) (Rainey and Bailey, 1996) was transformed with a fluorescent marker plasmid E1433 pGFP (Heeb et al., 2000). The resulting isolate was referred to as *P. fluorescens* SBW25 E1433. This was used as the model isolate for all subsequent experiments. The E1433 pGFP plasmid conferred tetracycline resistance to transformed bacteria. The E1433 pGFP plasmids was transformed into competent *P. fluorescens* isolate SBW25 by electroporation. Transformed bacteria were isolated by plating on LB agar containing tetracycline (25 μg ml^{-1}). To test the stability of the plasmid, *P. fluorescens* SBW25 E1433 was grown in liquid LB and RD-MOPS (Neidhardt et al., 1974) in the absence of tetracycline. Cultures were incubated at 27°C, with shaking (200 rpm). Every 24 h, for 7 days, fresh 1:100 subcultures were prepared, and a sample was taken (100 μl). Serial dilutions of each sample were plated on LB agar with and without tetracycline (25 μg ml^{-1}). These plates were incubated for 24 h at 27°C, then Colony Forming Units (CFU) were counted. Plates containing tetracycline were compared to those without to ensure there was no more than 10% difference in CFU number. A visual examination for fluorescence under the microscope was also carried out at each 24-hour timepoint. To facilitate a comparison between transformed and non-transformed bacteria, the growth of *P. fluorescens* SBW25 and *P. fluorescens* SBW25 E1433 was measured in LB and a rich defined RD-MOPS media using a micro plate reader (Multiskan Go, Thermo Scientific, United States). Measurements of optical density at 600 nm (OD_{600}) were taken every half hour. Bacteria were grown at 21 and 27°C with intermittent shaking. The emission spectrum of the transformed isolate was measured using a plate reader (Varioskan – Lux, Thermo Scientific, United States) with an excitation wavelength of 485 nm and emission range of 510–600 nm.

Bacterial Growth Conditions and Root Inoculation

Pseudomonas fluorescens SBW25 E1433 was removed from storage in 20% glycerol at −80°C, streaked on LB agar plates containing tetracycline (25 μg ml^{-1}) and incubated at 27°C for 24 h. A single colony was selected and cultured in liquid LB containing tetracycline (25 μg ml^{-1}) for 24 h at 27°C with shaking (200 rpm). A 1:100 sub-culture was then transferred into a rich defined RD-MOPS media containing tetracycline (25 μg ml^{-1}). This was incubated for a further 24 h at 18°C with shaking (200 rpm). Bacterial suspensions for inoculation of roots were prepared by diluting this liquid culture to an OD_{600} of 0.02 in 0.5 × MS media. This corresponded to an approximate bacterial density of 2×10^7 CFU ml^{-1}. For each treatment, an initial measurement of bacterial density was obtained (CFU ml^{-1}) by serial dilution and plating on Kings-B agar (Sigma Aldrich 60786) containing tetracycline (25 μg ml^{-1}). Kings-B agar enables the efficient counting of *P. fluorescens* as it encourages the production of fluorescent compounds. All inoculations were carried out at the same point in the light cycle of the growth-cabinet. Prior to inoculation, the 0.5 × MS was removed from microcosm wells using a Pasteur pipette. Approximately 1 ml of either bacterial suspension, or a negative control of 0.5 × MS, was then used to fill the well. Microcosms were returned to the growth-cabinet to await sampling or further treatment.

Root Sampling and Bacterial Counts

Bacterial colonization density was determined based on CFU counts. At the relevant timepoint for each experiment, plants were removed from microcosms. Each plant was dip washed three times in sterile Phosphate Buffer Saline (PBS). The phyllosphere, the region from the base of the hypocotyl upward, was removed using an ethanol-sterilized razor blade and discarded. Roots were weighed in 1.5 ml sample tubes using a (Ohaus PA214) scale, then homogenized aseptically using a micro-pestle in the sample tube, in 100 μl of PBS. Serial dilutions of each sample were plated on Kings-B agar containing tetracycline (25 μg ml^{-1}) and incubated at 27°C for 24 h prior to obtaining a CFU count.

Analysis of Bacterial Internalization

An assay was carried out to assess the influence of internalization on rhizoplane colonization density. Microcosms containing plants were inoculated as described above. Microcosms were sampled at 2, 24, 48, 72, and 96 h post inoculation. As above, roots were removed from microcosms and separated from the phyllosphere. Roots were then surface sterilized by placing them in 0.03% w/v sodium hypochlorite for 3 min at room temperature with gentle shaking. Imprints were made by placing the roots on Kings-B agar containing tetracycline (25 μg ml^{-1}) for ∼ 30 s, followed by incubation at 27°C for 24 h, to assess the effectiveness of the surface sterilization protocol. Internalized bacteria were quantified by CFU counts as above. A minimum of five plants were collected for each timepoint, along with an equal number of non-inoculated negative control plants.

Analysis of Bacterial Numbers on the Root Surface

To quantify total colonization density (y_c), which results from both attachment and proliferation on the root surface, bacterial counts were obtained for entire root systems. Counts were carried out at 2, 18, 24, 48, 54, 72, and 96 h post inoculation, over six runs. Further sampling was carried out every 2 h between 18 and 54 h. For each timepoint, a minimum of five inoculated and non-inoculated (control) microcosms were sampled. To quantify the contribution of bacterial proliferation on the root surface to total colonization density (y_p, **Figure 2**), plants were inoculated, then gently removed from their chambers 2 h post inoculation. Root systems were rinsed in PBS to remove unattached bacteria. Plants were then placed in fresh, sterile, microcosm chambers. The first set of samples was taken during transferal – 2 h post inoculation. Following this, microcosms were sealed and returned to the growth chamber prior to sampling at 24, 48, 72, and 96 h post

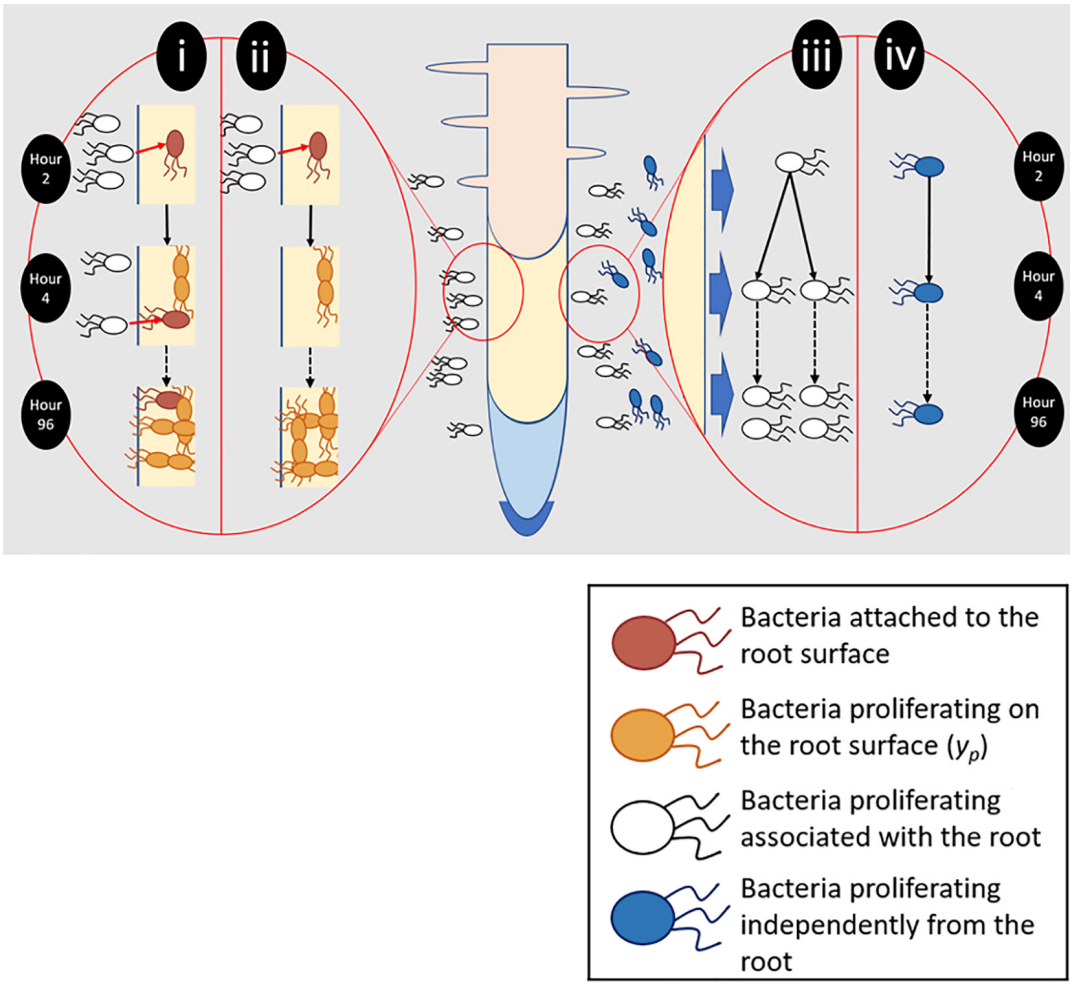

FIGURE 2 | Different aspects of root surface colonization were experimentally isolated and quantified. (i) Total bacterial numbers on the root surface (y_c) were the result of bacterial attachment (pink) and proliferation (orange). (ii) To quantify proliferation on the root surface (y_p), plants were transferred to sterile microcosms 2 h after inoculation. Sterile microcosms contained no bacteria in suspension (white) and as a result attachment could not occur. (iii) Bacteria proliferating in the presence of the root and root exudate were quantified. (iv) The ability of bacteria to proliferate in the absence of any root input was also determined.

inoculation. This experiment was repeated three times with a minimum of five inoculated microcosms per timepoint, along with an equal number of non-inoculated negative controls.

Analysis of Bacterial Proliferation Surrounding the Root

The ability of *P. fluorescens* SBW25 E1433 to proliferate in the medium surrounding the root was investigated by inoculation of a group of five chambers containing a plant, as well as a control group with no plants, as above (**Figure 2**). These were placed within the plant growth chamber. Sampling was carried out at 2, 24, 48, 72, and 96 h post inoculation with further sampling every 2 h between 2 and 24 h. At each timepoint, 100 μl of growth medium was taken from a minimum of five chambers from both groups. CFU counts were established based on plating of serial dilutions on Kings-B agar containing tetracycline (25 μg ml^{-1}), incubated at 27°C for 24 h.

Analysis of Bacterial Proliferation in Root Exudate

To study the ability of *P. fluorescens* SBW25 E1433 to proliferate in the exudates of roots grown in hydroponic, and potentially hypoxic conditions, exudates were collected from lettuce plants grown for 8 days in the microcosm system. During the 8 days, plant grew without replenishment of nutrient solution. The liquid solution from 30 microcosms was collected and pooled. Although it was assumed that non-inoculated microcosm chambers remained sterile, exudates were sterilized using a 0.45 μm filter (fisher scientific 10619672). Filtered exudate solution was plated on non-selective LB agar to test for contamination and found to be negative. Benedict's reagent (Sigma Aldrich 11945) was used for quantification of reducing sugars. Exudate solution was stored at −80°C between experiments. Bacterial growth was quantified in microcosms containing no plants. Liquid cultures of *P. fluorescens* SBW25 E1433 in rich defined RD-MOPS media

containing 25 μg ml^{-1} tetracycline were prepared as described above. Cultures were diluted to an OD_{600} of 0.02 in either 0.5 × MS or root exudate solution. 1 ml of either suspension was pipetted into eight microcosm chambers containing no plants. Each chamber corresponded to the exudate collected from a single plant, after pooling to control for plant-plant variation. An equal number of negative controls containing no bacteria were prepared. Microcosms were placed in the plant growth chamber. At 2, 24, 48, 72, 144, and 168 h post inoculation, 100 μl of solution was taken from each chamber. CFU counts were established based on plating of serial dilutions on Kings-B agar, containing tetracycline (25 μg ml^{-1}).

Models for Microbial Growth

Bacterial density on root surfaces (y, g^{-1}) was determined based on CFU counts and root weight (Wt, g). These were normalized based on bacterial density in the inoculant (CFU^0) to account for variations in initial conditions,

$$y = \frac{CFU}{CFU^0 Wt}. \tag{1}$$

Data relating to the proliferation of bacteria in the medium surrounding the root, in the absence of the root, and in root exudate were expressed as Log_{10}(CFU ml^{-1}).

Various classical models of microbial growth were tested on the data. These included the Logistic (Tsoularis and Wallace, 2002), Gompertz (Gibson et al., 1988), Baranyi (Baranyi and Roberts, 1994) and Richards models (Richards, 1959). Model selection, based on the lowest Akaike Information Criterion (AIC) value, led to the use of the Gompertz function for the proliferation of unattached bacteria in the presence of the root,

$$y = K^* e^{\left(^{(log\ \mathrm{y0}}/_{\mathrm{K}}\right) e^{-\mu time}}). \tag{2}$$

A separate model, representing logistic decline, was used for bacteria in the absence of a root or root exudate,

$$y = a + b\left(1 - e^{\left(^{-\mathrm{c}}/_{\mathrm{time}}\right)}\right). \tag{3}$$

The logistic model (Eqs. 4, 5) was the best fit for total increase of microbial density on the root surface (y_c), proliferation on the root surface (y_p) and proliferation in root exudate data. The logistic equation defines the change of bacterial density (y) as a function of the intrinsic growth rate (μ), and the carrying capacity of the medium (K).

$$\frac{dy}{dt} = \mu y\left(\frac{K - y}{K}\right). \tag{4}$$

The solutions of Eq. 4 are of the form

$$y = \frac{Ky_0}{y_0 + (K - y_0)\, e^{-\mu t}}, \tag{5}$$

with y_0 the initial bacterial density. The equation is fitted on experimental data to estimate growth parameter values (y_0, K, μ) from each experiment. Because the carrying capacity of the root is an intrinsic property of plant roots, it is assumed to be constant across all conditions will be considered constant in the system.

Measurement of Attachment and Time for the Recruitment of Bacteria

To determine the relative contribution of attachment and proliferation on overall bacterial density, bacterial density was monitored from two distinct experiments. The bacterial density due to proliferation (y_p) is defined as the bacterial density on the rhizoplane that result from inoculation at the start of the experiment before transferal to sterile microcosms. The total density due to bacterial colonization (y_c) is defined as the bacterial density on the rhizoplane resulting from both proliferation and attachment of bacteria present in the medium. y_p and y_c were both measured experimentally (**Figure 2**). Because the attachment rate R_a (g^{-1} h^{-1}) cannot be measured directly, it must be derived from the difference between the rate of total colonization R_c (g^{-1} h^{-1}) and rate of proliferation on the root surface (R_p):$R_c = R_p + R_a$. The rate of total colonization R_c (g^{-1} h^{-1}) is obtained by differentiation of Eq. 5,

$$R_c = \frac{K_c\left(^{K_c}/_{y_c^0} - 1\right) e^{-\mu_c}}{\left[1 + \left(^{K_c}/_{y_c^0} - 1\right) e^{-\mu_c t}\right]^2}. \tag{6}$$

R_c represents the combination of attachment to and proliferation on the root surface. Proliferation on the root surface depends on the density of bacteria on the root as described in Eq. 4. Therefore, in the second step, the contribution of proliferation to colonization rate was determined. This was as a function of y_c at time t, which, according to Eq. 4 is,

$$R_p = \mu_p y_c\left(\frac{K_c - y_c}{K_c}\right). \tag{7}$$

Here y_c is the bacterial density determined from Eq. 4 fitted on experimental data for total root colonization density. Finally, the rate of attachment (R_a) is defined as the difference between total colonization rate and proliferation rate,

$$R_a = R_c - R_p = \frac{K_c\left(^{K_c}/_{y_c^0} - 1\right) e^{-\mu_c}}{\left[1 + \left(^{K_c}/_{y_c^0} - 1\right) e^{-\mu_c t}\right]^2} - \mu_p y_c\left(\frac{K_c - y_c}{K_c}\right) \tag{8}$$

The attachment rate can therefore be expressed as a function of the total colonization density y_c using the proliferation coefficient μ_p (**Table 1**).

To characterize the role of timing in the success of a microbe colonizing the root surface, we quantified the relative contribution of attachment at any given time (t) to the total colonization of the rhizoplane at the end of the experiment. This was calculated as the proportion (p) of the final quantity of bacteria that originate from those attached at time t,

$$p(t) = \frac{R_a(t)}{K_c} \int_t^{96} \mu_p y_c\left(\frac{K_c - y_c}{K_c}\right) dt. \tag{9}$$

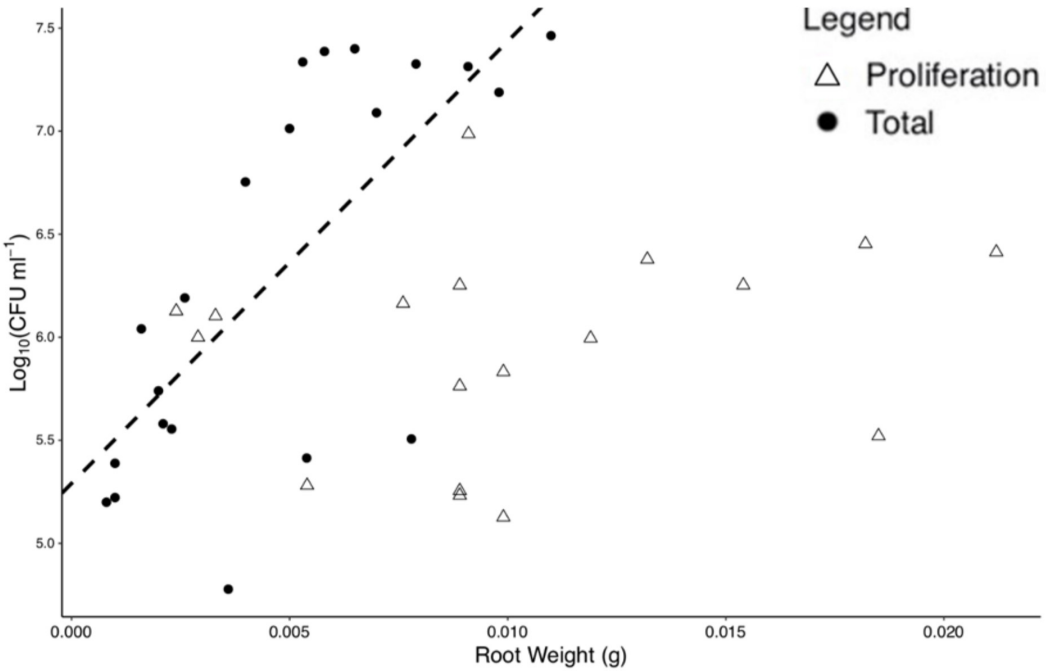

FIGURE 3 | Bacterial numbers on the rhizoplane and root weight are positively correlated for total colonization at 96 h. Each point represents an individual destructive measurement of root weight and Log_{10}(CFU) on the root surface. A significant linear relationship was found for total colonization density at 96 h but not for proliferation data. The dashed line represents the linear model for total colonization (slope = 214.551 Log_{10}(CFU) ml^{-1}, intercept = 5.291 Log_{10}(CFU) ml^{-1}).

Software and Statistical Analyses

Modeling and data analysis were carried out using R (R Development Core Team, 2018). Individual replicates of each treatment type were pooled and analyzed together. Models were fit to each data set using the R package, growthrates (Petzoldt, 2019). Time was given by hour for all data sets. Error for selected models was calculated by bootstrapping with 1000 replicates. To ensure bias was not introduced by the use of parametric forms in calculation of attachment parameters, the same calculations were also done with non-parametric cubic spline fitting on both total colonization density and proliferation data sets. Rate of change of total colonization density (R_c) and proliferation on the root surface (R_p) were calculated based on the finite difference approximation of the derivative of the splines. The relationship between root weight and Log_{10}(CFU ml^{-1}) per root, for total colonization density and proliferation data, was investigated by preforming a linear regression. This was carried out for data at 96 h. Source code can be downloaded at https://github.com/DaireCarroll2019/Root-Attachment-Modeling.

TABLE 1 | Model variables and parameters.

Wt	**Root weight (g)**
CFU^0	Bacterial density of inoculant (ml^{-1})
y	Bacterial density on root surfaces (g^{-1})
K	Carrying capacity (g^{-1})
y^0	Bacterial density on root surfaces at t = 0 (g^{-1})
μ	Intrinsic growth rate
y_c	Total colonization density (g^{-1})
K_c	Root surface carrying capacity (g^{-1})
y_c^0	Bacterial density on root surfaces at t = 0 for total colonization (g^{-1})
μ_c	Intrinsic growth rate for total colonization (g^{-1} h^{-1})
y_p	Colonization density in the absence of attachment (g^{-1})
y_p^0	Bacterial density on root surfaces at t = 0 in the absence of attachment (g^{-1})
μ_p	Intrinsic growth rate in the absence of attachment (g^{-1} h^{-1})
R_c	Rate of total colonization (g^{-1} h^{-1})
R_p	Rate of proliferation (g^{-1} h^{-1})
R_a	Rate of attachment (g^{-1} h^{-1})
$p(t)$	Contribution of attachment at t to the total colonization of the rhizoplane at t = 96 h

RESULTS

Root Exudation and Bacterial Proliferation on or Near the Rhizoplane Are the Main Factors Contributing to Colonization

Imprints of lettuce seeds on non-selective LB agar were found to be clean, indicating that surface sterilization was successful in removing bacteria from the surface of the seed. Plates containing homogenized roots from non-inoculated, negative control chambers for subsequent experiments were also found to be clean. This suggested that non-inoculated microcosms remained free of contamination and *P. fluorescens* SBW25

E1433 did not compete with other microorganisms during these experiments.

To identify the factors influencing the colonization of lettuce roots by *P. fluorescens* SBW25 E1433, plants were grown in a microcosm set up (**Figure 1**) enabling the quantification of bacterial numbers on the root surface. Roots were either grown continuously in one microcosm, enabling both attachment and proliferation on the root surface, or transferred to sterile microcosms 2 h post-inoculation enabling a quantification of proliferation in the absence of attachment (**Figure 2**).

For total colonization, root weight was found to be positively correlated with bacterial numbers at $T = 96$ h ($p < 0.01$, $N = 20$, $DF = 19$, $SE \leq 0.01$, $R^2 = 0.51$), with a slope of 214.55 Log_{10}(CFU) ml^{-1} and an intercept of 5.29 Log_{10}(CFU) ml^{-1}, based on a linear regression (**Figure 3**). No significant correlation was found for root weight and bacterial numbers for proliferation ($p = 0.43$, $N = 17$, $DF = 16$, $SE = 0.01$, $R^2 = 0.04$) alone. Root weight and total CFU count were used for the determination of normalized colonization density (y, Eq. 1) to control for variation in plant size across replicates.

Next, the presence of bacteria internalized within root tissue was quantified. All root imprints were found to be clean, indicating that surface sterilization was successful in removing bacteria from the rhizoplane. We found that internalization was limited to <0.2% of mean total colonization density (g^{-1}) in the root tissue at the final timepoint ($T = 96$ h). Internalization was therefore considered insignificant as a distinct contributing factor and so not included in further analyses.

The ability of bacteria to grow in the presence of root exudates, produced in hydroponic conditions, was also quantified. In the absence of a root or any other plant input, the bacterial count remained constant, with a mean value of 7.12 Log_{10}(CFU ml^{-1}) until $T = 96$ h at which point it began to decline, ending up at a mean value of 6.67 Log_{10}(CFU ml^{-1}) at $T = 192$ h (**Figure 4A**). In contrast, bacterial density increased in the presence of root exudates. Bacterial density increased up to a mean maximum value of 8.09 Log_{10}(CFU ml^{-1}) which was reached at $T = 72$ h (**Figure 4B**), rising from a mean of 7.85 Log_{10}(CFU ml^{-1}) at $T = 2$ h. Sterile root exudates (pooled) were found to have a reducing sugars content of 0.25% w/v. In the presence of a root, bacterial density increased up to a mean maximum value of 7.41 Log_{10}(CFU ml^{-1}), reached at $T = 24$ h, rising from a mean of 5.22 Log_{10}(CFU ml^{-1}) at $T = 2$ h. The best fit for growth in the absence of a root or root exudate was obtained with the logistic decline model (Eq. 3, AIC = −76.92, $r^2 = 0.82$, $SE = 0.08$, $N = 33$, **Figure 4B** and **Table 2**). The best fit for growth in root exudate obtained with the logistic model (Eq. 4, AIC = −16, $r^2 = 0.87$, $SE = 0.68$, $N = 46$, **Figure 4C** and **Table 2**). The best fit for growth in the presence of a root was obtained with the Gompertz model (Eq. 2, AIC = 123, $r^2 = 0.78$, $SE = 0.41$, $N = 112$, **Figure 4C** and **Table 2**).

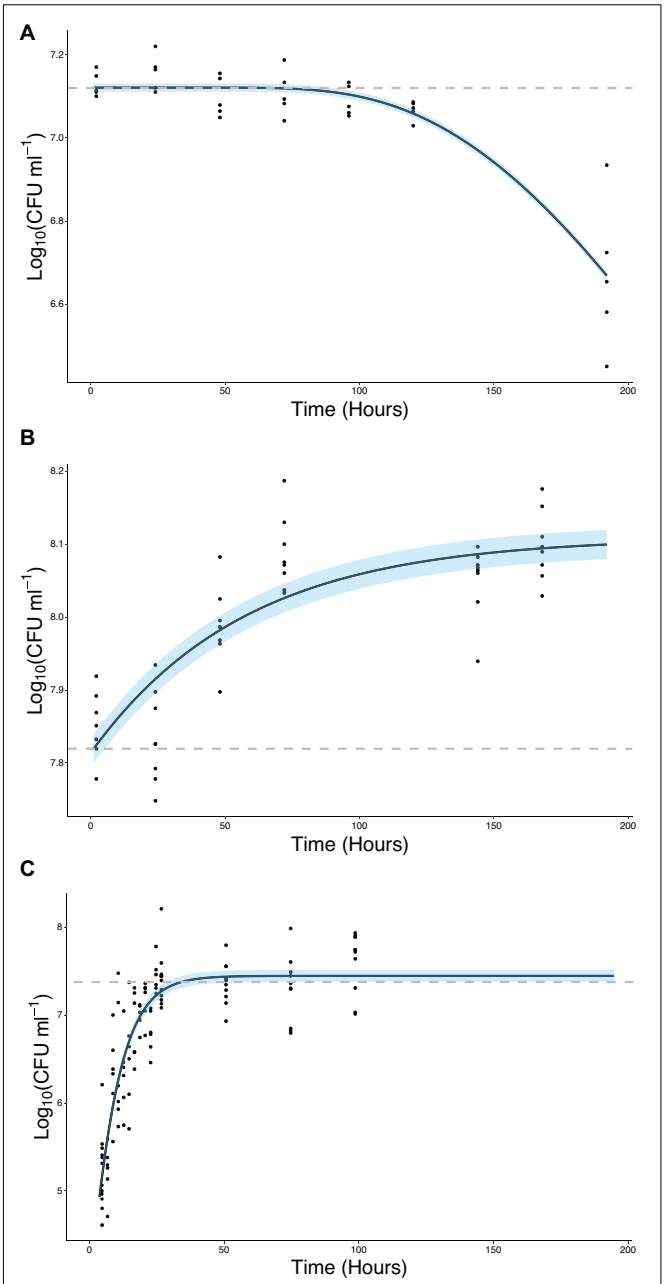

FIGURE 4 | Bacteria proliferate in the presence of the root or root exudate, but not in the absence of any root input. **(A)** The change in Log_{10}(CFU) of *P. fluorescens* SBW25 E1433 in a microcosm system over time in the absence of a plant root or root exudate. Here bacterial numbers in suspension were quantified in microcosm systems containing 1/2 MS plant growth media and no plant (**Figure 2** iii). **(B)** The change in Log_{10}(CFU) of *P. fluorescens* SBW25 E1433 in a microcosm system over time in the absence of a plant root but presence of root exudate. Here bacterial numbers in suspension were quantified in microcosm systems filled with exudate from lettuce roots (**Figure 2** ii). **(C)** The change in Log_{10}(CFU) of *P. fluorescens* SBW25 E1433 in a microcosm system over time in the presence of a plant root. Here bacterial numbers in suspension were quantified in microcosm systems containing a plant (**Figure 2** iv). Black lines represent the relevant fitted model (**Table 1**). Gray dashed lines represent the average initial value for Log_{10}(CFU) in the original inoculant across different replicates of the same treatment. Shaded regions represent bootstrap errors.

Attachment and Proliferation Contribute Differently to Rhizoplane Colonization

Experiments carried out to quantify the density of bacteria on the root surface (y_c) showed that there is consistent increase in

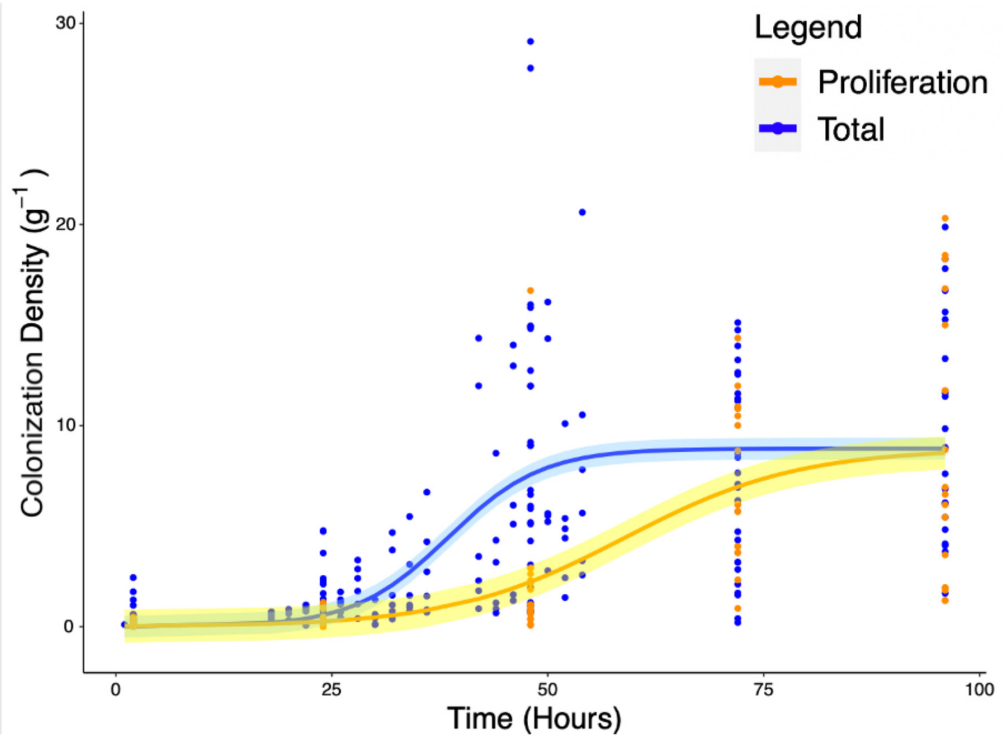

FIGURE 5 | Increase of total root surface colonization density (blue) as well as increase due to proliferation (orange) were modeled using the logistic equation. The accumulation of *P. fluorescens* SBW25 E1433 on root surfaces over time is shown with root surface colonization density represented by CFU normalized for original inoculant and root weight (Eq. 4). Each point represents a destructive measurement of CFU on the root surface of an individual plant. Lines represent fitted logistic models. The total values, shown in blue, represent the bacteria present on the root due to both attachment and proliferation (total colonization density). The proliferation values, shown in orange, represent bacteria proliferating on the root in the absence of attachment beyond 2 h. Shaded regions represent bootstrap errors.

microbial density with time as part of the colonization process. Total colonization of root surfaces reached a mean plateau of 9.97 CFU g^{-1} at T = 72 h, rising from a mean of 0.4 CFU g^{-1} at T = 2 h. A similar form of growth was observed in experiments carried out to quantify the density of bacteria (y_p) in the root proliferation experiment. When roots were inoculated at the start of the experiment and subsequently transferred to sterile microcosms, a consistent increase in bacterial density was observed on the rhizoplane. Although the extent of cell density increase declined marginally, it continued to increase up to T = 96 h with a mean value of 8.78 CFU g^{-1}, rising from a mean of 0.13 CFU g^{-1} at T = 2 h. The density of bacteria observed on the root remained variable between sample replicates, despite the normalization, showing that there was biological variation between bacterial populations and plants. Fitting of classic bacterial growth models on experimental data provided useful parameters for understanding the process of colonization (**Table 1**). The best fit for colonization and proliferation were obtained with the logistic model with respective fit parameters of AIC = 1286, r^2 = 0.42, SE = 0.56, N = 223) and AIC = 498, r^2 = 0.48, SE = 3.32, N = 88 (**Figure 5** and **Table 2**).

TABLE 2 | Models selected based on AIC value for each data set along with model parameters.

Data Set	Selected Model	Bootstrap (1000) Error	Parameters	*P* value
Total Colonization	Logistic	0.54	$K = 8.855974$ $y0 = 0.007333$ $\mu = 0.184677$	$p < 0.001$ $p = 0.559$ $p < 0.001$
Proliferation on root surface	Logistic	0.82	$K = 9.04029218$ $y0 = 0.02508862$ $\mu = 0.09949397$	$p = 0.001$ $p = 0.6409$ $p = 0.0117$
Proliferation in root presence	Gompertz	0.07	$K = 7.4503415$ $y0 = 4.6670427$ $\mu = 0.1240495$	$p < 0.001$ $p < 0.001$ $p < 0.001$
Proliferation in root absence	Logistic decline	0.01	$a = -5.151$ $b = 12.272$ $c = 633.798$	$p = 0.66632$ $p = 0.30719$ $p = 0.00181$
Proliferation in exudate	Logistic	0.02	$K = 8.109634$ $y0 = 7.814389$ $\mu = 0.017965$	$p < 0.001$ $p < 0.001$ $p = 0.177$

Mathematical Modeling Allows Decoupling of Proliferation From Attachment Rate on the Rhizoplane

Since attachment rate cannot be measured directly from experimental methods, a mathematical framework was developed (Eqs. 6–9) to estimate such parameters from experimental growth

curves y_c and y_p. Estimation of the attachment parameters was achieved by the following steps. First, growth curves y_c and y_p were used to determine the experimental colonization and proliferation rates. In a second step, the proliferation rate was expressed as a function of the density of bacterial colonization. The attachment rate was subsequently calculated as the difference between total colonization rate and the proliferation rate during the total colonization experiment (**Figure 6A**). Finally, the total quantity of bacteria present on the root surface due to recruitment from the surrounding media can be calculated from the attachment rate by integration (**Figure 6B**). Attachment rate estimated using this approach exhibited similar kinetics to microbial colonization. A peak of 0.18 g^{-1} h^{-1} in attachment rate is achieved at T = 38 h. This indicates that the level of colonization of the root affects the attachment rate of bacteria. Attachment rates calculated based on cubic splines did not show disagreement from those generated by treatment of parametric models. This suggests that no bias was introduced by the choice of growth models.

Factors Contributing to Attachment and Colonization

Attachment rate R_a (g^{-1} h^{-1}) was found to vary over time, rising from a starting value of 7.5 × 10^{-4} g^{-1} h^{-1} to a peak value of 0.188 g^{-1} h^{-1} at T = 38 h before declining to a value of 1.82 × 10^{-5} g^{-1} h^{-1} at T = 96 h (**Figure 6B**). To investigate the influence of total colonization density on attachment rate, R_a was expressed as a function of y_c, the total colonization density (g^{-1}) on the root surface. It was found that this relationship could be expressed as a quadratic equation (**Figure 7A**),

$$R_a = -1.19\ x\ 10^{-11} + 8.52\ x\ 10^{-2}\ y_c - 9.98\ x\ 10^{-3} y_c^2 \quad (10)$$

A peak of 0.19 g^{-1} h^{-1} in attachment rate was seen when total colonization density was at 4.26 g^{-1}. This corresponded to the attachment and colonization values at 38 h post inoculation (**Figure 7A**). Results also show the timing of attachment influenced the extent of successful colonization of the microbe on the root. Bacteria proliferation rate was used to calculate the contribution of attached bacteria at time t to the total density of bacteria at the end of the experiments (**Figure 7B**).

DISCUSSION

The experimental approaches proposed in our study are in line with a long series of past studies for measurement of root colonization (Hansen et al., 1997; Schmidt et al., 2018) and assessment of attachment of bacteria to roots (Mills and Bauer, 1985; Albareda et al., 2006). Destructive quantification of root colonization is generally carried out at a single timepoint or at very coarse time intervals (Unge and Jansson, 2001; Schmidt et al., 2018). Unattached bacteria can be removed by washing, with numbers of attached bacteria subsequently being determined through either plating or imaging. Such assays are commonly used in plant and bacterial sciences, however, as the destruction of the sample is required, they lack the temporal resolution necessary to map out the dynamic process of colonization. Our experimental system shares the same limitations, but significant effort was put into quantifying colonization at dense time intervals during the early stages of colonization for the data to capture the precise kinetics of attachment on the root.

Efficient colonization is a key component of plant growth promoting bacterial activity (Chin-A-Woeng et al., 2000; Kamilova et al., 2005). As a result, quantification of colonization is important for assessing plant growth promoting bacterial strains (Mendis et al., 2018), although it is often overlooked (Cipriano et al., 2016; Kour et al., 2019). Destructive quantification methods, similar to that used in our experimental system, can be used to assess colonization of roots by plant growth promoting bacteria (Bach et al., 2016; Hsu and Micallef, 2017).

We observed similar bacterial colonization levels to those reported previous studies. Noirot-Gros et al. (2018) reported 5 × 10^7 CFU g^{-1} of root after 5 weeks, on aspen (*Populus tremula*). Unge and Jansson (2001) studied the colonization of wheat roots by *P. fluorescens* isolate SBW25, a plant growth promoting bacterial isolate, at 6 days post inoculation and reported root colonization values between 1.15 × 10^8 and 4.29 × 10^8 CFU g^{-1} of root. We reported slightly lower mean colonization density 9.1 × 10^6 CFU g^{-1} of root at T = 96 h. The lower values we report are unlikely to be the result of shorter experimental times, as the logistic growth model predicted that carrying capacity would be reached during our experimental timeframe (**Figure 5**). Instead, differences in colonization levels are likely due to root maturity, plant species and quantification method. Studies based on short exposure of the root to bacteria have had limited scope because colonization rate is affected by a range of factors (Massalha et al., 2017; Schmidt et al., 2018).

Colonization assays are powerful because of their simplicity and ability to study large numbers of samples in one experiment. However, using such screens for characterization of attachment or proliferation rates is more difficult. They cannot distinguish attachment from proliferation on the root meaning individual rate parameters cannot be obtained directly. Attachment rate is a particularly difficult parameter to measure since direct observation and tracking of single bacterial cells is rarely achievable in the root environment. A typical approach is to quantify attachment by viable cell counts after a short period of exposure to bacteria (Shimshick and Hebert, 1979; Albareda et al., 2006), during which time increase in bacterial density due to proliferation is limited. Mills and Bauer (1985) preformed a quantification of the attachment of *Rhizobium trifolii* to white clover (*Trifolium repens*), using root sonication and enumeration to quantify attached cells. Variations of these approaches have been tested on a range of bacteria and plant species (Albareda et al., 2006).

In this study, we have addressed the limitations of colonization and attachment assays using both data with high temporal resolution and a suitable mathematical framework linking

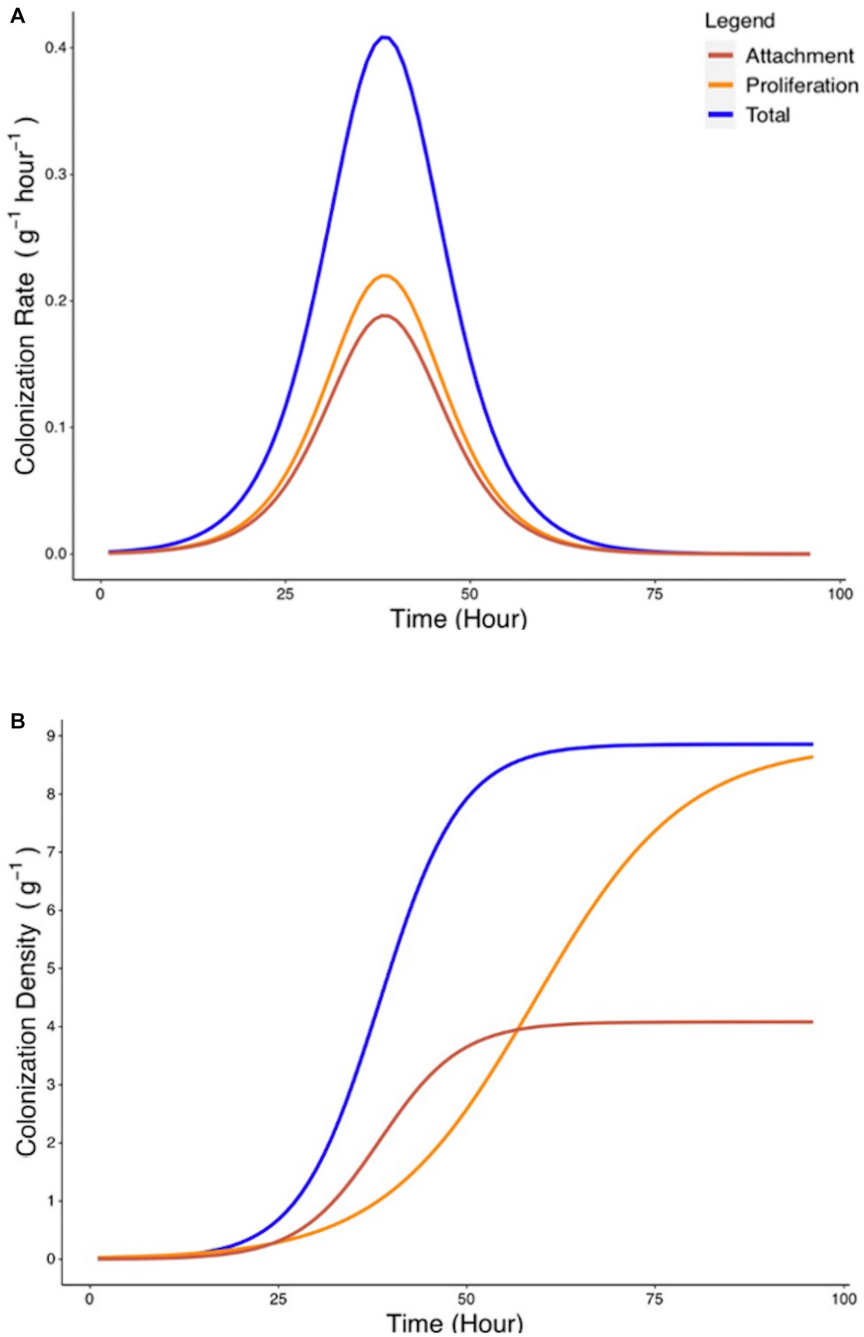

FIGURE 6 | Estimated rate of bacterial attachment to the rhizoplane. **(A)** The rates of total colonization (R_c, blue) and proliferation (R_p, yellow) were calculated based on Eqs. 6, 7. The rate of attachment (R_a, red) was calculated based on Eq. 8. **(B)** Colonization density due to attachment was estimated by integration of Eq. 8.

colonization and proliferation rates. This allowed the calculation of system parameters unobtainable using traditional methods. We observed a notable time lag required for permanent attachment (approximately 24 h). This was not detected in previous studies, probably due to differences in method of extraction that counted non-permanent attachment of bacteria. Our method also allows resolution of time variations in colonization rate not previously available. Attachment rate varies with time, due to the changing density of both attached and free moving bacteria, as well as transient and heterogeneous adherence factor gene expression profiles of bacteria. This was established by the early work of Shimshick and Hebert (1979), who proposed a dynamic model of attachment on roots based on adsorption-desorption theory. However, the scope of the study by Shimshick and Hebert (1979) is limited because the model did not consider the proliferation of

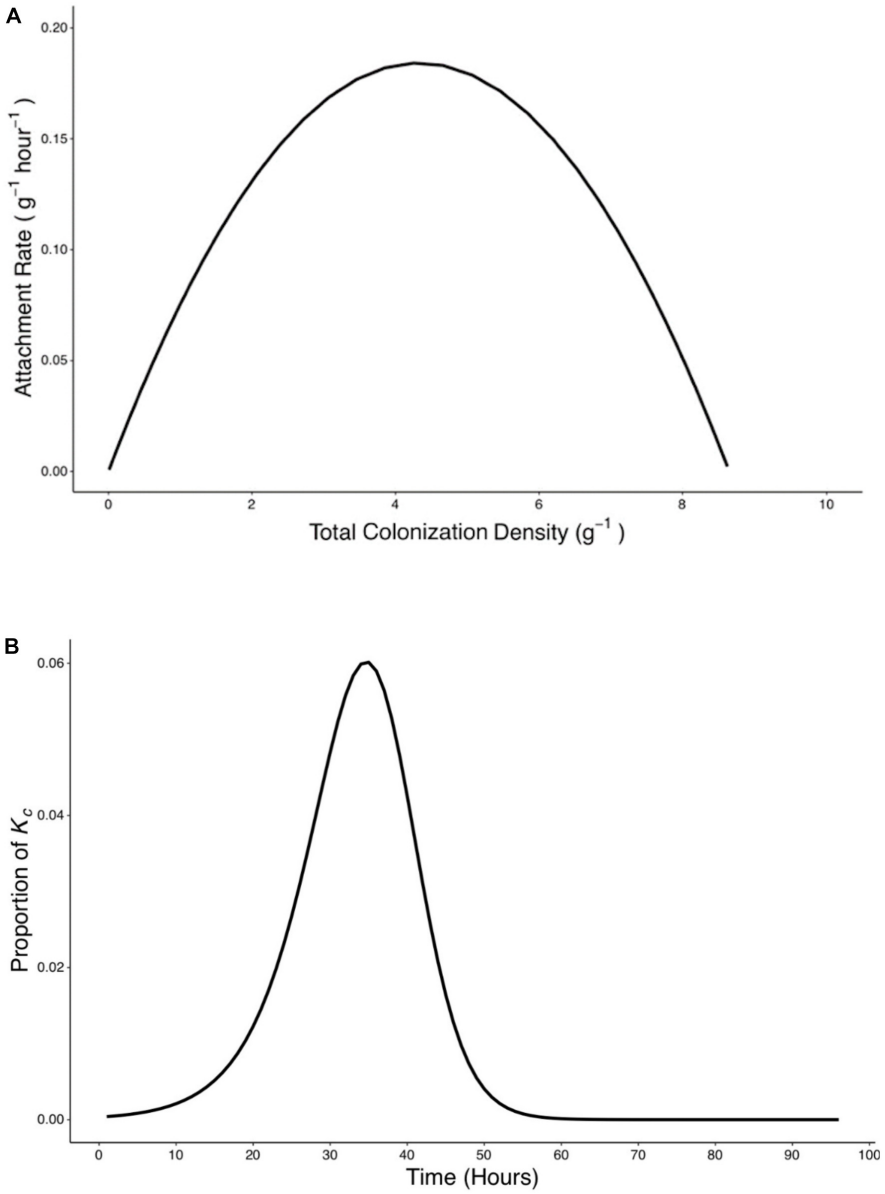

FIGURE 7 | Rate of attachment (R_a) expressed as a function of total colonization density (y_c) and the relative contribution of attachment to final colonization density was established. **(A)** The relationship between rate of attachment and total colonization rate can be expressed as a quadratic function ($R_a = -1.19\ x\ 10^{-11} + 8.52\ x\ 10^{-2}\ y_c - 9.98\ x\ 10^{-3} y_c^2$). **(B)** The proportion of K_c reached at 96 h by bacteria which attach at any time p(t) was calculated based on Eq. 9.

bacteria on the root surface itself, which we showed is not negligible (**Figure 4**).

Application of Mathematical Framework for Estimation of Attachment Rate During Bacterial Establishment

Our mathematical estimations of bacterial attachment rates have broad applicability. They rely on standard colonization assays commonly used in laboratories. The method does not require sophisticated live observations of bacteria, and calculations for estimation of growth and attachment coefficients are simple. The method also provides temporally resolved measurements of attachment rate, which is extremely time consuming in dedicated attachment assays. Currently, limitations are linked to the simplified experimental system and how quantification of bacterial density is achieved. The experimental system is highly simplified with comparison to rhizosphere development in natural environments. The lack of physical structure in the substrate is most likely a source of bias in the estimation of attachment rate. Reliably recovering and quantifying a bacterial strain in the field using a plating method is difficult, and only

culturable bacteria can be studied by plating. More specific molecular methods for quantification of specific strains, or taxa, are available (Mendis et al., 2018). There is evidence from the literature to suggest adequate data could be obtained from more sophisticated experimental system and modern analytical tools. Colonization data could be obtained from roots grown in natural soils by fluorescent *in situ* hybridization (Gamez et al., 2019), sequencing (Mitter et al., 2017) or qPCR (Mendis et al., 2018). Fluorogenic PCR assays, for example, have been used to quantify the presence of non-culturable *Pseudomonas* in natural soils (Lloyd-Jones et al., 2005). Hydroponic solutions can be replaced with transparent soil which has been shown to provide the physical structure of a soil while enabling direct observation of root and bacteria (Downie et al., 2015).

Because colonization assays rely on plating, they are destructive and require large replication numbers. Colonization assays do not provide maps of spatial variations in attachment rate and use of hydroponics neglect the role of transport to the root surface. Such limitation can be remediated to fully exploit the mathematical framework developed here. Modern live-microscopy can overcome the limitations of colonization assays (Downie et al., 2015; Noirot-Gros et al., 2018). For example, Gamez et al. (2019) compared the root colonization patterns of two plant growth promoting bacterial strains, Pseu*domonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006, on banana. They concluded that *B. amyloliquefaciens* was a faster colonizer. Modern microscopes provide the ability to image large samples in high throughput (Berthet and Maizel, 2016), to grow plants vertically with automated tracking of root tips (von Wangenheim et al., 2017), and simultaneously map the distribution of bacteria around the root (Massalha et al., 2017; Pavlova et al., 2017). Processing of data using artificial intelligence can automate the mapping of bacterial density along the root (Carbone et al., 2017). The ability to track bacteria has drastically improved since the early work of Shimshick and Hebert (1979), for example, observation of single bacterial cell and visualization of their attachment is now routinely achieved with modern microscopes (Duvernoy et al., 2018; Ipina et al., 2019). Mathematical frameworks will be essential to interpret such complex experimental data because they can establish links between attachment rates, root growth, bacterial proliferation, and the complex distribution of bacterial density along the root (Dupuy and Silk, 2016).

Microbial Establishment on Rhizoplanes

The exact attachment mechanisms of *Pseudomonas fluorescens* isolate SBW25 have not yet been determined. Exploring the dynamics of rhizoplane colonization gives clues as to what might be occurring when bacteria first interact with roots. Based on the results of this study, we can propose various stages of bacterial establishment on root surfaces. In the first step, roots and microbes come into contact. In the case of a hydroponic solution, root exudates diffuse, leading to bacteria detecting the presence of the root and rapidly moving toward it. Secondly, bacteria likely form weak, reversible attachments to the root surface. This establishes a large proportion of the bacterial population in close association with the root. During this stage, rate of proliferation of bacteria surrounding the root increases. This accounts for the low rate of colonization predicted by our model during T = 0–24 h. As a third step, strong, irreversible attachment to the root is established. At this stage, the recorded rate of attachment begins to rapidly increase. Attached bacteria proliferate, further increasing colonization rate during T = 24–38 h. We predicted that colonizers between T = 24–48 h would make the greatest contribution to final root colonization density at capacity, suggesting a dependence of attachment rate on colonization density. Based on our modeling, the involvement of these factors suggests a level of priming activity. Attachment and proliferation rates begin to decrease (T = 38 h) before reaching zero in the fourth and final stage (T = 38–72 h). At carrying capacity, the rate of new bacteria colonizing the rhizoplane through recruitment and proliferation will be balanced by death, dissociation, and dilution of colonies through root growth. Carrying capacity is the result of limiting factors on bacterial growth. The two most likely limiting factors are space and nutrient availability. The system reaches capacity when the rate of production of new regions, through root growth, is matched by the rate of colonization. The system will be maintained at capacity if root growth rate and colonization rates remain in equilibrium. Longer term, the capacity may also be determined by the rate of nutrient production and the availability of carbon and nitrogen within the rhizosphere has been linked to root size in previous studies (Guyonnet et al., 2018).

CONCLUSION

The ability to model rhizoplane colonization is a valuable tool for researchers. Modeling of bacterial interaction with plants is a complex process requiring a solid base in experimental data. Isolating and quantifying aspects of root surface colonization has been shown here to allow the contributions of attachment and proliferation of bacteria root maturity to be estimated, and thus this is an important step in understanding the process of rhizoplane colonization. Our experimental and mathematical frameworks provide a novel method for inferring attachment and proliferation rates during the early period of colonization. This has never previously been possible as these processes are not quantifiable through direct observation. The utilization of plant growth promoting and pathogen suppressing bacteria in agricultural systems will require a solid understanding of the colonization process which has not previously been available. Applications of these novel frameworks include the selection of traits promoting maintenance on the root of beneficial bacteria or limiting the impact of soil borne pathogens. The work presented gives new insight into the interaction between *Pseudomonas fluorescens* isolate SBW25 and Lettuce. It sets the groundwork for more targeted and in-depth studies of rhizoplane colonization, and a more holistic understanding of the interactions between bacteria and plant roots.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DC carried out the wet lab work and data collection along with design of the microcosm system, carried out the data analysis and modeling with advice from LXD, produced the figures, and wrote the initial manuscript draft and edited with advice from LXD, MLG, and NH. LXD gave advice and assistance in the modeling and downstream data analysis and suggested significant revisions to the text of the manuscript and figures. NH and MLG provided supervision of wet lab experiments and suggested significant revisions to the text of the manuscript and figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Microbial Interactions Within Multiple-Strain Biological Control Agents Impact Soil-Borne Plant Disease

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Major losses of crop yield and quality caused by soil-borne plant diseases have long threatened the ecology and economy of agriculture and forestry. Biological control using beneficial microorganisms has become more popular for management of soil-borne pathogens as an environmentally friendly method for protecting plants. Two major barriers limiting the disease-suppressive functions of biocontrol microbes are inadequate colonization of hosts and inefficient inhibition of soil-borne pathogen growth, due to biotic and abiotic factors acting in complex rhizosphere environments. Use of a consortium of microbial strains with disease inhibitory activity may improve the biocontrol efficacy of the disease-inhibiting microbes. The mechanisms of biological control are not fully understood. In this review, we focus on bacterial and fungal biocontrol agents to summarize the current state of the use of single strain and multi-strain biological control consortia in the management of soil-borne diseases. We discuss potential mechanisms used by microbial components to improve the disease suppressing efficacy. We emphasize the interaction-related factors to be considered when constructing multiple-strain biological control consortia and propose a workflow for assembling them by applying a reductionist synthetic community approach.

Keywords: microbial interaction, biological control agents, soil-borne disease, consortia, microbiome and community

INTRODUCTION

The interest in control of plant diseases by beneficial microbes, has increased recently due to the global need for environmentally friendly alternatives to chemical pesticides and fertilizers (Handelsman and Stabb, 1996; Fira et al., 2018; Syed Ab Rahman et al., 2018). A large number of bacterial and fungal strains, as well as viruses, nematodes, and insects have been employed as biological control agents (BCAs) in the management of soil-borne crop pathogens for decades. BCAs have become a crucial component of sustainable agriculture and forestry

(Cazorla and Mercado-Blanco, 2016; Alvarez and Biosca, 2017; Busby et al., 2017; Umesha et al., 2018). Although numerous beneficial microbial strains performed well against pathogens under controlled conditions in the laboratory or the greenhouse, examples of successful BCA application in commercial field-based crop production are rare (Xu et al., 2011; Mazzola and Freilich, 2017). This is mainly due to inadequate colonization of host rhizosphere connected with inefficient inhibition of soil-borne pathogen growth (Sarma et al., 2015; Mazzola and Freilich, 2017).

Different BCA consortia, consisting of two or more microbial strains [multi-strain biological control agents (MSBCAs)], are assembled to improve the stability and efficiency of disease-inhibition (Sarma et al., 2015; Mazzola and Freilich, 2017; Vorholt et al., 2017; Woo and Pepe, 2018). The biotechnological potential of microbial consortia was reviewed recently and examples for their possible applications in areas of biopolymers, bioenergy, biochemicals, and bioremediation have been presented (Bhatia et al., 2018). Here, we focus on the application of MSBCAs in sustainable agriculture. In several cases, superior disease suppression exerted by MSBCAs has been reported (**Table 1**). Diverse modes of action were proposed: (i) diversity in biocontrol mechanisms offered by each microbial component (Pierson and Weller, 1994; Sarma et al., 2015), (ii) occupation of distinctive niches by probiotic microorganisms resulting in more stable communities (Pierson and Weller, 1994; Pliego et al., 2008; Thomloudi et al., 2019), (iii) enhanced modulation of genetic elements acting in the community (Lutz et al., 2004), and (iv) a broader spectrum of targeted phytopathogens (Sarma et al., 2015; Thomloudi et al., 2019) may contribute to greater biocontrol activity in communities. However, our understanding of the mechanisms underlying the reinforcement of their disease-inhibitory effects by MSBCAs is still very limited.

Interactions in communities of plant-associated microbes are essential for plant health (Whipps, 2001; Frey-Klett et al., 2011; Kemen, 2014; Hassani et al., 2018). A well-known example is disease suppressive soil. They are defined by their ability to suppress plant diseases such as "take-all" disease in wheat caused by the fungal pathogen *Gaeumannomyces graminis*. The suppressive effect is due to the presence of 2,4-diacetylphloroglucinol produced by a group of soil-borne *Pseudomonas* spp. (Kwak and Weller, 2013).

The interplay among the members of MSBCAs might be relevant to their elevated disease-suppressing effect. It is necessary to pay attention to the microbe−microbe interplay-related elements when constructing MSBCAs because microbial interactions within the plant microbiome are important selective forces forming complex microbial assemblages (Hassani et al., 2018). In general, two different methods can be distinguished when BCA consortia are prepared: (i) mixing existing single-strain biological control agents (SSBCAs) according to empirical experience or (ii) preparing MSBCAs as a reductionist synthetic community (RSC) (Liu et al., 2019). In the RSC approach, defined synthetic communities (SynCom) are assembled using a limited number of isolates from the natural microbiome. In the following we prefer to use the term "SynCom" given that synthetic communities contain usually a limited number of isolates.

In this review, we provide a brief overview of the current state of the use of MSBCAs in the management of soil-borne diseases and describe potential mechanisms used by their microbial components to improve disease-suppression. We describe interaction-related factors to be considered when constructing MSBCAs and propose a workflow for assembling them as a reductionist synthetic community (Vorholt et al., 2017; Liu et al., 2019).

UTILIZATION OF MSBCAS IN MANAGEMENT OF SOIL-BORNE DISEASES

Selection of novel biocontrol microbial strains via isolation and screening is a permanent approach to improve the disease-controlling efficiency of BCAs. Although novel disease-suppressive strains might overcome inadequate colonization of the host rhizosphere and inefficient inhibition of soil-borne pathogen growth, the discovery of taxonomically novel isolates possessing biological disease control activity becomes more difficult over time even after extensive searches. Another promising approach, exploiting genetically modified microbial strains with improved antagonistic function has been restricted or prohibited worldwide (Migheli, 2001; Stemke, 2004). When applying BCAs in natural settings, BCAs do not act independent of their environment but interact with many indigenous microbes to become components of local microbial communities. The members of such consortia may evolve niche−specific microbial interactions to influence plant health (Whipps, 2001). There is growing interest in the use of disease-suppressing microbial communities, specifically MSBCAs, for controlling soil-borne pathogens.

Multi-strain biological control agents have successfully controlled soil-borne diseases of valuable crops caused by fungi, oomycetes, bacteria and nematodes (**Table 1**). Several microbial combinations are possible, such as fungus to fungus, fungus to bacterium, and bacterium to bacterium. Similar to the single-strain biological control agents (SSBCAs), MSBCAs employ diverse modes of action for control, e.g., competition for resources and niches (McKellar and Nelson, 2003; Wei et al., 2015; Hu et al., 2016), production of antimicrobial compounds (Thakkar and Saraf, 2014; Santhanam et al., 2019), induction of systemic resistance (Sarma et al., 2015; Solanki et al., 2019), and regulation of microbial communities (Zhang L.-N. et al., 2019). MSBCAs appear to have higher efficiency for control of soil-borne disease than SSBCAs (**Figure 1A**).

Synergistic and/or additive effects exerted by carefully selected microbial consortia might explain their superior efficacy compared to single SSBCAs. In simple cases, MSBCAs consist of only two strains, e.g., a fungus and a bacterium where one or both have biocontrol activities. A consortium consisting of *Trichoderma asperellum* GDFS1009 and *Bacillus amyloliquefaciens* ACCC1111060 was found to be more efficient against infection by *Botrytis cinerea* (the agent of gray mold disease) than the individual strains (Wu et al., 2018). Similarly, when *Trichoderma virens* GI006 was combined

TABLE 1 | List of multiple-strain biological control agents (MSBCAs) against soil-borne pathogens.

Number	Multiple-strain biological control agents	Mode of application	Disease	Pathogens	Potential mode of action	Host	References
1	*Trichoderma harzianum* CECT 2413 and *Streptomyces rochei* Ziyani	Soil inoculation	Root rot	*Phytophthora capsici*	Disintegration of the hyphae and production of 1-propanone, 1-(4-chlorophenyl)	Pepper	Ezziyyani et al., 2007
2	*Bacillus cereus* AR156, *Bacillus subtilis* SM21 and *Serratia* sp. XY21	Seedling treatment	*Phytophthora* blight	*Phytophthora capsici*	Alternation of the soil bacterial community	Sweet pepper	Zhang L.-N. et al., 2019
3	*Pseudomonas aeruginosa* MBAA1, *Bacillus cereus* MBAA2 and *Bacillus amyloliquefaciens* MBAA3	Seed bacterization	Stem rot and charcoal rot	*Sclerotinia sclerotiorum* and *Macrophomina phaseolina*	Production of ammonia, siderophore and enzymes like β-1,3 glucanase, chitinase and cellulase	Soybean	Thakkar and Saraf, 2014
4	*Pseudomonas aeruginosa* PJHU15, *Trichoderma harzianum* TNHU27 and *Bacillus subtilis* BHHU100	Seed coating	White rot	*Sclerotinia sclerotiorum*	Induced systemic resistance and enhanced oxygen species managenment	Pea	Jain et al., 2015
5	*Pseudomonas* sp. S1, *Bacillus* sp. S2, *Azotobacter* sp. S3, *Azospirillum* sp. S4 and *Pseudomonas fluorescens* S5	Seedling treatment	Vascular wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Induced systemic resistance	Tomato	Kannan and Sureendar, 2009
6	*Glomus intraradices*, *Pseudomonas* sp. and *Trichoderma harzianum*	Seed soaking	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Production of siderophore and rhamnolipid	Tomato	Srivastava et al., 2010
7	*Bacillus subtilis* S2BC-1 and *Bacillus subtilis* GIBC-Jamog	Seed bacterization and soil application	Vascular wilt	*Fusarium oxysporum* f. sp. *lycospersici*	Direct biocontrol and induced systemic resistance	Tomato	Shanmugam and Kanoujia, 2011
8	*Trichoderma* sp. NRCB3 and *Trichoderma asperellum* Prr2	Soil inoculation and root treatment	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Inhibition of spore germination and mycelial growth due to antibiosis and antifungal metabolites production	Banana	Thangavelu and Gopi, 2015b
9	*Bacillus subtilis* GB03, *Bacillus amyloliquefacien* IN937a and *Pseudomonas fluorescens* CECT 5398	Media inoculation and seed drenching	*Fusarium* wilt and *Rhizoctonia* damping off	*Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Rhizoctonia solani*	Production of siderophores and induced systemic resistance	Pepper and tomato	Domenech et al., 2006
10	*Bacillus* sp. EPB10, *Bacillus* sp. EPB56 and *Pseudomonas fluorescens* Pf1	Root soaking	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Enhancement of the expression of defense related enzymes	Banana	Mathiyazhagan et al., 2014
11	Mixture of uncultivated endophytes derived from healthy banana plants	Root drenching	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Antagonism and induction of the activities of host defense-related enzymes	Banana	Lian et al., 2009
12	*Glomus mosseae*, *Trichoderma harzianum* and *Pseudomonas fluorescens*	Soil inoculation	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Physical modifications in the cell wall, growth promotion and induction of disease resistance	Banana	Mohandas et al., 2010
13	*Pseudomonas putida* C4r4, *Pseudomonas putida* Jrb2, *Bacillus cereus* Jrb1, *Bacillus cereus* Jrb5, *Bacillus flexus* Tvpr1, *Achromobacter* spp. Gcr1 and *Rhizobium* spp. Lpr2	Root dipping and soil application	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Production of siderophores, protease enzymes, chitinase and hydrogen cyanide	Banana	Thangavelu and Gopi, 2015a
14	*Bacillus subtilis* EPB56, *Bacillus subtilis* EPB10 and *Pseudomonas fluorescens* Pf1	Root soaking	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Increasement of the activity of defense enzymes	Banana	Kavino and Manoranjitham, 2017
15	*Pseudomonas aeruginosa* DRB1 and *Trichoderma harzianum* CBF2	Soil inoculation	*Fusarium* wilt	*Fusarium oxysporum* f. sp. *cubense*	Production of 2,4-diacetylphloroglucinol and chitinase	Banana	Wong et al., 2019
16	*Pseudomonas* sp. UPMP3 and *Burkholderia* sp. UPMB3	Soil drenching	*Fusarium* Wilt	*Fusarium oxysporum*	Increase of resistance-related enzymes, lignithioglycolic acid and pathogenesis-related proteins	Banana	Mohd Fishal et al., 2010

(Continued)

TABLE 1 | Continued

Number	Multiple-strain biological control agents	Mode of application	Disease	Pathogens	Potential mode of action	Host	References
17	*Bacillus subtilis* GBO3, *Bacillus subtilis* MBI600 and *Rhizobium tropici*	Seed application	Root rot	*Fusarium oxysporum*, *Fusarium solani f. sp. phaseoli* and *Rhizoctonia solani*	Production of siderophores	Dry bean	Estevez de Jensen et al., 2002
18	*Pseudomonas fluorescens* LPK2, *Sinorhizobium fredii* KCC5 and *Azotobacter chroococcum* AZK2	Seed bacterization	Fusarial wilt	*Fusarium udum*	Production of metabolites against the conidial germination and germ tube growth	Pigeon pea	Choure et al., 2012
19	*Stenotrophomonas maltophilia* AA1, *Ochrobactrum pituitosum* AA2, *Curtobacterium pusillum* AA3, *Enterobacter ludwigii* AA4, *Chryseobacterium indologenes* AA5, *Herbaspirillum frisingense* AA6 and *Pseudomonas putida* AA7	Seed soaking	Seedling blight	*Fusarium verticillioides*	Inhibiting fungal colonization and arresting hyphal expansion growth	Maize	Niu et al., 2017
20	*Xanthobacter agilis*, *Microbacterium* sp., *Paracoccus denitrificans*, two Enteric bacterium strains and five Coryneform bacterium strains	Seed soaking	*Pythium* damping-off	*Pythium ultimum*	Fatty acid metabolism	Cotton	McKellar and Nelson, 2003
21	*Trichoderma viride* and *Streptomyces* sp.	Media inoculation	Sudden wilting	*Pythium aphanidermatum*	-	Poinsettia	Bolton, 1980
22	Chitinophaga sp. 94, and *Flavobacterium* sp. 98	Root drenching	Damping off	*Rhizoctonia solani*	A NRPS-PKS gene cluster from *Flavobacterium* was essential for disease suppression	Sugar beet	Carrión et al., 2019
23	*Streptomyces atrovirens* N23 and *Trichoderma lixii* NAIMCC-F-01760	Soil inoculation and root treatment	Root rot	*Rhizoctonia solani*	Activation of plant defense	Tomato	Solanki et al., 2019
24	*Trichoderma virens* GI006 and *Bacillus velezensis* Bs006	Soil inoculation	Fusarium wilt	*Fusarium oxysporum* f. sp. *phaseoli*	Formation of biofilms and production of antimicrobial compounds	Cape gooseberry	Izquierdo-García et al., 2020
25	*Bacillus cereus* AR156, *Bacillus subtilis* SM21 and *Serratia* sp. XY21	Seedling and soil drenching	Verticillium wilt	*Verticillium dahliae*	Induced systematic Resistance and secretion of anti-fungal metabolites	Cotton	Yang et al., 2014
26	*Pseudomonas* sp. CHA0, *Pseudomonas* sp.PF5, *Pseudomonas* sp.Q2-87, *Pseudomonas* sp.Q8R1-96, *Pseudomonas* sp.1M1-96, *Pseudomonas* sp. MVP1-4, *Pseudomonas* sp.F113, and *Pseudomonas* sp. Phl1C2	Root drenching	Bacterial wilt	*Ralstonia solanacearum*	Competition for resources and interference with the pathogen	Tomato	Hu et al., 2016
27	*Ralstonia* spp. QL-A2, *Ralstonia* spp. QL-A3, *Ralstonia* spp. QL-A6, *Ralstonia* spp. QL-117 and *Ralstonia* spp. QL-140	Root drenching	Bacterial wilt	*Ralstonia solanacearum*	Resource competition	Tomato	Wei et al., 2015
28	*Serratia plymuthica* A294, *Enterobacter amnigenus* A167, *Rahnella aquatilis* H145, *Serratia rubidaea* H440, and *S. rubidaea* H469	Tuber soaking	Potato soft rot	*Pectobacterium spp., Dickeya spp.*	Production of antibiotic compounds, biosurfactants and siderophores	potato	Maciag et al., 2020
29	Tomato rhizosphere microbiome	Transplantation	Bacterial wilt	*Ralstonia solanacearum*	*Flavobacteriaceae* sp. TRM1 could suppress *Ralstonia solanacearum* disease development	Tomato	Kwak et al., 2018
30	Eggplant and cucumber rhizosphere microbiome	Root drenching	Root knot	*Meloidogyne* spp.	Direct antagonism and/or induction of plant resistance	Tomato	Zhou et al., 2019
31	Root associated synthetic multikingdom assemblages	Soil inoculation	–	Fungal pathogens	Bacterial microbiota suppresses fungal pathogens	Arabidopsis	Durán et al., 2018

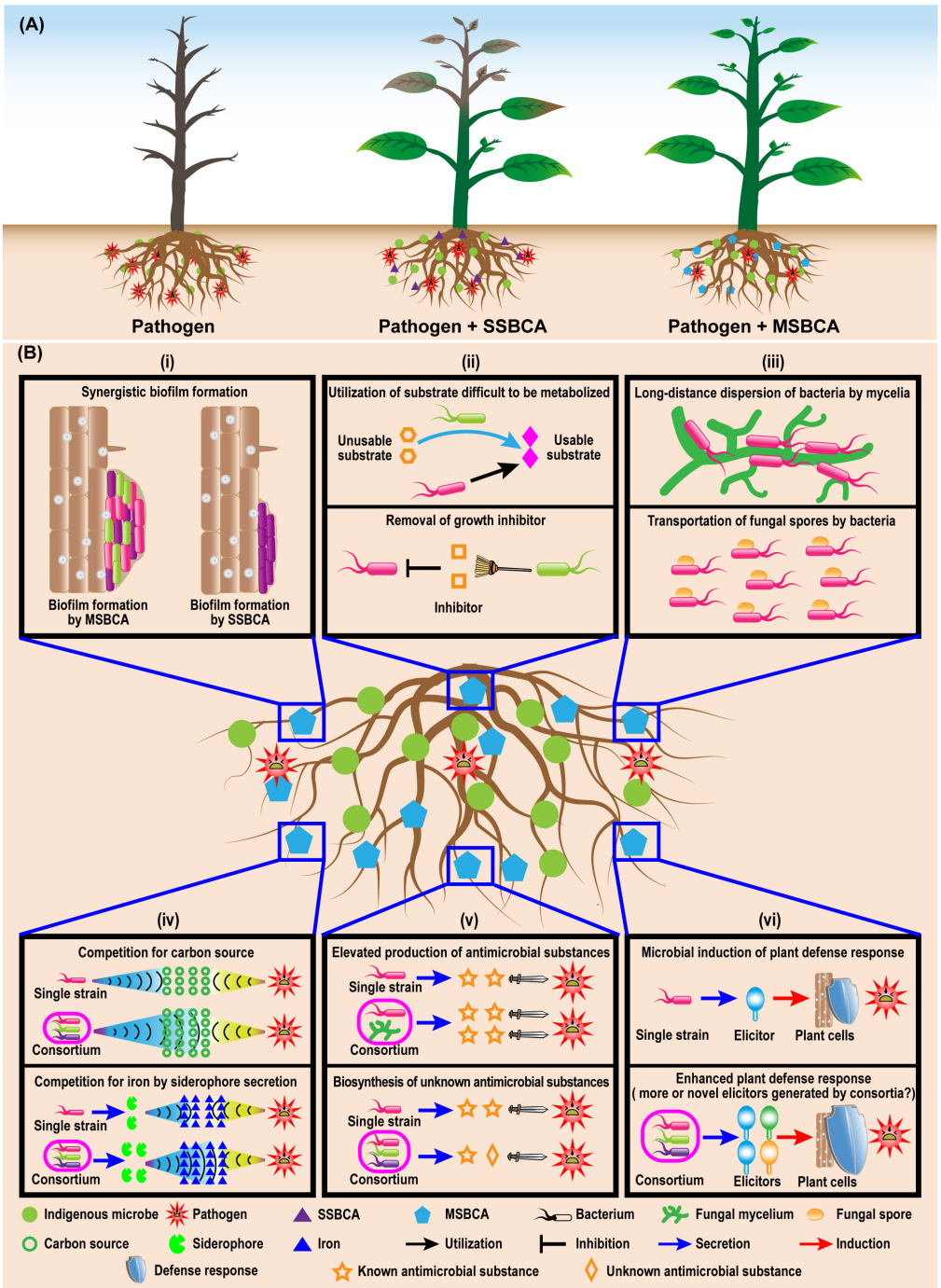

FIGURE 1 | Enhanced biocontrol effects of multiple-strain biological control agents (MSBCA) against soil-borne pathogen **(A)** and the potential mechanisms underlying the elevated disease-suppressive efficacy **(B)**. (i) enhanced biofilm formation, (ii) syntrophic microbial growth promotion, (iii) facilitated migration, (iv) boosted competition for resources, (v) stimulated antimicrobial substance biosynthesis, and (vi) elevated plant defense response induction.

with *Bacillus velezensis* Bs006, efficiency against *Fusarium* wilt of cape gooseberry was enhanced (Izquierdo-García et al., 2020). A bacterial consortium of *Chitionophaga* sp. 94 and *Flavobacterium* sp. 98 conferred more consistent protection against the infection of root rot, the infection of sugar beets by *Rhizoctonia solani* than the individual community members (Carrión et al., 2019). Thus, MSBCAs are capable of providing more effective protection of the hosts than inoculation with single-strains. A model resident bacterial community composed of five non-virulent *Ralstonia* spp. strains was more efficient

at reducing the spread of the bacterial wilt of tomato caused by *Ralstonia solanacearum* than the single non-virulent strains (Wei et al., 2015). Further examples documenting the superior action of MSBCAs against soil-borne plant pathogens are listed in **Table 1**.

The enhancement of disease inhibition by MSBCAs is widely thought to be due to the addition of different features for control (Pierson and Weller, 1994; Sarma et al., 2015). Occupation of distinct niches in the rhizosphere may avoid competition among probiotic microorganisms, resulting in more stable rhizosphere communities (Pierson and Weller, 1994; Pliego et al., 2008; Thomloudi et al., 2019). Modulation of genetic elements (Lutz et al., 2004) and suppression of a broader range of phytopathogens (Pierson and Weller, 1994; Thomloudi et al., 2019) may account for the elevated biocontrol activity in microbial communities. In addition, some key features related to the disease-controlling effect of BCAs, including rhizosphere colonization and suppression of pathogen growth, can be promoted in consortia via a complex network of microbe−microbe interactions. This interplay might serve as the selective force building plant-associated microbial communities (Hassani et al., 2018). Members of the MSBCAs apply interspecies communication as a strategy to improve their control of soil-borne diseases.

MICROBIAL INTERACTIONS PROMOTE RHIZOSPHERE COLONIZATION

Colonization in the Plant Rhizosphere

Efficient colonization of the rhizosphere is the first and fundamental step to protect plants from soil-borne pathogens by BCAs. Insufficient rhizosphere colonization can impair the beneficial effects of the biocontrol microbial strains, resulting in reduction or failure of disease control. Inoculation with MSBCAs may enhance the colonization of the rhizosphere by biocontrol microbes. The rhizoplane colonization ability of a five-strain bacterial consortium suppressing a sudden wilt disease of *Nicotiana attenuata* was enhanced compared to that of each single community member (Santhanam et al., 2019). Survival of *Pseudomonas* species communities inhibiting bacterial wilt disease of tomato increased with increasing diversity (Hu et al., 2016). In addition, the total bacterial abundance on bean root tips rose when a two-membered biocontrol *Pseudomonas* species consortium for anthracnose was added (Bardas et al., 2009). Thus, using disease-controlling microorganisms as multi-strain consortia can indeed promote rhizosphere colonization by BCAs (**Figure 1B**). Such positive effects on microbial colonization may be due to positive regulation of some colonization-related biological processes, such as biofilm formation, growth and migration, by the interactions among microorganisms within consortia.

A promising example of successful use of empirical mixtures of BCA is the combination of the fungus *Trichoderma* spp. and the biocontrol bacterium *Bacillus velezensis.* According to *in vitro* observations the microbes appear to be incompatible. *B. velezensis* FZB42 produces an arsenal of antifungal compounds. The lipopeptides bacillomycin D and fengycin act antagonistically against filamentous fungi (Chowdhury et al., 2015) and it is to be expected that the bacilli might inhibit growth of *Trichoderma* when applied together. However, supernatants of *B. velezensis* stimulated growth of *Trichoderma virens* under *in vitro* conditions. *Vice versa*, addition of *Trichoderma* conidia did not affect viability of *B. velezensis* suggesting high compatibility of both microbes. Adhesion of *B. velezensis* spores to the conidia of *T. virens* without affecting their morphology was observed, supporting compatibility of both soil inhabitants (Izquierdo-García et al., 2020).

Germination of fungal conidiospores and *Bacillus* endospores, is a critical step in successful colonization of BCAs. The contact of *Trichoderma* conidia with *Bacillus* biofilms did not impair the ability of fungal spores to germinate and establish the fungus in soil (Izquierdo-García et al., 2020). The mycelia of *Trichoderma* can serve as a supporting layer for formation of bacterial biofilms and can aid bacterial migration in the soil. Growth of bacteria was supported by nutrients present in the fungal exudates (Warmink et al., 2011; Triveni et al., 2012).

Enhanced Biofilm Formation

Microbial colonization of plant roots can be promoted by the formation of biofilms (Fan et al., 2011; Beauregard et al., 2013). Biofilms are communities of surface-associated microorganisms encased in a self-produced extracellular matrix composed of exopolysaccharides, proteins and sometimes DNA (Vlamakis et al., 2013). Beside the well-studied single-species biofilms, rhizosphere microorganisms belonging to multiple taxa are able to form multi-species biofilms, of which the formation might be elevated by the microbe−microbe interactions within biofilm communities (**Figure 1B**). In a previous study, a consortium of five native bacterial isolates was found to be able to colonize the roots of *N. attenuata* by forming multiple-taxa biofilms on the root surfaces. Furthermore, under both *in vitro* and *in vivo* conditions, the amount of biofilm produced by each individual strain was significantly less than the biofilms formed by the five-membered bacterial community, which indicating a synergistic biofilm formation by the consortium (Santhanam et al., 2019). Similarly, a three-species biocontrol community composed of *Xanthomonas* sp. WCS2014-23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259 showed synergy, as the combination of three formed more biofilm than the single strains. Moreover, colonization of host roots by this community was stimulated by enhanced biofilm formation (Berendsen et al., 2018). Although the mechanisms of such positive effects on biofilm production are unclear, the improved efficacy can be attributed to the cooperative microbial interactions in consortia, triggering increased extracellular matrix deposition and cell-to-cell signaling (Santhanam et al., 2019).

Syntrophic Microbial Growth Promotion

Colonization of the rhizosphere requires robust microbial growth, which can be greatly improved by syntrophy, a nutritional situation in which multiple microorganisms combine their metabolic abilities to catabolize a substrate that cannot be

degraded by either one of them alone (Morris et al., 2013; Mee et al., 2014). For example, *Azospirillum brasilense*, a well-known plant growth-promoting rhizobacterium (PGPR), is not able to use certain sugars and polysaccharides as carbon sources for growth *in vitro*. However, it is capable of associating with sugar- or polysaccharide-degrading bacteria, establishing a metabolic association where the sugar- and/or polysaccharide-degrading bacteria degrade the metabolites to products, which can be utilized as carbon source by *A. brasilense*. In turn *A. brasilense* provides the sugar- or polysaccharide-degrading bacteria with nitrogen by fixing the atmospheric N_2 (Bashan and Holguin, 1997; Bashan, 1998). Such a synergistic catabolic effect on sugars and polysaccharides may boost the survival of *A. brasilense* in the rhizosphere, because plant roots release 5−21% of their photosynthetically fixed carbon as root exudates (Bais et al., 2006; Huang et al., 2014), including sugars and polysaccharides, which are used by the rhizosphere microbial communities. Beside nutrients, there are always microbial growth-inhibiting materials in the rhizosphere. While methanol can suppress the growth of methanotrophs in the rhizosphere, the methanotrophs are able to survive by coexisting with *Hyphomicrobium* spp. to build a rhizospheric microbial association, in which the *Hyphomicrobium* spp. is capable of removing methanol (Liechty et al., 2020). Therefore, the promotion of the growth of rhizosphere microbes can be achieved by syntrophic interactions leading to effective nutrient utilization and removal of harmful substances (**Figure 1B**).

Facilitated Migration

Another crucial microbial trait for rhizosphere colonization is motility, defined as the ability of microorganisms to move or to perform mechanical work at the expense of metabolic energy (Harshey, 2003). There are six different categories of surface motility including swimming, swarming, gliding, twitching, sliding and darting (Harshey, 2003). According to Allard-Massicotte et al. (2016), motility is required for early root colonization by BCAs. The migration of microorganisms can be enhanced by the interactions among community members. For example, fungal hyphae are capable of serving as vectors for the dispersion of bacteria in the rhizosphere, which is known as a "fungal highway" (Kohlmeier et al., 2005; Warmink et al., 2011; **Figure 1B**). In a recent study, Zhang et al. (2020) showed that rhizobia use mycelia of *Phomopsis liquidambaris* as dispersal networks to migrate into legume rhizospheres and to trigger nodulation. Extraradical mycelium formed by the mycorrhiza fungus *Glomus formosanum* CNPAB020 can facilitate the translocation of *Bradyrhizobium diazoefficiens* USDA 110 in the rhizosphere (de Novais et al., 2020) in addition to its main activity in nutrient transfer. Prokaryotic cells are able to facilitate dispersal of non-motile asexual fungal spores as well (**Figure 1B**). Conidia of *Aspergillus fumigatus*, a non-motile rhizosphere fungus, can be transported by the rhizobacterium *Paenibacillus vortex* from niches of adverse growth conditions. Fungal mycelia may act as bridges to allow *P. vortex* to cross air gaps, which can be mutually facilitated dispersal, benefiting the life cycles of both of these very different rhizosphere inhabitants (Ingham et al., 2011). The enhanced dispersal may also occur between distinct bacterial species. An ampicillin-sensitive *P. vortex* strain was capable of swarming and colonizing on ampicillin plates using non-motile ampicillin-resistant *Escherichia coli* as cargo, dispersing both bacteria (Finkelshtein et al., 2015; Venieraki et al., 2016). Co-swarming or transporting other bacterial species may expand the abilities of the partners in occupying and exploiting ecological niches in diverse environments including the rhizosphere (Venieraki et al., 2016). Hence, interactions among the microbial components of a given community may bring about facilitated microbial migration, essential for efficient rhizosphere colonization.

In brief, microbe−microbe interactions can play a positive role in promoting rhizosphere colonization by beneficial microorganisms through boosting biofilm formation, microbial growth, migration inside of the microbiome, and interacting with plant roots. Thus, utilization of MSBCAs performing active interactions among their members may improve survival of disease-suppressing microbes, and their adaption to complex and changeable environmental conditions. In consequence, they may be able to stabilize their beneficial effects for the inhibition of soil-borne diseases.

MICROBIAL INTERACTIONS ENHANCE GROWTH SUPPRESSION OF SOIL-BORNE PATHOGENS

Multi-strain biological control agents are able to exhibit stronger suppressive efficacy on the growth of soil-borne pathogens than SSBCAs. For instance, a bacterial strain mixture involving *Bacillus subtilis* S2BC-1 and GIBC-Jamog showed greater anti-fungal activity against the tomato vascular wilt pathogen, *Fusarium oxysporum* f.sp. *lycopersici*, than each of the individual strains (Shanmugam and Kanoujia, 2011). Similarly, *Pseudomonas fluorescens* T5 showed no inhibition against *Rhizoctonia solani in vitro*. However, when it was applied together with four non-antagonistic bacterial strains isolated from the rhizosphere of *Tamarindus*, this five-species bacterial community exhibited strong suppression of growth of *R. solani* (Kannan and Sureendar, 2009). Although the understanding of the enhanced pathogen-inhibiting effect of biocontrol consortia is limited, changes in resource competition and secretion of antimicrobial compounds triggered by microbial interactions may contribute to the enhanced suppression (**Figure 1B**).

Boosted Competition for Resources

Resource competition is a basic mechanism by which BCAs may protect plants from pathogens, implying that the beneficial microorganisms are able to rapidly and efficiently utilize the limited resources in the vicinity of the plant hosts to restrict or suppress the growth of phytopathogens. Plant exudates on root surfaces and in their surrounding rhizosphere, are the primary sources of nutrients for the rhizosphere microbiome. Successful suppression depends on the competition for nutrients in root exudates by biocontrol microbes and soil-borne pathogens. This contest can be elevated by the microbial interplay inside MSBCAs (**Figure 1B**). Two biocontrol consortia for tomato

bacterial wilt caused by *R. solanacearum*, consisting of eight *Pseudomonas* and five non-virulent *Ralstonia* strains, exhibited much stronger inhibiting effects on the population density of *R. solanacearum* than each individual strain. The enhanced inhibition is caused by an increase of niche overlaps exerted by these consortia with *R. solanacearum.* Niche overlaps may be defined as 'likeness' between the communities and *R. solanacearum* in the catabolism of 48 different single-carbon resources found in tomato root exudates (Wei et al., 2015; Hu et al., 2016). The more diverse soil bacterial communities are, the better they are able to acquire many of the 31 individual carbon sources typical for soil, than the pathogen *E. coli* O157:H7 (van Elsas et al., 2012). Limited assimilatable iron resources remain in the rhizosphere, following the competition between disease-suppressing microorganisms and soil-borne pathogens (Gu et al., 2020). Many soil microbes scavenge iron by secreting siderophores, a chemically diverse group of secondary metabolites with a high affinity for iron, because iron predominantly occurs in soil in its insoluble ferric Fe (III) form (Traxler et al., 2013; Traxler and Kolter, 2015). The siderophores can both, to facilitate and suppress competitors, depending on whether the competitors possess the transporters or channels for siderophore uptake. The production of siderophores can be positively regulated by interspecies interactions among soil microbes. The interplay of *Streptomyces coelicolor* with five other soil actinobacteria increased the diversity of siderophores. Production of desferrioxamines by *S. coelicolor,* was triggered by siderophores from neighboring strains (Traxler et al., 2013). Therefore, the disease-inhibiting microorganisms in the rhizosphere may acquire elevated capability to utilize resources through microbial associations (**Figure 1B**).

Stimulated Synthesis of Antimicrobial Compounds

Microorganisms are able to synthesize a multitude of compounds with antimicrobial activity, which is an important mode of action for direct inhibition or lethality on the microbial opponents in environments. So far, there have been a large number of reports of the antimicrobials produced by BCAs exhibiting suppressing effects on the growth of phytopathogens. These studies mainly focus on the biocontrol strains from the genera *Bacillus*, *Pseudomonas*, and *Trichoderma*, well known for the production of antibiotics including lipopeptides, polyketides, bacteriocins, phenazines, 2,4-diacetylphloroglucinol (DAPG) and chitinase (Ghisalberti and Sivasithamparam, 1991; Haas and Keel, 2003; Chen et al., 2007). Some metabolites with inhibitory functions are found in low concentration or are not expressed in pure culture but may be upregulated in a community (Nützmann et al., 2011; Brakhage, 2013; Pishchany et al., 2018). Lutz et al. (2004) examined the molecular interactions between bacterial and fungal BCAs, the DAPG-producing *P. fluorescens* and chitinase-producing *Trichoderma atroviride* P1. DAPG enhanced the expression of the *nag1* chitinase gene, indicating that the positive regulation of key biocontrol genes may take place while mixing antagonists. Co-culturing the endophytic fungus *Fusarium tricinctum* with *Bacillus subtilis*, resulted in as much as a 78-fold increase in the accumulation of secondary metabolites including compounds with antimicrobial efficacy (Ola et al., 2013). Therefore, specific interactions among microorganisms belonging to different domains may enhance production of antimicrobial compounds. Not only are the microbial interactions able to upregulate the production of known antimicrobial compounds, but interactions may also activate the biosynthesis of hitherto unknown compounds with antimicrobial activity (**Figure 1B**). A novel antibiotic named amycomicin has been recently described (Pishchany et al., 2018). The production of this compound is dependent on the interaction between two soil-dwelling actinobacteria, *Amycolatopsis* sp. AA4 is the producer strain and *Streptomyces coelicolor* M145 is an inducer. According to these examples the synthesis of antimicrobial compounds can be stimulated or activated through both, inter- and intra-domain microbial interactions.

Therefore, the modulating effect of microbial interactions on resource competition and production of antimicrobial compounds may contribute to strengthening the inhibition of growth of pathogens (**Figure 1B**). Thus, applying BCAs as multi-strain mixtures can elevate the ability of biocontrol microorganisms to compete for the resources needed for rhizosphere survival with soil-borne pathogens and to stimulate the production of compounds toxic to specific pathogens. The increased niche overlaps and biosynthesis of novel antimicrobial compounds induced by microbe−microbe interactions may facilitate the BCAs to suppress a broader range of phytopathogens. The positive impact of interactions within MSBCAs may result in more efficient growth suppression of soil-borne pathogens, and improve the efficiency of soil-borne disease control by disease-inhibiting microbes.

INTERACTIONS OF MICROBIAL COMMUNITIES WITH PLANTS AND SOIL

Plants rely on rhizosphere microbiota to facilitate nutrient acquisition, in exchange for carbon-rich root exudates for bacterial nutrition. In addition, the rhizosphere microbiome is important for plant health and fitness (van der Heijden et al., 2008). The plant root microbiome consists of prokaryotic bacteria, eukaryotic filamentous fungi, and oomycetes. Besides a core microbiome ubiquitous in a multitude of hosts and geographical regions, a variable part of the microbiome is shaped by secretion of species-dependent plant secondary metabolites, which belong to diverse classes, such as coumarins, benzoxazinoids, phytoalexins and triterpenes (Jacoby et al., 2020). Consequently, diversity of species along the bulk-soil to root microbiota was found gradually decreasing. Positive correlations dominate within each of the three kingdoms. Reconstitution experiments performed with synthetic mono- or multi-kingdom microbial consortia and germ-free *Arabidopsis* plantlets revealed that the bacterial microbiota protects plants against potentially pathogenic fungi and oomycetes by mainly negative factors exerted against filamentous fungi (Durán et al., 2018). Widely distributed members of the core microbiota such as *Variovorax,* a gram-negative beta-proteobacterium, and

Pseudomonas appeared to be important for plant protection but individual members of other bacterial taxa could overtake their function in biocontrol. Therefore, addition of an SSBCA or MSBCA might have positive effects in complex systems of agriculture and forestry.

The plant immune system also affects the composition of the microbiota in the vicinity of plant roots. The root-microbiome may expand plant immunity and acts as an additional layer of defense against plant pathogens (Teixeira et al., 2019). Interaction of beneficial microbes with plant roots can result in systemic host resistance to pathogens, which may be due to the activation of induced systemic resistance (ISR) (Sarma et al., 2015). In addition to promoting rhizosphere colonization and suppressing soil-borne pathogen growth, inducing enhanced plant defense responses to pathogens has been described in many studies as another important feature employed by the MSBCAs for their elevated disease-controlling effect. The additive or synergistic efficacy of the biocontrol consortia on the induction of elevated host immunities to plant pathogens is directed by activating several distinctive metabolic and signaling pathways against a given pathogen (Jain et al., 2012; Alizadeh et al., 2013; Sarma et al., 2015). However, how interactions among the members of MSBCAs can effectively boost specific systemic resistance to soil-borne pathogens remains to be better illustrated. One possible hypothesis is that the microbe-microbe interplay within the biocontrol consortia might lead to the production of larger amounts of specific elicitors and potent compounds capable of more efficiently eliciting ISR (**Figure 1B**).

Many root-associated gram-positive and gram-negative bacteria are able to produce plant growth hormones, such as indole-3-acetic acid (IAA) and thus promoting plant root growth, when auxin production does not exceed a critical level (Vessey, 2003; Lugtenberg and Kamilova, 2009). In case of some pathogenic bacteria, IAA production exceeds the critical threshold needed for plant growth and may negatively affect plant health (Spaepen et al., 2007; Subramoni et al., 2014; Segev et al., 2016). Some beneficial root-associated microbes such as *Variovorax* possess the IAA catabolic gene cluster and can reverse root growth inhibition occurring at high IAA concentrations by degrading IAA (Fitzpatrick et al., 2020).

Soil not only supports plant and animal life, but also hosts myriad microorganisms inside, referred to collectively as the soil microbiome (Berg and Smalla, 2009; Fierer, 2017; Jansson and Hofmockel, 2018; Thakur and Geisen, 2019), which governs biogeochemical cycling of macronutrients, micronutrients and other elements vital for the growth of plants and animals (Jansson and Hofmockel, 2020). The interactions between microbes and soil have always drawn the attention of microbiologists and ecologists. It has been widely accepted that microbial communities inhabiting soil are capable of alternating its physicochemical properties by organic litter deposition and metabolic activities (Jacoby et al., 2017; Jansson and Hofmockel, 2020), for example, by improving water retention (Naylor and Coleman-Derr, 2017), increasing carbon storage (Jansson et al., 2018) and mineral nutrition contents (van der Heijden et al., 2008; Jacoby et al., 2017). Vice versa, the variability in soil traits may impact the composition and function of soil microbial communities (Peiffer et al., 2013; Yang et al., 2019; Chen et al., 2020; Wang et al., 2020). Our increasing awareness of the influences of soil-feature changes on the microbiome has resulted in an emerging urgency to elevate the suppressing effect of soil microbiota against phytopathogens by managing the soil properties. Wang et al. (2020) demonstrated that the addition of biochar to the soil not only raises the pH and the available nutrient content, but also augments fungal richness and diversity, especially the abundance of potential biocontrol fungi, which led to the inhibition of *Phytophthora* blight of pepper. Similarly, biochar amendment controlled bacterial wilt through changing soil chemistry and the composition of the microbial community. The application of biochar specifically enriched beneficial bacteria and decreased pathogen abundance (Chen et al., 2020). Furthermore, Yang et al. (2019) showed that wheat straw return significantly increased soil nitrogen and reduced the relative abundance of pathogenic fungal genera in the soil microbial community, indicating a potential for disease control. Thus, promoting the biocontrol effects of the soil microbial community against soil-dwelling pathogens by manipulating soil features is a promising strategy for soil-borne disease management. Moreover, understanding the interplay between the soil and its associated microbiota will expand our knowledge about the impact of abiotic factors on biological soil-borne pathogen control.

MICROBIAL INTERACTIONS AND CONSTRUCTION OF MSBCAS

Application of BCAs in the community context as MSBCAs can increase the ability to control soil-borne diseases of crops through interaction-mediated promotion of rhizosphere colonization outcompeting soil-borne pathogens. Thus, construction and utilization of MSBCAs could augment soil-borne disease control in sustainable agriculture and forestry. So far, there are at least two strategies for preparing effective MSBCAs, (i) mixing the existing SSBCAs according to empirical criteria, and (ii) assembling MSBCAs by applying the reductionist SynCom approach, also named RSC (Liu et al., 2019). Using either one of the two strategies, microbe-microbe interactions need to be taken into account.

Mixing the Compatible and Diverse SSBCAs According to Empirical Criteria

Combining beneficial microbial isolates that may enhance the effect achieved by single isolates dates back to the discovery of PGPR (Kloepper et al., 1980). Selecting proper strains is critical. We noted that microorganisms used for developing biocontrol consortia were often selected according to their individual disease suppressive capacity. However, except for this property, no precise selection standards have been adopted to choose microbial components (Sarma et al., 2015; Thomloudi et al., 2019). This approach often results in equal or even lower efficacy of the multi-strain mixtures compared to the individual strains (Sarma et al., 2015). Thus, it is necessary to carefully evaluate the compatibility and interactions of the candidate strains before the

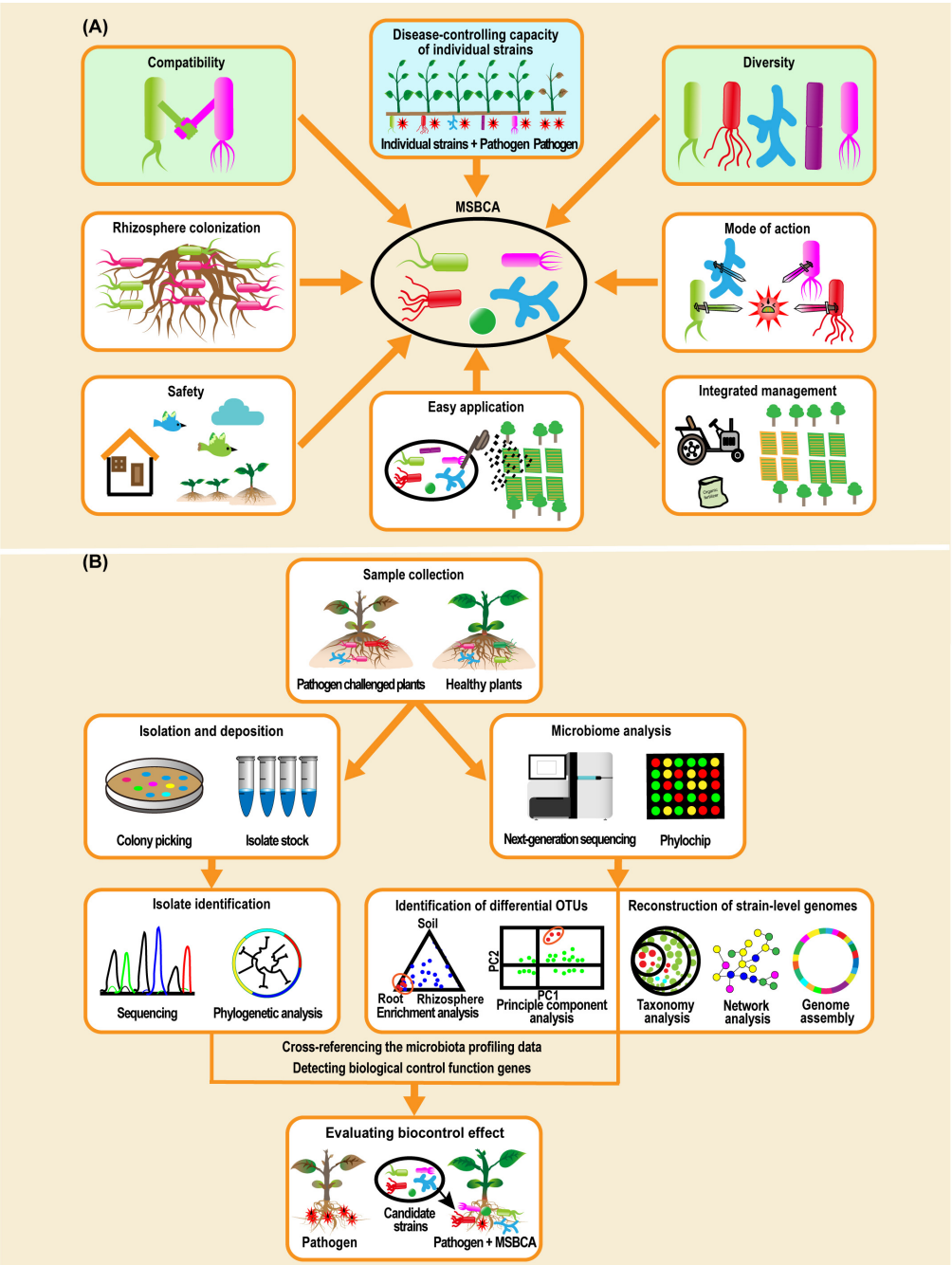

FIGURE 2 | Biological features need to be considered when constructing multiple-strain biological control agents (MSBCAs) and workflow of assembling MSBCAs using a reductionist synthetic community (Vorholt et al., 2017; Liu et al., 2019). **(A)** Biological features involving disease-suppressive effects of each individual strain, compatibility, diversity, microbial colonization of rhizosphere, mode of action for disease control, safety to humans and the environment, easy application and convenience to be incorporated into an existing management system (Raupach and Kloepper, 1998; Sikora et al., 2010; Bashan et al., 2013; Grosskopf and Soyer, 2014; Ahkami et al., 2017), need to be taken into account when establishing the MSBCAs for soil-borne diseases. **(B)** In general, methodology of constructing MSBCAs by a reductionist synthetic community approach is built on the conception of host-mediated selection of plant-associated microbiota (Mueller and Sachs, 2015). Microbiome analysis by 16S rRNA gene amplicon sequencing or metagenomics sequencing, or by PhyloChip analysis, in parallel to the extended microbial strain isolation to achieve as much diversity as possible, is an early step to pick the potential disease-suppressing species by identifying the differential OTUs between the microbiome of the samples collected from pathogen challenged and control plants (Berendsen et al., 2018), or by reconstructing strain-level genomes based on functional diversity (Carrión et al., 2019). Then, after cross-referencing the microbiota profiling data with the taxonomic identities of the isolates in comprehensive culture collections (Niu et al., 2017; Berendsen et al., 2018), or by detecting the genes encoding the functions of biological control in the genomes of cultivated isolates (Carrión et al., 2019), the candidate strains will be characterized and selected for the multi-strain community, of which the disease-reducing effects will be further evaluated.

MSBCA consortium is established. We propose, in addition to the disease-inhibiting activity of individual strains, to consider two interaction-related properties, (i) compatibility, and (ii) diversity.

The members of a probiotic consortium are considered to be compatible when they do not inhibit growth of each other during their *in vitro* co-culture and/or in rhizosphere colonization competition assays (Liu et al., 2018; Thomloudi et al., 2019). Co-inoculation with incompatible isolates might hinder one or more microbial agents from reaching the appropriate population threshold for plant disease control (Haas and Defago, 2005). The results of the *in vitro* co-culture compatibility tests often represent the interactions occurring among the members of the consortium. However, variation in media used to test *in vitro* compatibility (Lyons and Kolter, 2017), the colonization of different ecological niches on roots (Pliego et al., 2008), and interference among mechanisms for disease control (Stockwell et al., 2011) can lead to inconsistent compatibility assays. Thus, compatibility among members of a synthetic microbial community should be considered as a prerequisite in the engineering of MSBCAs applied to plants, and should be verified by further assays.

In addition, the degree of microbial diversity affects the assembly, survival, and functionality of BCAs in the rhizosphere and their ability to inhibit soil-borne diseases (Hu et al., 2016). First, a high level of species diversity can increase the resources that microbial species can collectively use as a community (the niche breadth), and enable microorganisms to survive in the rhizosphere more efficiently (Wei et al., 2015). Second, the amount and number of secondary metabolites that suppress pathogen growth increase with increasing taxonomic diversity in MSBCAs (Raaijmakers and Weller, 1998; Jousset et al., 2014). A combination of different secondary metabolites produced jointly by diverse microbes may strengthen the antagonistic effect against pathogens (Loper et al., 2012). Therefore, MSBCAs of high diversity could be more adaptive to the pressure of rhizosphere environments and act more efficiently against soil-borne plant diseases.

In summary, compatibility and diversity are two interaction relevant factors (**Figure 2A**) that may determine the success of MSBCAs. Some additional traits, such as microbial colonization of the rhizosphere, mode of action for disease control, safety to humans and the environment, ease of application and convenience of management systems need to be considered, when establishing the biocontrol microbial communities for soil-borne diseases (Raupach and Kloepper, 1998; Sikora et al., 2010; Bashan et al., 2013; Grosskopf and Soyer, 2014; Ahkami et al., 2017).

Building MSBCAs by the Reductionist SynCom Approach

Although empirically combining existing microbial isolates with biocontrol activity is useful, it is nearly impossible to predict efficiency of such consortia in suppressing plant disease and strengthening plant growth in the context of the whole plant microbiome. In contrast, utilizing a reduced number of representative members of the target host microbiota to build SynCom (Vorholt et al., 2017; Liu et al., 2019) will likely simplify handling and production of such MSBCAs. SynCom analysis performed in gnotobiotic systems allows us to study the effect of the plant microbiota on host fitness under different environmental circumstances. It also allows us to investigate microbe−microbe interactions and microbial gene functions (Carlström et al., 2019; Liu et al., 2019), and to construct novel MSBCAs.

Several microbial communities able to suppress plant diseases have been assembled via the reductionist SynCom approach based on microbiome analysis and comprehensive culture collections (Liu et al., 2019). A synthetic bacterial consortium was constructed, able to reduce the severity of the maize seedling blight caused by *Fusarium verticillioides* (Niu et al., 2017). The biocontrol effect of the synthetic community against *F. verticillioides* was stronger than that of each individual strain. To prepare this synthetic community, Niu et al. (2017) started from microbiota established by maize roots, which were identified by 16S rRNA gene amplicon sequencing and additional strain cultivating methods. A greatly simplified SynCom was obtained, consisting of seven strains, *Enterobacter ludwigii*, *Stenotrophomonas maltophilia*, *Ochrobactrum pituitosum*, *Herbaspirillum frisingense*, *Pseudomonas putida*, *Curtobacterium pusillum*, and *Chryseobacterium indologenes*, representing three of the four most dominant phyla found in maize roots.

A three-membered bacterial community able to induce systemic resistance in *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis* (downy mildew) was constructed (Berendsen et al., 2018) via host-mediated microbiome selection (Mueller and Sachs, 2015). Carrión et al. (2019) showed that infection of sugar beets by a fungal pathogen, *Rhizoctonia solani*, is hindered by an endosymbiotic community of bacteria living inside plant roots. This endophytic community was enriched for Chitinophagaceae and Flavobacteriaceae harboring chitinase genes and biosynthetic gene clusters encoding non-ribosomal peptide synthetases and polyketide synthases. A MSBCA consortium of *Chitinophaga* and *Flavobacterium* strains was established, which consistently suppressed fungal root disease. Carrión et al. (2019) concluded that endophytic root microbiomes may harbor many functional traits that can protect synergistically their host plants (Carrión et al., 2019).

CONCLUSION AND FUTURE PERSPECTIVES

Building MSBCAs by a reductionist SynCom approach (**Figure 2B**) offers the chance to accurately and rapidly pick out the microbial strains qualified for establishing the MSBCA from thousands of isolates found in the natural host microbiome. In this way, the crucial disease control-interactions present in the plant microbiome (Hassani et al., 2018) can be mirrored in the few selected strains used for the MSBCA. Establishing SynComs should be the method of choice. SynComs represent a helpful complement to pesticides, and might be combined in future application with effective empirical mixtures and/or single representatives of existing SSBCAs.

Utilization of selected beneficial microorganisms in the community is an effective approach to improve the efficiency of BCA (**Figure 1A**; Sarma et al., 2015; Mazzola and Freilich, 2017; Vorholt et al., 2017; Woo and Pepe, 2018). A necessary precondition for its success is the analysis of the microbial interactions among the members and the effect exerted by the MSBCA on plant health (**Figure 1**). When designing a MSBCA, two crucial interaction-related factors, compatibility and diversity, need to be considered (**Figure 2A**). Constructing MSBCA by combining microbes with great taxonomic distance appears desirable. We recommend a reductionist SynCom approach based on the principle of host-mediated microbiome selection (Mueller and Sachs, 2015), and selection of representative microbes to form efficient biocontrol consortia. This allows us to assemble customized MSBCAs depending on the specific requirements of disease management in different crops and environments. This strategy will result in protecting against distinct pathogens and might be comparable to the concept of "precision medicine" for human health (Berg et al., 2020), that advocates treatments of patients on a personalized level (Collins and Varmus, 2015) based on the patient's genome sequence and their specific genome-environment interaction. Beside the practical use of MSBCAs as biopesticides, they may also serve as useful tools for investigating how microbial interspecies interactions affect plant microbiome assembly (Niu et al., 2017), and how evolutionary processes act on the plant holobiont (integrating the plant, the microbiome and the environment) (Hassani et al., 2018).

In this review, we summarize the potential mechanisms deployed by microbial components of communities to improve their disease-suppressing functions. Our understanding of these processes at the level of molecular mechanisms is rudimentary, especially the mechanisms of the initiation of rhizosphere colonization and the resulting elevated host immunity. Next, the technology of functional genomics, transcriptomics, proteomics and metabolomics will need to be applied to elucidate the genetic basis of enhanced biofilm formation, syntrophic microbial growth promotion and migration, and enhanced ISR. Although a 16S rRNA gene amplicon sequencing-based reductionist SynCom approach is useful to characterize MSBCAs, the relatively short reads may not achieve the taxonomic resolution needed to distinguish related strains (Edgar, 2018; Fuks et al., 2018). Thus, beside the high cost of a metagenomics approach, the utilization of modified 16S rRNA gene amplicon sequencing-based methods with improved resolution, such as full-length 16S rRNA gene amplicon sequencing (Callahan et al., 2019), may be expanded in the future when constructing the SynComs with biocontrol activity. In addition to the disease-suppressing function of the SynComs, their plant growth-promoting effects are worth further investigation (Zhang J. et al., 2019; Zhuang et al., 2020). So far, most MSBCAs have been applied in agriculture (**Table 1**), Using BCAs in forestry for plant disease control should be recommended. Finally, as agrochemical companies such as BASF, Syngenta and Bayer have developed and launched several MSBCA-based commercialized products for sustainable management of soil-borne pathogens, application of MSBCAs should bring more efficient control of soil-borne diseases in agriculture, horticulture, and forestry.

AUTHOR CONTRIBUTIONS

BN and RB conceived the idea, designed the outlines of the review, and wrote the manuscript. WW and ZY prepared the figures and the table. VC, RS, and HS revised the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Determinants of Host Range Specificity in Legume-Rhizobia Symbiosis

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Leguminous plants possess the almost unique ability to enter symbiosis with soil-resident, nitrogen fixing bacteria called rhizobia. During this symbiosis, the bacteria physically colonize specialized organs on the roots of the host plant called nodules, where they reduce atmospheric nitrogen into forms that can be assimilated by the host plant and receive photosynthates in return. In order for nodule development to occur, there is extensive chemical cross-talk between both parties during the formative stages of the symbiosis. The vast majority of the legume family are capable of forming root nodules and typically rhizobia are only able to fix nitrogen within the context of this symbiotic association. However, many legume species only enter productive symbiosis with a few, or even single rhizobial species or strains, and vice-versa. Permitting symbiosis with only rhizobial strains that will be able to fix nitrogen with high efficiency is a crucial strategy for the host plant to prevent cheating by rhizobia. This selectivity is enforced at all stages of the symbiosis, with partner choice beginning during the initial communication between the plant and rhizobia. However, it can also be influenced even once nitrogen-fixing nodules have developed on the root. This review sets out current knowledge about the molecular mechanisms employed by both parties to influence host range during legume-rhizobia symbiosis.

Keywords: specificity, rhizobia, legume, host range, symbiosis, nodulation

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INTRODUCTION

The legume family is almost unique amongst plants in that its members are able to interact with soil dwelling bacteria called rhizobia. This leads to nitrogen-fixing symbiosis, during which specialized structures called root nodules form on the plant root. These nodules are colonized by the rhizobia, then reduce atmospheric nitrogen to ammonia, in a process known as biological nitrogen fixation (BNF). This fixed nitrogen is utilized by the host plant for growth whilst photosynthates, in the form of dicarboxylic acids, are provided to the rhizobia as a carbon source in exchange (White et al., 2007).

Nitrogen (N)-fixing symbiosis begins with molecular cross-talk between the plant root and rhizobia. During times of nitrogen paucity, polyphenolic compounds called flavonoids are exuded by the root into the rhizosphere. These compounds are able to diffuse across the membranes of rhizobia in their vicinity (Fisher and Long, 1992). Upon flavonoid perception, rhizobia respond by activating transcription of symbiosis-related (Nod) genes and are chemoattracted into closer proximity to the plant root. Nod gene expression is orchestrated by the activity of nodulation

protein D (NodD) binding to the *nod* box found upstream of these genes. The key effect of expression of Nod genes is the production and secretion of lipochitooligosaccharide (LCO) compounds called Nod factors (Schlaman et al., 1992). Some rhizobia possess multiple copies of *nodD* (Perret et al., 2000) and/or a copy of a repressor of *nod* gene expression called *nolR* (Kiss et al., 1998).

Nod factors are then responsible for driving the host plant toward symbiosis. They are recognized by membrane-localized proteins called Nod factor receptors, leading to root hair deformation and activation of nodulation-related genes. Nod factor perception is mediated by co-receptors NFR1/NFR5 in *Lotus japonicus* (Radutoiu et al., 2003) and LYK3/NFP in *Medicago truncatula* (Amor et al., 2003; Smit et al., 2007). The exact processes that occur as a consequence of this can differ substantially between different legume-rhizobia partnerships; for a detailed overview of this process, see (Gage, 2004; Sprent, 2009). Most commonly, localized inhibition of growth at the tips of root hair cells induces curling of root hairs, creating a pocket in which rhizobia may become trapped (Esseling et al., 2003). Localized cell wall degradation (Xie et al., 2012), cytoskeletal re-arrangement and vesicle trafficking give rise to a tubular cell wall and membrane-lined invagination called an infection thread (IT), which the rhizobia gain access to Murray (2011). As the IT extends through the epidermis and ultimately into the underlying cortex, bacterial cells close to the growing tip of the IT grow and divide, in effect enabling the bacteria to traverse the IT. Concomitant with IT formation, there is de-differentiation and division of underlying cortical cells, resulting in the formation of a nodule meristem (Patriarca et al., 2004). Rhizobia then internally colonize the plant root, first gaining access to the intracellular space, and then infecting cells of the nodule primordia.

Bacteria in the developing nodule primordia are enclosed within a host-derived membrane, obtained as they exit the infection thread, giving rise to an organelle called the symbiosome (Brewin, 2004). Bacteria in the symbiosome differentiate into specialized nitrogen fixing bacteroids, losing their ability to replicate in the process (Oke and Long, 1999). There is a distinction between legumes in which the meristem is transient (determinate nodulators) or maintained (indeterminate nodulators). The nodules of indeterminately-nodulating species are able to persist indefinitely whilst determinate nodules eventually senesce (Gibson et al., 2008). Many legumes forming indeterminate nodules belong to a clade termed the inverted repeat-lacking clade (IRLC) on the basis of the absence of one of two 25 kb inverted repeats in their chloroplast genome. In the case of nodules of IRLC legumes, bacteroids are almost always unable to resume vegetative growth should they be released from the nodule (terminal bacteroid differentiation, TBD). TBD is associated with more extreme cell enlargement and genome endoreduplication and is thought to lead to enhanced efficiency of N-fixation [reviewed in Alunni and Gourion, 2016]. Inside the nodule, bacterial nitrogenase breaks down atmospheric dinitrogen into ammonia which is provided to the host.

Incompatibility during legume-rhizobia symbiosis may manifest in different ways, depending on the stage at which it occurs. Whilst some legume-rhizobia pairings may not symbiotically interact at all [for instance, *M. truncatula* and *Mesorhizobium loti* (Radutoiu et al., 2007)], other ultimately incompatible pairings can progress through the very early stages of the symbiosis, such as inducing root hair curling, only to fail to colonize the root or form nodules (known as a *nod*$^-$ outcome) e.g., *M. truncatula* F83005.5 and *Sinorhizobium meliloti* Rm41 (Liu et al., 2014). It is also possible that an interaction can result in normal nodule morphogenesis only for the resultant nodules to be either uninfected or deficient in nitrogen fixation (known as a *fix*$^-$ outcome$^-$) e.g., *M. truncatula* A17 and *S. meliloti* Rm41 (Liu et al., 2014). Even interactions resulting in nitrogen fixation may not represent maximum compatibility. For instance, the widely used model organism *S. meliloti* 1021 is a natural symbiont of *Medicago sativa* (alfalfa) but is also able to form *fix*$^+$ nodules with some accessions of closely related *M. truncatula*. However, the efficiency of nitrogen fixation during the interaction with *M. truncatula* is substantially lower than natural *M. truncatula* symbionts such as *S. meliloti* 1022 or *Sinorhizobium medicae* 419 (Terpolilli et al., 2008; Kazmierczak et al., 2017).

Nodule formation incurs a significant cost for the host plant in terms of photosynthates to supply N-fixing nodules, and therefore an optimal strategy is to only participate in symbiosis with bacteria that fix nitrogen efficiently in return. However, a lifestyle closer to parasitism, sometimes termed "cheating" may be more beneficial from the perspective of the bacteria if it is able to sequester carbon, whilst providing little or no nitrogen fixation in return. Cheating is especially a consideration in some legume-rhizobia interactions where bacteroids are subject to TBD and therefore where the process of fixing nitrogen occurs a huge fitness cost to the rhizobial population as a whole (Denison and Kiers, 2004). It is possible that cheating happens more frequently in legume species where bacteroid differentiation is less well-advanced, since terminal bacteroid differentiation could be considered to enable a greater degree of host control. Studying a wider range of host-symbiont combinations across the legume phylogeny would certainly help to explore this interesting question.

Although N-fixing symbiosis is often considered in terms of an interaction between a legume and a single strain of rhizobia, the root is typically exposed to mixed populations of rhizobia in the rhizosphere. Therefore, it is crucial that the host is able to not only discern "friend" vs. "foe" (i.e., between a compatible symbiont and a cheater or a *bona fide* pathogen) but also between "friend" and "best friend" (i.e., between a poorly matched and an efficient symbiotic partner) in order to optimize N-fixation to satisfy the nutritional needs of the host. This review considers the mechanisms that legumes and rhizobia employ to identify each other, and how these can facilitate partner selection.

FLAVONOIDS AS THE PRIMARY DETERMINANT OF RHIZOBIAL HOST RANGE

The initial signaling events during legume-rhizobia associations provide the first opportunity for partner choice by both parties. The first step of this exchange is the exudation of flavonoids

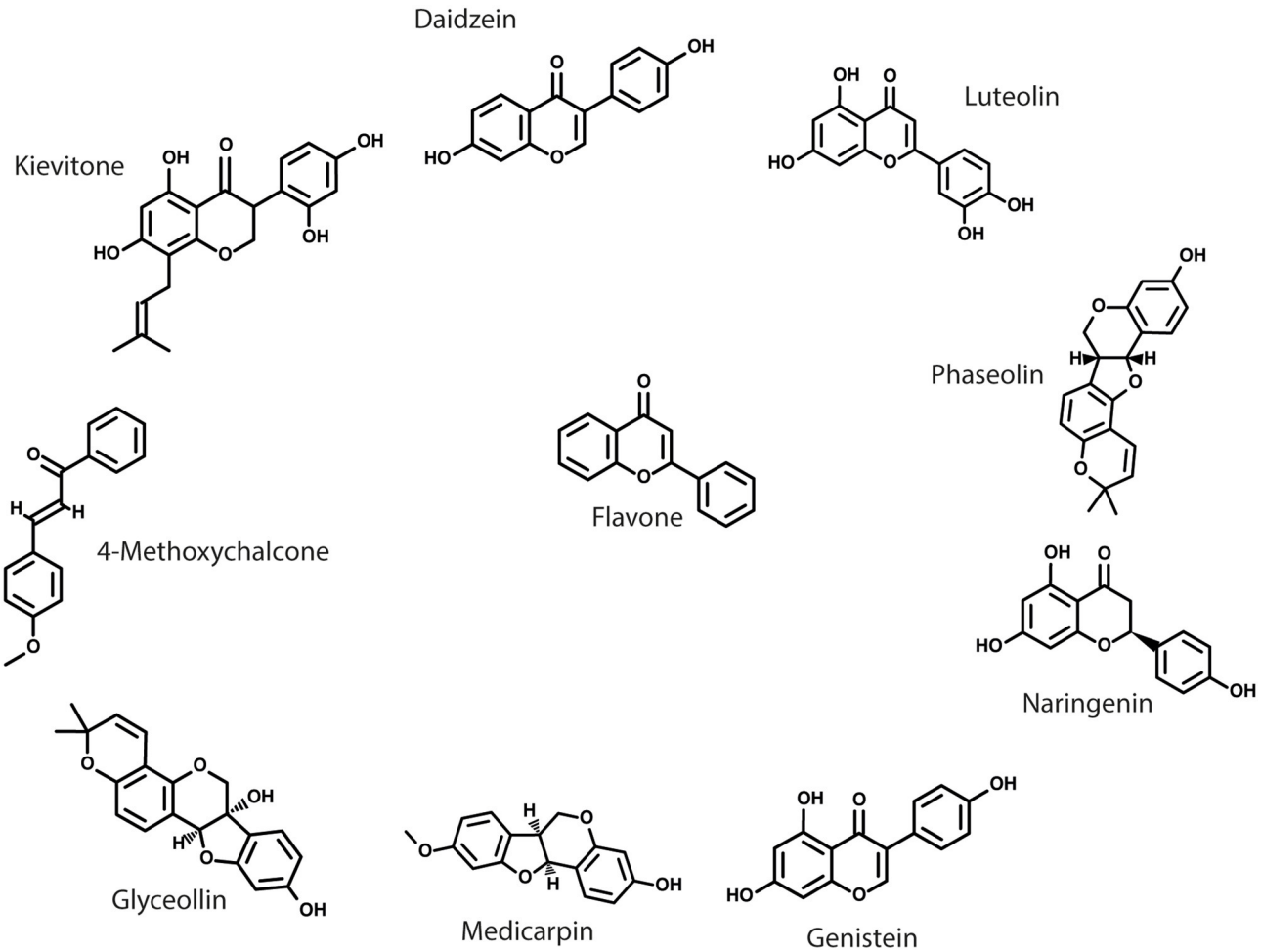

FIGURE 1 | Structures of select flavonoids associated with legume nodulation. Flavonoids are plant secondary metabolites of plants that have many diverse functions. Many distinct flavonoids are found in plants, consisting of chemical modifications to a core flavone structure.

from the root of the prospective host plant. Legumes possess an enormous diversity of flavonoids, although evidence so far suggests that only a subset of these are involved in symbiosis (**Figure 1**). Flavonoids are additionally responsible for many developmental and allelopathic processes in legume and non-legume plants alike [reviewed in Weston and Mathesius, 2013]. The presence of flavonoids may influence rhizobial host range by two mechanisms [reviewed in Liu and Murray, 2016]; either acting as an infection signal and stimulating rhizobial *nod* gene expression or acting as a phytoalexin and eliciting antimicrobial activity.

For a flavonoid to serve as an infection signal, it must not only possess the ability to elicit Nod factor production *in vitro* but must also be physically present in the root exudate. For instance, the flavonoid luteolin is a potent inducer of *nodD* expression in *S. meliloti* but is naturally absent from the root exudate of its natural host *M. sativa* (Maxwell et al., 1989). It has long been known that flavonoids that act as infection signals induce *nodD* activity only in specific species or strains of rhizobia (Spaink et al., 1987). There is strong evidence that methoxychalcone is the primary flavonoid infection signal in *M. sativa*/*truncatula* whilst genistein and daidzein are responsible for *nodD* activation in *Glycine max* (soybean)/*Bradyrhizobium* symbiosis [reviewed in Liu and Murray, 2016]. The role of flavonoids as a primary host range determinant has been elegantly demonstrated by expressing *nodD* from various donor strains in a strain of rhizobia with no endogenous functional *nodD* genes. Expression of *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii *nodD* in *S. meliloti* strain A2105 (which has insertions inactivating all three of its endogenous copies of *nodD*) allows activation of *nod* gene expression in the presence of naringenin or 7-hydroxyflavone and, only in the case of bv. viciae, eriodyctiol (Peck et al., 2006).

Some flavonoid compounds show increased production following rhizobial inoculation but do not themselves activate *nod* gene expression, instead acting as antimicrobials. The release of these phytoalexin flavonoids specifically in response to rhizobia would suggest that they are still involved in

legume-rhizobia symbiosis. The *Phaseolus vulgaris* symbiont *Rhizobium etli* is able to grow in the presence of some legume phytoalexin flavonoids, including kievitone and phaseolin, which were first isolated from its natural host. *S. meliloti* is able to tolerate medicarpin, which is found in the exudates of the compatible host *M. truncatula* (Pankhurst and Biggs, 1980). This would suggest that tolerance to antimicrobial flavonoids produced by a potential host is a prerequisite for symbiotic compatibility. Given that these flavonoids are still exuded into the rhizosphere, their presence is likely to have a significant effect on the community composition, which could, in the longer term, lead to formation of a niche for favored symbionts to thrive within. There is also evidence that resistance to flavonoids may sometimes be under the control of additional host flavonoids. For instance, glyceollin is toxic to the *G. max* symbionts *B. japonicum* and *Sinorhizobium fredii* but pre-incubation with genistein and daidzein induces resistance. The mechanism of this resistance is independent of *nod* gene activation by flavonoids because the effect is replicated in bacteria that do not have a functional *nodD* gene (Parniske et al., 1991).

Distinct legume species exude different combinations of flavonoids. By only recognizing a specific flavonoid profile, a rhizobial strain is able to reciprocate symbiosis signals only in the context of a compatible host. Moreover, the resistance of compatible strains to antibiotic flavonoids may create a niche within the rhizosphere where the compatible strain(s) can replicate in an environment of reduced community competition. Thus, flavonoids serve as a first mechanism for both host and rhizobia to find their favored partners. This mechanism may be reinforced by production of phytoalexins from the host plant that are able to suppress the growth of less compatible rhizobia.

REGULATION OF HOST RANGE BY RHIZOBIAL NOD FACTORS

The ability of the host plant to recognize Nod factors during the initial cross-talk between the two parties is also a determinant of host range as subsequent events depend on the activity of nodulation-related genes such as *NIN* (Vernié et al., 2015), which are themselves expressed in response to Nod factor signaling. Although Nod factors share the same basic structure of a chitooligosaccharide chain connected to a fatty acid, they can be extensively modified by the bacteria. This has given rise to exceptional diversity of Nod factors across rhizobial species (Long, 1996). Specifically, variations in the extent of chitooligosaccharide polymerization, the nature of the attached fatty acid and chemical substitutions at either terminus of the molecule [reviewed in Mergaert et al., 1997] allow for different rhizobial species to produce chemically distinct combinations of Nod factors.

In much the same way that the ability to recognize flavonoids is the primary rhizobial determinant of host range, the ability to recognize Nod factors is the primary determinant of symbiosis specificity from the perspective of the host. Transfer of Nod factor genes between rhizobial strains may allow the recipient to colonize natural hosts of the donor strain. For instance, strains of *R. leguminosarum* with extensive deletions in their symbiosis plasmids are unable to form nodules with their natural host *Trifolium repens*. Subsequent transfer of a plasmid bearing the *nod* genes from *S. meliloti* permits the formation of *fix*$^-$ nodules with *M. sativa*, the natural host of the donor rhizobia (Debellé et al., 1988). Additionally, expression of the fucosyl-transferase encoded by the *nodZ* gene of *Bradyrhizobium japonicum* in *Rhizobium leguminosarum* permits the formation of *fix*$^-$ nodules in the tropical legumes *Macroptilium atropurpureum* (siratro), *Glycine soja*, *Vigna unguiculata* and *Leucaena leucocephala* (López-Lara et al., 1996). These experiments show that the Nod factor structure is at least partially responsible for determining the range of plant species (or accessions) that a given rhizobia is able to associate with.

Strains of *S. fredii* possess the Nod factor repressor *nolR* as well as two functional copies of *nodD*. Wild type *S. fredii* HH103 is able to associate with *Lotus burtii* via crack entry infection, independently of infection thread formation (Acosta-Jurado et al., 2016). *nodD1* mutants fail to form nodules with *L. burtii*, whilst *nodD2* or *nolR* inactivation leads to an extension of host range to *L. japonicus* Gifu. Interestingly, both the *nodD2* and *nolR* mutants produced Nod factors in higher quantities than the wild type upon genistein exposure and infected both *L. burtii* and *L. japonicus* via infection threads rather than crack entry (Acosta-Jurado et al., 2019). Subsequent study has revealed that inactivation of *SyrM*, another transcription factor involved in regulation of early Nod gene expression, is also sufficient to replicate the *fix*$^+$/infection thread-dependent phenotype in *L. japonicus* and *L. burtii* (Acosta-Jurado et al., 2020). *nolR* (Vinardell et al., 2004), *nodD2* (Acosta-Jurado et al., 2019) and *SyrM* (Acosta-Jurado et al., 2020) mutants all exhibit diminished ability to infect *G. max* relative to wild type HH103. Despite its broad potential host range, HH103 may therefore represent an example of a rhizobial strain that has evolved to restrict interactions with legumes that it is less well-suited to colonize, in favor of its natural host, *G. max*.

Nod factors are perceived by Nod factor receptors to instigate the symbiosis pathway and thus Nod factor receptors themselves represent an additional component of host range specificity The role of these receptors in symbiosis specificity has been demonstrated by transformation of *M. truncatula* with the *LjNfr1*/*LjNfr5* genes of *L. japonicus*. Inoculation of *M. truncatula* *Nfr1*$^+$/*Nfr5*$^+$ with strains of the *L. japonicus* symbionts *M. loti* or *R. leguminosarum* DZL modified to constitutively express NodD (thus producing Nod factors independently of flavonoid signaling) led to nodule formation, which does not occur in wild type *M. truncatula*. However, these nodules had a *fix*$^-$ phenotype and their infection was arrested before symbiosome formation (and hence N-fixation) could occur (Radutoiu et al., 2007).

Recent work by Bozsoki et al. (2020), in which chimeric receptors comprising domains from *Lj*NFR1, the related chitin receptor *Lj*CERK6 and *Mt*LYK3 were generated, has led to a significant enhancement in our understanding of Nod factor recognition. These chimeric proteins were expressed in *nfr1 L. japonicus* mutants inoculated with *M. loti*, allowing the sensitivity of the engineered receptor to *M. loti* Nod factor to be assessed by the extent of nodule formation. Nod factor specificity of NFR1

was mapped to two regions within the LysM1 domain of the extracellular domain of the protein but the transmembrane and kinase domains were also found to influence the efficiency of nodulation. Substitution of residues associated with Nod factor recognition in the LYK3 LysM1 domain with corresponding residues from NFR1 still permitted nodulation of *nfr1* plants. Similarly, expression of a chimeric receptor with the NFR1 LysM1 domain but substituting the Nod factor recognition regions with those of LYK3 (and an additional region of the LYK3 LysM1 domain) allows recognition of *S. meliloti* by *lyk3 M. truncatula*. Interestingly, receptors combining the CERK6 ectodomain with the NFR1 transmembrane and kinase domains still permitted nodulation of *nfr1* plants, provided the ligand specificity regions of the LysM1 domain were substituted with corresponding regions from NFR1 (Bozsoki et al., 2020).

RECOGNITION OF RHIZOBIAL POLYSACCHARIDES UNDERPINNING SYMBIOTIC INTERACTION

Extracellular polysaccharides produced by rhizobia, such as exopolysaccharides (EPS) and lipopolysaccharides (LPS), are indispensable during many legume-rhizobia interactions due to their roles in root attachment, signaling and the suppression of host immunity [reviewed in Downie, 2010]. The identification of the *L. japonicus* receptor kinase *Epr3* and the demonstration that it directly binds to and enables perception of the EPS of the compatible symbiont *M. loti* R7A (Kawaharada et al., 2015) has suggested that rhizobial exopolysaccharides moderate symbiosis by regulating receptor-ligand interactions. Given that there is extensive diversity in polysaccharide structure across rhizobia species, it is therefore plausible that specificity in polysaccharide recognition may represent an additional aspect of host range regulation, akin to Nod factor recognition.

The exopolysaccharide succinoglycan of *S. meliloti* is required for infection of its natural host *M. sativa* (Cheng and Walker, 1998) and in interactions with compatible accessions of *M. truncatula* (Simsek et al., 2007; Liu et al., 2014). Transfer of a segment of the succinoglycan-coding *exo* gene from the A17-compatible *S. meliloti* 1021 into the usually incompatible Rm41 strain results in a fix^+ phenotype in A17 that is comparable to the 1021 donor strain (Simsek et al., 2007). In addition to its role in facilitating infection during the early stages of the symbiosis, there is also evidence that succinoglycan acts to protect *S. meliloti* from the bactericidal effects of NCR247, a peptide belonging to the nodule-specific cysteine-rich (NCR) family of peptides (discussed later in this review) during later stages of symbiosis. Both overexpression and exogenous application of succinoglycan leads to dramatically enhanced survivability of cells in the presence of otherwise toxic concentrations of NCR247 (Arnold et al., 2018).

A possible role for LPS in symbiosis specificity is supported by observations of the broad host range *S. fredii* strain HH103. This strain is able to form nodules with many legume species including its natural host *G. max, Cajanus cajan* (pigeon pea) and IRLC member *Glycyrrhiza uralensis* (liquorice) (Crespo-Rivas et al., 2016). Remarkably, in the latter example, the endoreduplication and poor external survivability of bacteroids, which is seen as a hallmark of TBD in nodules of clade members, is absent. Whilst LPS signatures of HH103 bacteroids isolated from non-IRLC members *G. max* and *C. cajan* nodules do not exhibit alterations relative to free-living bacteria, modifications were observed in bacteroids isolated from *G. uralenis* nodules (Crespo-Rivas et al., 2016). Further study is needed to clarify the contribution of these LPS modifications to the unusual absence of terminal differentiation seen in this interaction.

STRAIN-SPECIFIC RESTRICTION BY EFFECTOR-TRIGGERED IMMUNITY

Effector-trigger immunity (ETI) is a layer of plant innate immunity directed against effector proteins used by microorganisms to enhance virulence or circumvent host immunity. Resistance (R) proteins are receptors that are responsible for either recognizing pathogen effectors directly or detecting modification to endogenous proteins as a result of the activity of pathogen effectors [reviewed in Cui et al., 2015]. Many pathogens use a specialized apparatus called a secretion system to translocate effector proteins directly into the cytoplasm of host cells. Some rhizobia are also known to use type III or IV secretion systems (T3SS/T4SS) to deliver effector proteins into target cells to aid infection [reviewed in Soto et al., 2009]. Delivered rhizobial effectors have been found to have both positive and negative effects on symbiosis, often depending on the species or accession of the host. For instance, *Bradyrhizobium* sp. DOA9 (Songwattana et al., 2017) and *M. loti* MAFF303099 (Okazaki et al., 2010) mutants deficient in protein secretion show an increased ability to form nodules with some hosts but decreased ability with others. Meanwhile, knockout of *Bradyrhizobium* sp. ORS3257 effectors leads to enhanced nodulation or symbiotic defects in *Aeschynomene indica* depending on the specific effector (Teulet et al., 2019). It has been theorized that some legumes may have evolved R proteins that detect specific rhizobial effectors and activate defense responses to prevent colonization, thus serving as another mechanism of control of host range.

The role of ETI during symbiosis has been best studied in soybean (*G. max*) which possesses at least eight known genes involved in strain specific restriction of nodule formation (Hayashi et al., 2012). Amongst these, the dominantly-acting *Rj2*, *Rfg1* and *Rj4* genes have been best characterized and are involved in restricting symbiosis with certain strains of *Bradyrhizobium* spp. and *S. fredii*. More specifically, *Rj2* and *Rfg1* are classical R proteins that have been shown to restrict nodulation by *B. japonicum* USDA122 or *S. fredii* USDA257, USDA205 and USDA193 respectively (Yang et al., 2010; Fan et al., 2017). Inactivation of the T3SS of USDA122 permits the formation of functional N-fixing nodules in the previously incompatible wild type Hardee accession (Tsukui et al., 2013), suggesting that ETI likely precludes symbiosis in the wild type strain and that effector secretion is dispensable for symbiosis. The active variant of the *Rj4* allele is inferred to be an antimicrobial thaumatin

protein rather than a classical R protein and prevents nodulation by *B. japonicum* Is-34 and *B. elkanii* USDA61. Through the employment of transposon insertions, the genetic basis of this incompatibility has been mapped to an inferred T3SS effector in *B. japonicum* Is-34 (Tsurumaru et al., 2015) and a region containing six genes in USDA61, including one with homology to a known *Xanthomonas campestris* pathogen effector (Tang et al., 2016). A role for T3SS activity in *Rj4*-USDA61 incompatibility is further supported by the finding that the expression of defense-related genes in the incompatible BARC-2 accession induced by wild type USDA61 is abolished in a strain with a non-functional T3SS (Yasuda et al., 2016).

Although the role of ETI during N-fixing symbiosis is best studied in soybean, there is evidence that this is used as a means of symbiont selection in other legume species. In addition to its incompatibility with accessions of soybean carrying the *Rj4* allele, *B. elkanii* USDA61 also interacts poorly with some accessions of mung bean (*Vigna radiata*). Five genes have been identified (*innA-E*) in USDA61 that are associated with *V. radiata* incompatibility. Knockout of any one these is sufficient to restore symbiotic compatibility with the *V. radiata* accession KPS1. Remarkably, the knockout of four of these genes was also sufficient to allow nodulation of *G. max* BARC-2 (Nguyen et al., 2017), suggesting that a common mechanism of symbiont restriction is conserved between the two legume species. The fifth gene, *innB*, encodes a T3 effector which is induced by genistein. Given that *innB* does not interfere with nodulation in *G. max* and positively regulates nodulation in the closely related *V. mungo* (Nguyen et al., 2018) it is likely that specific accessions of *V. radiata* possess an R protein directed against *innB* which precludes symbiosis with this strain.

A recent study of the ability of USDA61 to infect *Lotus* species has also implicated the T3SS of this strain as a source of incompatibility in *L. japonicus* and *L. burtii*. Inoculation using a mutant USDA61 strain that is deficient in effector secretion led to significantly enhanced infection of nodules, although infected nodules exhibited early senescence-like responses regardless. By mutating specific T3 effectors, *nopF* was identified as being responsible for inhibition of infection in *L. japonicus* Gifu whilst *nopM* was found to affect early nodulation senescence in *L. burtii* and the Gifu and MG20 accessions of *L. japonicus*. A third effector, as yet unidentified, is likely to prevent nodule maturation in *L. burtii* and *L. japonicus* Gifu. This suggests that some *Lotus* species and accessions employ ETI to prevent symbiosis with *B. elkanii* USDA61, including *L. japonicus* MG20, which has at least three ETI-based checkpoints to reinforce its incompatibility with USDA61 (Kusakabe et al., 2020).

Less is known about a potential role for ETI in symbiont selection in leguminous species that form indeterminate nodules (such as *M. truncatula*). One reason for lack of study here is the knowledge that the NCR family possessed by IRLC members within this group already exhibits substantial influence over symbiont compatibility, as described later. By monitoring compatibility between *S. meliloti* Rm41 and different accessions of *M. truncatula*, Liu et al. (2014) have identified a locus containing eight genes associated with enabling nodulation specificity. Experiments crossing the *fix*$^+$ A20 accession with *nod*$^-$ F83005.5 suggest that this specificity is regulated by a single dominant gene, termed *NS1*, and thus it is possible that this represents an example of ETI. However, Rm41 is not known to possess a T3SS and the precise nature of the gene underpinning this phenotype remains unknown (Liu et al., 2014).

ETI provides a mechanism by which legume species could be able to restrict interactions with less favored rhizobial strains with exquisite specificity. Because ETI depends on both host (R proteins) and rhizobial (effector proteins) factors, it is often the basis of incompatibility between specific legume accessions and rhizobial strains. Secreted effector proteins used by the rhizobia to aid infection make ideal targets for ETI because they cannot easily escape recognition by mutation without losing their activity. Given the resemblance of the role of ETI during legume-rhizobia symbiosis to what occurs during some pathogen interactions, it could be speculated that rhizobial strains targeted by ETI in this way could interfere with R-gene mediated recognition of their effectors through the use of additional effectors and thus continue to infect a legume host, although this has not yet been demonstrated.

REGULATION OF RHIZOBIAL HOST RANGE BY NODULE-SPECIFIC CYSTEINE-RICH (NCR) PEPTIDES IN THE INVERTED REPEAT LACKING CLADE (IRLC) OF LEGUMES

Nodules belonging to the inverted-repeat lacking clade (IRLC) of legumes are marked by more extreme bacteroid differentiation, and this is mediated, at least in part, by antimicrobial peptides belonging to the nodule-specific cysteine-rich (NCR) family (Van de Velde et al., 2010; Roy et al., 2020). The role of NCR peptides is best understood in *M. truncatula*, which has over 700 inferred NCRs to date (Maróti et al., 2015), although it is unclear if all NCR family members are involved in regulating symbiosis since their expression levels and pattern can vary [reviewed in Roy et al., 2020]. Patterns of NCR peptide expression vary greatly between nodules of *M. truncatula* accessions but show little variation within accessions in response to different strains of rhizobia (Nallu et al., 2014). Given their large number and that the expression of NCR peptides is not tailored to the symbiont, expression of NCR family members is an ideal means to discern between potential symbiotic partners alongside their role in enforcing TBD of nodule-resident rhizobia.

In *M. truncatula*, certain NCR peptides have been demonstrated to have negative effects on the viability of specific rhizobial strains within nodules. *S. meliloti* strain Rm41 is able to infect and form nodules in both the DZA315 and A17 accessions of *M. truncatula* but a *fix*$^-$ phenotype is observed in the latter case. The basis for this incompatibility with A17 has been mapped to two loci, named *NFS1* (Yang et al., 2017) and *NFS2* (Wang et al., 2017), which both encode NCR peptides. The peptide sequences of NFS1 and NFS2 in *M. truncatula* A17 differ by one and three amino acid substitutions, respectively from the corresponding DZA315 sequences. In either case, the A17 isoform of the peptide exhibits antimicrobial activity

against Rm41 *in vitro*. However, this is not sufficient to explain the *fix*$^-$ phenotype in A17; the DZA315 variant of NFS1 is also bactericidal against Rm41 (Yang et al., 2017) yet this pairing still results in N-fixing symbiosis. Furthermore, A17 is able to form *fix*$^+$ nodules with *S. medicae* strain ABS7, despite this strain also being susceptible to A17 NFS2 *in vitro*. Knockout of *NFS1* is also sufficient to allow Rm41-infected nodules to fix nitrogen in A17 plants that still possess a functional copy of *NFS2* (Wang et al., 2017). Given that knockout of the A17 variants of *NFS1*/*NFS2* results in *fix*$^+$ nodules, the role of the DZA315 variants of these NCRs is unclear. The *S. meliloti* strain A145 also forms *fix*$^+$ nodules with DZA315 and *fix*$^-$ nodules with A17, with the A17 variant of the *NFS1*/*NFS2* genes acting dominantly to preclude nitrogen fixation with strain A145 (Wang et al., 2018). Therefore, some isoforms of NCR peptides appear to restrict symbiosis with specific rhizobial strains.

Despite the antimicrobial activity of many NCR peptides, the expression of some NCR family members is essential for symbiosis between *M. truncatula* and some rhizobial strains. Knockout of the *M. truncatula* gene encoding NCR211 results in plants that are symbiotically ineffective in partnership with the normally compatible *S. meliloti* 1021 (Kim et al., 2015). Although infected plants are still able to form nodules, the resulting organs fail to elongate and fix nitrogen, despite expression of bacterial *nif* genes and normal accumulation of leghemoglobin. Bacteria within mutant nodules are rarely able to fully differentiate and are unable to persist intracellularly (Kim et al., 2015), suggesting that NCR211 is required for long-term rhizobial viability within nodules. Similarly, perturbing the expression of *M. truncatula* NCR169 also interferes with nodule viability following infection with *S. meliloti* 1021 or *S. medicae* 419. Substitution of any of the four cysteine residues present in the mature NCR169 peptide sequence is sufficient to produce a *fix*$^-$ phenotype (Horváth et al., 2015). Given the large size of the NCR family in *M. truncatula*, the finding, in two distinct cases, that removal of a single peptide is sufficient to abolish successful symbiosis is remarkable. Despite being so numerous, some NCR peptides are clearly not functionally redundant and do not simply influence symbiosis on the basis of their antimicrobial activity.

There is strong evidence to suggest that rhizobial tolerance of NCR peptides depends on the activity of BacA and BacA-like proteins. These are membrane transport proteins that have been found to be essential for rhizobia to survive within the symbiosome of legume species belonging to the IRLC (reviewed in Roy et al., 2020), although their presence in rhizobia that do not interact with IRLC legumes and also many other bacteria besides, indicates they are likely to have functions outside of symbiosis. Deletion of the *bacA* gene of *S. meliloti* Rm2011 alone is sufficient to result in a *fix*$^-$ phenotype in previously compatible nodules of *M. sativa* and *Melilotus alba*. In *M. sativa*, a *fix*$^+$ phenotype cannot be recovered by complementation of Rm2011 *bacA* mutants with the *bacA* gene of *R. leguminosarum* bv. viciae 3841 or *S. fredii* NGR234. In contrast, expression of either the 3841 or the NGR234 *bacA* genes under the native Rm2011 promoter leads to a *fix*$^+$ phenotype in which the extent of N-fixation was comparable to wild type Rm2011 or roughly half, respectively (diCenzo et al., 2017). These results suggest that the *bacA* gene of *S. meliloti* has evolved to interact specifically with *M. sativa*. This is supported by phylogenetic analysis indicating that the *bacA* gene of *S. meliloti* Rm2011 has undergone rapid evolution and its sequence now resembles the *bacA* gene of pathogenic genera *Klebsiella*, *Brucella* and *Escherichia* more closely than it resembles many other rhizobial *bacA* orthologs (diCenzo et al., 2017). This suggests that BacA and BacA-like proteins possessed by rhizobia may be a determinant of host range when infecting legumes belonging to the IRLC and that this is likely mediated by interactions with host NCR peptides.

Taken together, the above data suggest that NCR peptides have roles in both encouraging symbiosis with favorable partners and restricting symbiosis with less favored rhizobia. However, it is possible that some rhizobial strains may have evolved mechanisms to interfere with the activity of NCR peptides, thus providing them with a means of moderating their own host range. *S. meliloti* strain B800 is able to form *fix*$^+$ nodules in *M. truncatula* accession A17 but not A20, with the latter outcome dependent on the expression of the pHRB800 accessory plasmid possessed by the bacteria (Crook et al., 2012). More specifically, this phenotype has been mapped to the activity of a single gene on the plasmid, the peptidase *hrrP*, the expression of which results in enhanced bacterial proliferation in both A17 and A20 nodules. Given that this peptidase has been demonstrated to cleave some NCR peptides *in vitro*, it is likely that it interferes with the activity of host NCR peptides and thus their effects on the proliferation and differentiation of nodule-resident rhizobia. The formation of *fix*$^-$ A20 nodules is likely a side effect of this (Price et al., 2015). This suggests a mechanism by which less-favored rhizobial strains or even cheaters are able to colonize hosts by directly targeting a host mechanism of control of symbiont selection.

The finding that certain components of *S. meliloti* EPS provide protection against the antimicrobial effects of NCR247 (Arnold et al., 2018) may serve as another mechanism by which rhizobia may resist moderation of their differentiation by their hosts. It is currently unclear if this is specific to NCR247, or if this mechanism provides a more generalized defense against NCR family activity. There is also an association between polysaccharide alterations and the absence of TBD observed during *S. fredii* HH103-*G. uralensis* symbiosis. Given the relatively low sensitivity of HH103 to *M. truncatula* NCR247 and NCR335 (Crespo-Rivas et al., 2016) and the small number of known NCRs (seven) possessed by *G. uralensis* (Montiel et al., 2017), it is possible that blanket resistance to NCR activity provides a mechanism by which HH103 is able to escape imposition of TBD by its host plant during this interaction. Further research is required to address this question and the role, if any, of LPS modifications in it.

Despite their relatively recent discovery, there is now an abundance of evidence that NCR peptides are key determinants of symbiont compatibility in *M. truncatula* and likely other members of the IRLC of legumes. Although NCR peptides are thought to mediate TBD by interfering with regulators of the bacterial cell cycle (Mergaert, 2018), little is known about their targets, aside from NCR247 (Farkas et al., 2014; Penterman et al., 2014). Therefore, for the NCR peptides which promote symbiosis with select strains, identifying their bacterial targets would

provide great insight into their activity. Given the exceptional number (hundreds) of NCR family members possessed by some IRLC species, it is likely that that specificity in bacterial targets is one component that has enabled diversification of NCR family members. This also allows symbiotic compliance to be reinforced by multiple mechanisms throughout the symbiosis. Given the fast-evolving nature of this area of research, it is hoped that the functions of other NCR family members outside *M. truncatula* will also be elucidated in the near future.

HOST SANCTIONS ON THE BASIS OF NITROGEN FIXATION EFFICIENCY

Although symbiont selection generally occurs during initial rhizobia-legume interactions, some legumes also possess mechanisms to discriminate between symbionts after colonized nodules have formed. In a natural rhizosphere ecosystem a root nodule will likely contain mixed populations of rhizobia in addition to other symbionts, parasites and other commensals [reviewed in Martínez-Hidalgo and Hirsch, 2017]. Whilst co-inoculation experiments suggest that legumes form more and larger nodules with preferred rhizobia (Heath and Tiffin, 2009), whether or not the plant actively penalizes nodules that do not fix nitrogen efficiently is less clear. In this context, sanctions are distinct from partner selection. Partner selection describes the preferential formation of nodules with a particular strain of rhizobia from a population of multiple compatible strains (and may be facilitated by any of the previously discussed mechanisms), whilst host sanctioning describes a mechanism to discriminate between and regulate the function of nodules (such as preferentially allocating photosynthates to productive nodules) once symbiosis is already established (**Figure 2**). Such control could impact the viability of rhizobia in these nodules and thus allow underperforming or cheating rhizobia strains to be "punished" by the host plant.

Host sanctions were first demonstrated by exposing nodules of *G. max* infected with *B. japonicum* to a modified atmosphere in which nitrogen was replaced with argon, thus preventing nitrogen fixation. Bacterial proliferation was dramatically reduced in plants, roots and even individual nodules exposed to the nitrogen-free atmosphere, and this was apparently mediated by reducing the oxygen permeability of offending nodules (Kiers et al., 2003). The approach of eliminating atmospheric nitrogen from nodules has also been used to demonstrate sanctions in *P. sativum* and *M. sativa*, which form indeterminate nodules (Oono et al., 2011).

The above experiments do suggest that some legumes have the ability to sanction the occupants of ineffective nodules. However, these studies occurred in an artificial environment in which BNF is almost completely eliminated. Other studies (with active BNF) have found evidence for partner selection but not sanctions in the *M. truncatula*-*S. meliloti* mutualism (Heath and Tiffin, 2009; Gubry-Rangin et al., 2010). Additionally, during some legume-rhizobia interactions that provide only low levels of N-fixation, such as *M. truncatula* and *S. meliloti* 1021, infected nodules persist regardless (Terpolilli et al., 2008). There are a number

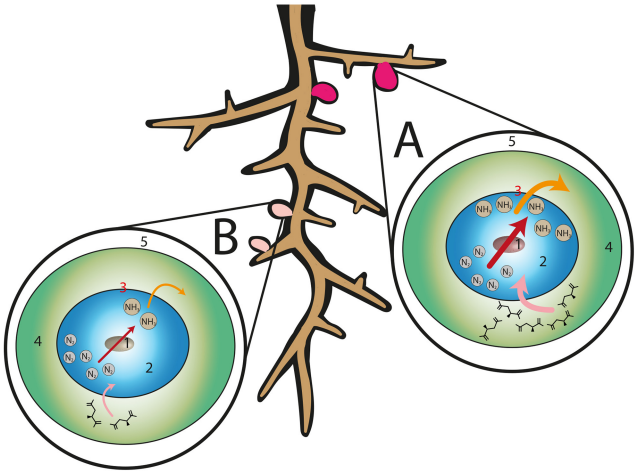

FIGURE 2 | Host sanctioning in legume-rhizobia symbiosis. In the event of a compatible plant-rhizobia interaction, root nodules can be colonized by rhizobia that have different N-fixation efficiencies. For example, nodules may be colonized by rhizobia that are **(A)** high efficiency and thus favored from the perspective of the host plant, or **(B)** poorly matched but not incompatible. Inside root nodules **(A,B)** atmospheric nitrogen is converted to ammonia by symbiosome-resident rhizobia (red arrows) which is then transferred to the host plant (orange arrows). Photosynthates are provided to rhizobia residing in nodules in the form of malate (pink arrows). In the event that a nodule contributes little or no nitrogen fixation **(B)** the host plant may deprive the offending nodule of resources, such as photosynthates, to impede the development of that nodule. Labels: 1–bacteroid, 2–peribacteroid space, 3–peribacteroid membrane, 4–cytosol, 5–infected cell.

of possible explanations for this; firstly, sanctions may not be universal amongst all legumes. Secondly, further work in *G. max* suggests sanction severity inversely correlates with the extent of N-fixation, and as such, rhizobia that contribute even small amounts of N-fixation could escape the strongest sanctions (Kiers et al., 2006). Thirdly, some rhizobia may possess mechanisms to avoid host sanctioning despite performing poorly with regards to N-fixation. Finally, previous experiments that did not find evidence for host sanctioning did so by comparing non-isogenic strains of rhizobia (Heath and Tiffin, 2009; Gubry-Rangin et al., 2010). These strains would likely exhibit differences, besides their ability to fix nitrogen in a given host, that influence their ability to colonize the host and this may have confounded the results of these studies. This last point has been convincingly addressed by Westhoek et al. (2017) who infected *P. sativum* with a strain of *R. leguminosarum* with a disrupted *nifH* gene. This mutant strain was therefore unable to participate in BNF but was otherwise identical to its parental strain. The authors assessed if the host plant was able to discern between the parental *fix*$^+$ strain and the *nifH* mutant (and thus exercise partner choice), by co-inoculating plants with both strains, each possessing a distinct marker gene allowing them to be distinguished by staining, to visualize rhizobial presence in nodules. The proportion of nodules infected with the *fix*$^+$ strain accurately reflected the proportion of the inoculum made up by this strain, demonstrating the absence of partner choice between the two strains by the host plant.

However, nodules infected with the *nifH* mutant *fix*$^-$ strain were significantly smaller than those infected with the wild type strain. This provides further evidence that *P. sativum* is able to penalize poorly performing nodules and consequentially is capable of sanctioning although the effect of any sanctions on the fitness of rhizobia within any sanctioned nodule remains unclear (Westhoek et al., 2017).

From an evolutionary perspective, it can be considered preferable for a host plant to accommodate the most efficient rhizobia (in terms of N-fixation) present in its surroundings; termed partner choice. This requires the compatibility of a putative symbiont to be assessed prior to the onset of N-fixation, and this could be mediated by a combination of the signaling factors discussed previously (for instance, Nod factor recognition and ETI). In situations where differences between rhizobial strains may not be perceivable by the host, e.g., in the experiments carried out by Westhoek et al. (2017), host sanctioning could provide an additional layer of security that is much harder for less efficient rhizobia to cheat. Outstanding questions relating to sanctions include how the N-fixing contribution of individual nodules is assessed, and how (or if) sanctioning occurs in nodules with mixed populations of rhizobia of varying N-fixation efficiency where the extent of N-fixation is likely to be intermediate. In such an instance, the absence of sanctioning would allow cheaters to thrive but tightening of sanctions would punish those individual rhizobia which are delivering optimal N-fixation.

TRANSFER RNA-DERIVED SMALL RNA FRAGMENTS PROVIDE A NOVEL MECHANISM BY WHICH RHIZOBIA CAN MANIPULATE HOST GENE EXPRESSION

The identification of transfer RNA (tRNA)-derived small RNA fragments (tRFs) involved in the regulation of N-fixing symbiosis provides another mechanism by which host specificity in the legume-rhizobia symbiosis is likely enabled. tRFs are generated by cleavage of tRNAs at specific regions, giving rise to small RNAs that may be able to silence the expression of target genes in a manner analogous to microRNAs (Sobala and Hutvagner, 2011). Ren et al. (2019) identified 25 distinct tRFs produced by *B. japonicum* USDA110 and inferred 52 putative targets of these in the soybean genome. Of these, three tRFs were found to suppress the expression of five host (soybean) genes that were putative homologs of proteins involved in root hair development in *A. thaliana*. Abolishing expression of these tRFs or overexpression of their targets resulted in attenuation of root hair curling and reduced nodule formation. Conversely, increased nodulation was observed if the host target genes were mutated, suggesting these host genes are negative regulators of nodulation. These tRFs were demonstrated to associate with the soybean ARGONAUTE-family protein GmAGO1b (Ren et al., 2019), suggesting they masquerade as host small RNAs and hijack the host RNA interference machinery to achieve silencing of target genes (as is a known function of the ARGONAUTE family, [reviewed in Mallory and Vaucheret, 2010].

Ren et al. (2019) also investigated conservation of this silencing mechanisms amongst other rhizobia and legumes. No variation was found in any of the three tRF sites in the eight *B. japonicum* strains tested or in the binding sites of their five target genes in 699 *G. max* accessions. This would suggest that this mechanism is universal in *B. japonicum*-*G. max* symbiosis. Of the target genes that had orthologs in *M. truncatula*, *P. vulgaris* or *L. japonicus*, the tRF target sites were absent or the corresponding tRFs were not known to exist in any compatible symbionts. However, the authors did infer the existence of ten *R. etli* tRFs that are predicted to target 14 host (*P. vulgaris*) genes (Ren et al., 2019). Therefore, it is likely that other rhizobial strains also use tRFs to control the expression of host genes, which would otherwise antagonize nodulation, in order to aid infection.

This novel silencing mechanism provides a remarkable example of gene expression within a legume host being directly molded by a rhizobial signal to promote symbiosis. In order to affect mRNA expression of host plant genes, rhizobial tRFs must be exported by an unknown mechanism to target cells where they interact with host machinery (Baldrich and Meyers, 2019). Given the recent nature of these findings, other examples of moderation of host gene expression by tRFs are likely to exist that have not yet been identified. If this silencing mechanism is conserved in other legume-rhizobia interactions, the diversity of tRFs possessed by rhizobial strains may also represent a broader additional layer in the regulation of host range during symbiosis. Another outstanding question is whether the plant host is itself able to influence rhizobial symbionts using similar mechanisms. Given that examples of both host-to-parasite and parasite-to-host trans-kingdom RNA signaling are now being found in plant-pathogen interactions [reviewed in Zhao and Guo, 2019], this possibility cannot be excluded.

CONCLUDING REMARKS

Compatibility between plant and rhizobial pairings during legume-rhizobia symbiosis is determined by many factors deriving from or being expressed in both host and symbiont. Both parties have evolved sophisticated mechanisms to recognize one another amongst the diversity of plant and bacterial life in the soil (**Figure 3**).

Compatibility may be reinforced at multiple stages throughout the symbiosis–for instance *S. meliloti* Rm41 is able to fix nitrogen with some accessions of *M. truncatula*, but whilst it is able to induce nodule organogenesis on A17 roots, resulting nodules fail to fix nitrogen and senesce early (Liu et al., 2014). At least two mechanisms underlie this incompatibility; the succinoglycan of this strain does not appear to correctly promote infection in A17 (Simsek et al., 2007) and the isoforms of NCR peptides NFS1/NFS2 in this accession preclude symbiosis (Wang et al., 2017; Yang et al., 2017). Interestingly, a *fix*$^+$ phenotype is observed when either expressing a compatible succinoglycan or when *NFS1* is knocked out, which would suggest that neither mechanism is insufficient to constrain symbiosis by itself. Another interesting example of host-rhizobial control is provided by *S. fredii* HH103 which is normally

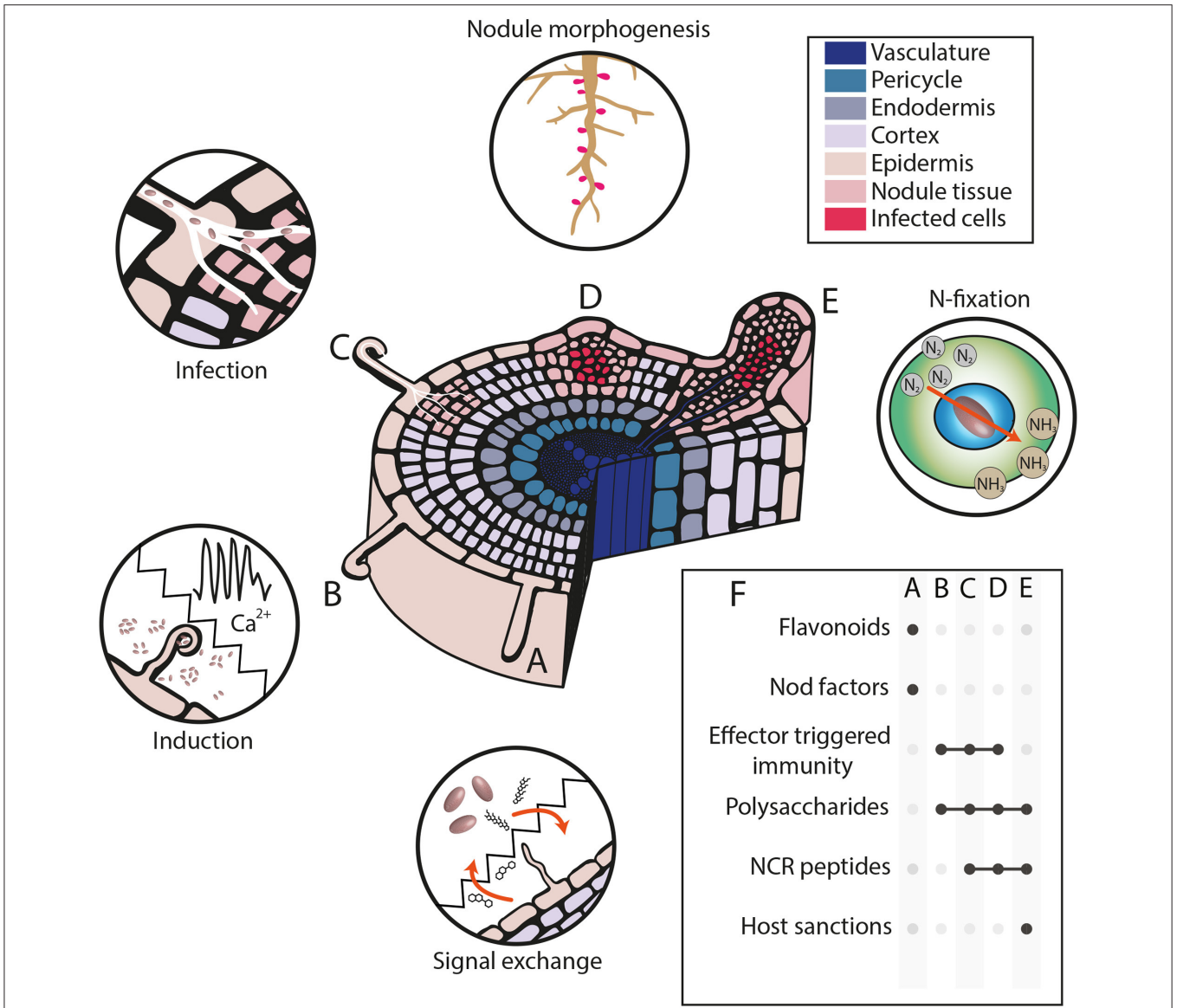

FIGURE 3 | Determinants of host range specificity in legume-rhizobia symbiosis. **(A–E)** Cross-section of a legume root depicting typical stages of nodule formation during a compatible rhizobial interaction. **(A)** Flavonoids are exuded into the rhizosphere by the legume and are detected by rhizobia which reciprocate through Nod factor secretion. **(B)** Nod factor recognition induces changes in the plant including fluctuations in Ca^{2+} and curling of root hairs which can trap nearby rhizobia. **(C)** Rhizobia are able to invade the plant root through the formation of an infection thread; simultaneously, cell divisions in the cortex give rise to a nodule meristem. **(D)** The developing nodule enlarges due to continued cell divisions and its cells are colonized by rhizobia. **(E)** Within colonized cells, rhizobia are enclosed within specialized structures called symbiosomes where they differentiate into bacteroids and convert nitrogen obtained from the atmosphere into ammonia which is used by the host plant. **(F)** Summary of factors that influence host compatibility at each of the previously described stages, with stages highlighted with a black dot where a factor is a key determinant.

associated with legumes that form determinate nodules, such as *G. max*. The early Nod gene expression of *S. fredii* HH103 seems to inhibit symbiosis with *Lotus* spp. (Acosta-Jurado et al., 2019), whilst this same strain is capable of symbiosis with IRLC member *G. uralensis*. *S. fredii* HH103 possesses a mechanism to escape TBD imposition by *G. uralensis*, possibly on the basis of modifications to its LPS (Crespo-Rivas et al., 2016).

This complexity of legume-rhizobia compatibility has implications for engineering symbioses (Pankievicz et al., 2019). Past attempts to transfer signaling components between rhizobia or legumes have allowed infection or even nodule formation between previously incompatible hosts. However, in spite of this, the resulting symbioses rarely result in nitrogen fixation and symbiosis is often terminated at a later stage (e.g., Debellé et al., 1988; López-Lara et al., 1996; Radutoiu et al., 2007). This

supports the notion that host range is not simply based on compatibility in molecular dialogue pre-infection, but is instead reinforced at multiple steps throughout the symbiosis. Therefore, if nodulation is to be transferred to other plant species, a complete understanding of the determinants of symbiotic compatibility is necessary to optimize nitrogen fixation.

Currently, much of our understanding about host range determination is derived from approaches based on rhizobial genetics, due to the relative ease of genetically manipulating bacteria. Whilst this has been invaluable for our understanding of how partner selection occurs before and during infection, much more research is needed into the processes that govern symbiont compatibility in the later stages of symbiosis. Amongst the outstanding questions that need to be addressed is the prevalence of ETI and host sanctioning and the role of NCR peptides in IRLC legume species besides *M. truncatula*. Additionally, the mechanisms that facilitate symbiont selection against compatible but inefficient rhizobia merit further study. Together this will enable a more complete understanding of how host range of nitrogen fixing rhizobia is controlled which could help engineer rhizobia for use as agricultural inoculants or symbiotic partners for non-legumes.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Revisiting Plant–Microbe Interactions and Microbial Consortia Application for Enhancing Sustainable Agriculture: A Review

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The present scenario of agricultural sector is dependent hugely on the use of chemical-based fertilizers and pesticides that impact the nutritional quality, health status, and productivity of the crops. Moreover, continuous release of these chemical inputs causes toxic compounds such as metals to accumulate in the soil and move to the plants with prolonged exposure, which ultimately impact the human health. Hence, it becomes necessary to bring out the alternatives to chemical pesticides/fertilizers for improvement of agricultural outputs. The rhizosphere of plant is an important niche with abundant microorganisms residing in it. They possess the properties of plant growth promotion, disease suppression, removal of toxic compounds, and assimilating nutrients to plants. Utilizing such beneficial microbes for crop productivity presents an efficient way to modulate the crop yield and productivity by maintaining healthy status and quality of the plants through bioformulations. To understand these microbial formulation compositions, it becomes essential to understand the processes going on in the rhizosphere as well as their concrete identification for better utilization of the microbial diversity such as plant growth–promoting bacteria and arbuscular mycorrhizal fungi. Hence, with this background, the present review article highlights the plant microbiome aboveground and belowground, importance of microbial inoculants in various plant species, and their subsequent interactive mechanisms for sustainable agriculture.

Keywords: rhizosphere interactions, microbial inoculants, plant growth promotion, sustainable agriculture, microbial community analysis

INTRODUCTION

Plants have dense inhabitation of the variety of microbes both belowground and aboveground that serve for their mutualistic benefits. The microbes that colonize the plants can be categorized into epiphytes that are present on the surface, endophytes that are located inside the plant tissues, phyllospheric that grow on leaf surfaces, and rhizospheric that inhabits into the soil close to the roots. Among them, rhizosphere is considered the most dynamic to significantly impact the nutritional status of plant and its growth (Bakker et al., 2013; Mendes et al., 2013; Lakshmanan et al., 2014). The term *rhizosphere* is defined as the narrow region of soil surrounding the roots

and directly influenced by microbes and root secretions. The underground system comprises mainly soil and primary roots along with lateral developments and root hairs, which establish their interactions with countless microbial diversity in the rhizosphere, thereby significantly influencing the plant growth stages and resistance against variety of stresses (**Figure 1**) (Panke-Buisse et al., 2015; Bandyopadhyay et al., 2017). This whole system with plant roots interacting with the rhizomicrobiome constitutes the plant–root microbiome (Philippot et al., 2013).

Knowing the hugely diversified speciation, complexity in interactions, and structural composition of communities, the need of comprehending the root architectural biology and associated microbiome as an interactome becomes essential. The intertwining nature of host and microbes opens the possibility of numerous interactions such as plant root–root interactions and root–microbe interactions. Apart from this, root–nematode interactions also serve as an essential mode to understand the behavior of plants in response to such factors. Plant hosts and associated microbes possess inseparable ecological properties, which functions as metaorganism or holobiont (Hacquard and Schadt, 2015; Hacquard, 2016).

With the advancements in the techniques with respect to genome and proteome identification and analysis, studies are conducted to explore the mutual association between plant and microbes and understand related mechanisms for improved crop production (Bakker et al., 2013; Oldroyd, 2013). If the characteristics that are responsible for forming microbial community in the rhizosphere and its influence on plants are unraveled, these can be utilized for probable sustainable alternative in agroecosystem for enhanced stability and crop productivity in longer run (Quiza et al., 2015; Knapp et al., 2018). Hence, with this background, the review focuses on belowground microbial communities that start from their establishment to their interactions in the rhizosphere and mechanistic approaches and also highlights the aboveground plant microbiome.

ABOVEGROUND PLANT MICROBIOTA

Unique environments for endophytic and epiphytic microbial diversities have been provided by different aboveground plant tissues such as vegetative foliar tissues, leaves, and floral parts, but the major differences in ecology of endospheric (endosphere is inside the environment of plant where microbes survive and may or may not be harmful to the plants; Hardoim et al., 2015; Compant et al., 2020) and phyllospheric (phyllosphere refers to the aerial region of the plant colonized by microbes) bacterial diversity exist. Systematic distribution of endophytes to different compartments such as stem, leaves, and fruits is facilitated by xylem (Compant et al., 2010), but it is observed that their entry to plant tissues can also take place through aerial parts such as fruits and flowers (Compant et al., 2011). Different compartments of plants possess distinct communities of endophytes, depending on source allocation of plant. The movement of phyllospheric bacteria is reportedly seen from soil environment that is driven by plant and various environmental parameters (Vorholt, 2012; Wallace et al., 2018). This leads to subsequent distribution of various microorganisms at genus and species level in endospheric and phyllospheric regions. For example, upon analyzing the structure of phyllosphere or carposphere of the grapevine, it was observed that *Pseudomonas*, *Sphingomonas*, *Frigoribacterium*, *Curtobacterium*, *Bacillus*,

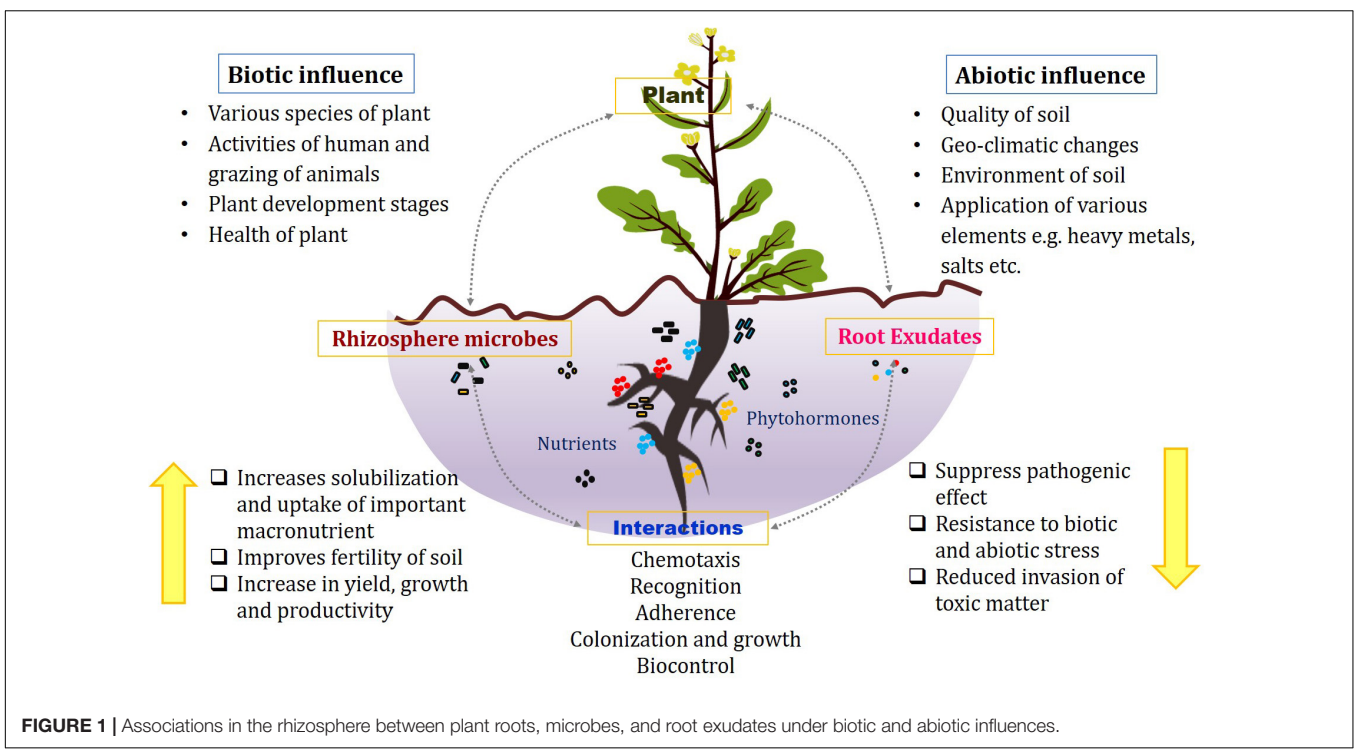

FIGURE 1 | Associations in the rhizosphere between plant roots, microbes, and root exudates under biotic and abiotic influences.

Enterobacter, *Acinetobacter*, *Erwinia*, *Citrobacter*, *Pantoea*, and *Methylobacterium* are predominant genera (Zarraonaindia et al., 2015; Kecskeméti et al., 2016), whereas when endophytes of grape berries were analyzed, the dominant genera found were *Ralstonia*, *Burkholderia*, *Pseudomonas*, *Staphylococcus*, *Mesorhizobium*, *Propionibacterium*, *Dyella*, and *Bacillus* (Campisano et al., 2014).

A study conducted on microbiome of maize leaf across 300 plant cell lines showed that *Sphingomonads* and *Methylobacteria* are the predominant taxa (Wallace et al., 2018). It was also established that environmental factors play a major role in deriving microbial composition of the phyllosphere. Another study done by Steven et al. (2018) on apple flowers showed the dominance of *Pseudomonas* and *Enterobacteriaceae* taxa. Moreover, *Pseudomonas* has been observed to be an abundant genus in numerous studies conducted on flowers of apple, grapefruit, almonds, pumpkin, and tobacco (Aleklett et al., 2014). Recent studies were facilitated to assess the seed microbes, and it was observed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* are the dominant ones (Liu et al., 2012; Barret et al., 2015; Rodríguez et al., 2018). The relation of seed microbiota has been seen with soil microbiota, and it is also evidenced that they can also be related to those of flowers and fruits (Compant et al., 2010; Glassner et al., 2018). The aboveground bacterial diversity originates from soil, seeds, and air followed by their inhabitation on or inside the plant tissues. Their existence on tissues is further shaped by various factors such as soil, environmental, and agricultural management practices. The strength of relationship between plant and its aboveground bacterial composition is specific to the host and the specific compartment where diversity exists; however, detailed knowledge of this relationship requires more research-based studies. These endophytes and aboveground microbiota are potentially known for promotion of plant growth, improvement of disease resistance, and alleviation of stresses (Hardoim et al., 2015; Vishwakarma et al., 2020).

BELOWGROUND MICROBIAL OCCURRENCE AND INTERACTIONS

Microorganisms are ubiquitously present on the surfaces of plant along with their presence in the soil and are recruited by the plant from the surroundings, which then serve as microbial reservoirs (Hardoim et al., 2015). The root microbiome can be transferred in two different ways, i.e., horizontal and vertical. The dynamic communities of microbes associated with the plant roots generally undergo horizontal transfer, which means that they are enriched from the soil rich in diversified bacterial communities predominated by *Acidobacteria*, *Bacteroidetes*, *Proteobacteria*, *Planctomycetes*, and *Actinobacteria* (Fierer, 2017). The transfer of bacterial communities can also take place in vertical direction by seeds, representing an essential source of proliferating microbes from roots of a plant to its development (Hardoim et al., 2012). Distinct and interesting soil microbial niches are provided by the plant roots that allow their colonization in the rhizosphere and root, as well as aboveground areas to a certain limit (Hartmann et al., 2009). The narrow layer of soil in the vicinity of the plant roots (rhizosphere) is thought to be a highly active area for microbial movement, making it one of the most intricate environments (Hiltner, 1904). In a study, it was demonstrated by using culture-based technique, i.e., terminal restriction fragment length polymorphism, that abundant microbial community was present in the rhizosphere in comparison to the bulk soil in an extensive wheat cropping system (Donn et al., 2015).

Root exudation is defined as the secretion of several compounds of importance by the roots into the rhizosphere, for example, organic acids, sugars, amino acids, polyphenols, flavonoids, hormones, and nutrients, which act as source of nutrients for the microorganisms surrounding the roots (Mendes et al., 2013; Compant et al., 2019). This phenomenon is known as the rhizosphere effect. Nevertheless, the association of plant roots with microbiome involves the formation of selective niches for microbial development (**Figure 2A**). With the help of phytochemicals and root exudates, several microbial groups fail to grow in the rhizospheric niche. The population able to grow by utilizing root-secreted compounds forms a niche for themselves and also helps in recruiting other microbes by cross-feeding approach, thereby generating a new niche for rest of the microbes (Jacoby and Kopriva, 2019). The niche selection process is specific for the plant species and the compounds being secreted. For example, several secondary metabolites with defense properties such as benzoxazinoids discharged from the maize roots change the structure of root microbiome and influence the group of *Actinobacteria* and *Proteobacteria* the most (Hu et al., 2018). Moreover, the dynamics of structural composition of bacterial communities in the *Avena barbata* roots and their mechanisms were researched in a recent study (Zhalnina et al., 2018). It was observed that the amalgamation of root exudate composition and substrate selectivity significantly modified the assemblage of bacterial population in rhizosphere. Fitzpatrick et al. (2018) revealed various rhizobacterial species of *Pseudoxanthomonas* depicting differential patterns of occurrence across 30 angiospermic species. Moreover, the niche specifications and the huge diversity of the rhizospheric microbiota are also governed by the spatiotemporal organization of the rhizosphere and changes in physicochemical conditions (Vetterlein et al., 2020). On the whole, variety of plant species and related genotypes and components of root exudates affect the structure and alignment of rhizospheric microbiome (Vishwakarma et al., 2017a,b).

The internal colonization of roots also takes place by a variety of endophytic microbes. Their distribution in plants is dependent on several parameters such as the distribution of plant assets and the capability of endophytes in colonizing plants. One of the important and symbiotic root endophytes, *Piriformospora indica*, has been significantly used in agriculture for its function. The endophyte *P. indica* increases phosphorous (P) uptake and protects the crop from variety of stress factors (Lahrmann et al., 2013). It has been reported that a cyclophilin A–like protein from *P. indica* was overexpressed for protecting the tobacco plant against salt stress (Trivedi et al., 2013). It has been also observed that *Azotobacter chrococcum* can facilitate the modulation of *P. indica* physiology and helps

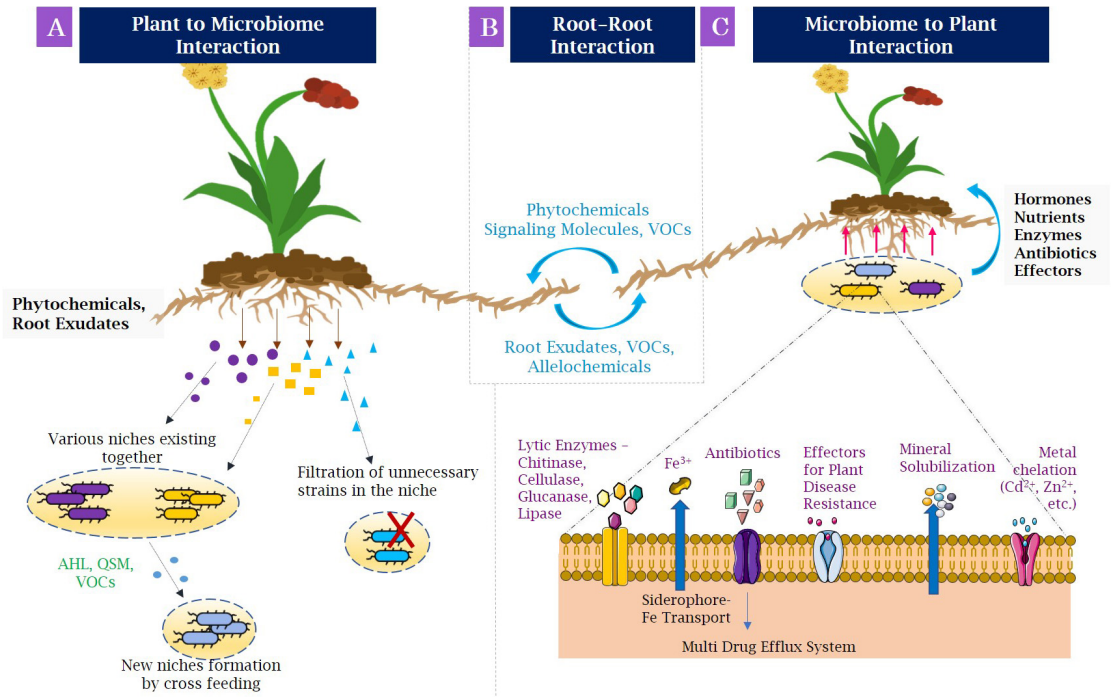

FIGURE 2 | Interactions in the rhizosphere, **(A)** Plant–microbiome interactions: Plant roots secrete root exudates and phytochemicals that engage microbial populations in developing niches. Some metabolites filter out the unnecessary microbial strains occupied in niches (indicated by red cross), whereas some metabolites allow the different microbial population to coexist in same niche that may secrete compounds needed for growth of other microorganisms. **(B)** Root–root interactions: The neighboring plants may associate with other forming beneficial, as well as competing interactions by allelochemicals, root exudates, and volatile organic compounds. **(C)** Microbiome–plant interactions: Beneficial bacteria allow the promotion of plant growth by various mechanisms, such as making nutrients available by chelating them and transporting to plants (for example, siderophore-Fe transporter to carry utilizable iron); and producing phytohormones, such as indole acetic acid, secreted effectors, and antibiotics to protect plants from pathogens. AHL, *N*-acyl homoserine lactone; QSM, quorum-sensing molecules; VOCs, volatile organic compounds; Fe, iron; Cd, cadmium; Zn, zinc.

in improving its nutrient acquisition through their synergistic action (Bhuyan et al., 2015).

Many endophytic fungi have been shown to exhibit chemotaxis for root-exuded chemicals. For instance, in a non-pathogenic *Fusarium oxysporum* when tested for activity against root knot nematode (*Meloidogyne incognita*) in tomato plants, it was found that the tomato exudates have facilitated the colonization of *F. oxysporum*, whereas it reduced the occurrence of nematode (Sikora and Dababat, 2007), suggesting that root exudates preferentially select the microbes in its vicinity. Nevertheless, root exudate–mediated chemotaxis also causes attraction for the pathogenic microbes. In a study by Gu et al. (2017), fine biochar was utilized to suppress bacterial wilt disease in tomato. The mechanism that biochar followed was absorption of root exudates that exerted strong chemotactic signal toward *Ralstonia solanacearum*, and as a result, its activity and swarming motility were suppressed. In a recent study, this bacterial pathogen has also been shown to follow chemotaxis for tomato root–exuded secondary metabolites (hydroxylated aromatic acids) (Hasegawa et al., 2019). *Pratylenchus coffeae* is an endoparasitic nematode that causes disruption of root tissues mechanically followed by invasion in plants (Das and Das, 1986). The molecular and gene expression studies on *Pratylenchus coffeae* have been conducted to specify the genes (related to cell wall degrading enzyme) regulated in the presence and absence of root exudates, and it was observed that their activity changed with respect to the host-specific root exudate components provided for the assay (Bell et al., 2019). The protozoan parasite *Trypanosoma brucei* generally displays its movement away from the other inhabited microbial groups; however, DeMarco et al. (2020) have recently observed their positive chemotactic effect toward the colonized area of *Escherichia coli*. It is due to the presence of attractant that is a soluble, diffusible signal dependent on actively growing *E. coli*.

Root–Root Interactions

Because of the coexistence of different plants in the same soil, a competition is formed in the overlapping root systems for required resources that are limited in the soil. This coexistence has been thought relative to differentiation of niche because of different rooting patterns of plant species (Parrish and Bazzaz, 1976; Berendse, 1982). However, this theory supports competitive interactions occurring belowground. The surprising knowledge of coexistence also helps in showing the interactions that are competitive as well as facilitative between the co-occurring roots. The communication between roots of neighboring plants takes place by secretion of several signaling molecules such as root exudates and allelochemicals (**Figure 2B**)

(Mommer et al., 2016a). Among them, allelopathy is the frequent communication process where phytotoxins such as catechin are released by plants. Catechin is capable of mediating both interspecific and intraspecific association by inhibiting growth of adjacent plant species, thereby enabling reduced competition and enhanced nutrient availability (Mommer et al., 2016b). Volatile organic compounds (VOCs) are also allelochemicals that mediate rhizospheric signaling by mycorrhiza networks among plants and increasing their transmission.

Apart from this, different experiments were carried out to prove different evidence in relation to interactions between plant roots with differential niches. For instance, Semchenko et al. (2018) showed that vertically distributed roots are related to competitive interactions between plants rather than integral niche. Their results have shown that there is strong competition between the plant species, which spread their roots largely leading to the suppression of neighboring species, whereas species having deeper and less branched root system are extensively able to withstand such competition. Further, using genetically transformed plants, Weidlich et al. (2018) showed facilitative interactions between the roots of legume and non-legume species belowground. These interactions are limited not only to different species but also between the genotypes. Stepping from interactions between species to interactions between genotypes, Montazeaud et al. (2018) experimented on some species and observed the productivity of rice plants (*Oryza sativa*), which was grown in pairs, and it was observed that with increase in between-genotype distance, there was increase in mixture productivity in crops, which was attributed to resource-use complementarity. Moreover, mixing of two different species of trees was performed to explore soil by their fine roots. The species used were *Acacia mangium* and *Eucalyptus grandis*, where soil was more exploited by tree species as compared to the trees that were grown in the monoculture (Germon et al., 2018). These results further helped in supporting the importance of direct competition over the niche complementarity hypothesis.

Root–Microbe Interactions

The identity of plant species largely influences variety of diverse organisms living in soils and particularly those living in close region to plant (Kowalchuk et al., 2002). Thus, organisms present in the soil can impact plant development and execution (Van der Putten et al., 2013; Jones et al., 2019). For establishing symbiotic association with the plants, microbes engage in releasing many beneficial compounds in the rhizosphere for plant's uptake. Such molecules facilitate the regulation of plant's transcriptome. In addition to production of hormones by plants, several cytokinin, auxins, and gibberellins are secreted by microbial population residing near plant roots (**Figure 2C**) (Fahad et al., 2015).

Interaction Between Root and Microbe via Root Exudates

Plant-specific root exudates display the specific selection of rhizospheric microbial communities; for instance, cucumber plant secreted citric acids from its roots, which then influenced the attraction of *Bacillus amyloliquefaciens* and banana root–exuded fumaric acid, which attracted *B. Bacillus subtilis* toward roots leading to biofilm formation (Zhang et al., 2014). Some compounds have displayed the ability of inducing nodule formation in roots like flavonoids, which are the derivatives of 2-phenyl-1,4-benzopyrone, cause induction of bacterial *nod* genes, and lead lipochitooligosaccharides (LCOs) to initiate root nodule formation. These compounds have classified role in mimicking quorum sensing in bacteria and hence impact the bacterial metabolism (Hassan and Mathesius, 2012). Apart from these, several other compounds help in synthesizing phytohormones required by bacteria for plant growth–promoting rhizobacterium (PGPR) activities like tryptophan that biosynthesizes indole acetic acid (IAA) (Haichar et al., 2014). Additionally, aminocyclopropane-1-carboxylic acid (ACC) is also exuded by roots for synthesis of ethylene (ET, a stress hormone) and as carbon and nitrogen source for bacterial growth, which is evident from the expression of *acd*S gene in microbes inhabiting the roots and involved in root exudate assimilation (Haichar et al., 2012). Through this, ACC deaminase–producing PGPRs help in utilization of ACC to decrease the level of ACC outside the plants to equalize with that of inside levels (Glick et al., 1998).

Influence of Climatic and Soil Conditions on Root–Microbe Interaction

The role of plant species is dependent on the soil feedback and climatic alterations. For instance, concentrating on how climatic conditions impact plant-soil inputs, Legay et al. (2017) showed that the inheritance impact of a past dry spell supported the resistance of *Lolium perenne* to another dry season occasion. This beneficial outcome was then credited to the choice of microorganisms during the primary dry season. Concentrating on severely phosphorous drained soils, Zemunik et al. (2017) showed that the extent of non-mycorrhizal plant species expanded directly with phosphorous deprivation in soils. The authors recommend that in severely phosphorous-exhausted soils, retaining the phosphorous through the influx of carboxylates is supported over the broadly spread beneficial interaction between arbuscular mycorrhizae and plant roots. In another study, Gang et al. (2018) deliver the constructive outcomes of the rhizobacterium *Klebsiella* SGM 81 on the development and improvement of root hairs by *Dianthus caryophyllus*. A mutualistic connection between *Klebsiella* SGM 81, living and forming IAA in close region to the establishment of *D. caryophyllus*, was distinguished as the fundamental mechanism clarifying the improved root hair generation and plant development. Rutten and Gómez-Aparicio (2018) demonstrated that soil and plant feedback depended on different species as well as on the related soil microbial communities, by using precipitation gradient that showed climatic change.

These examinations work to translate the complex and frequently setting wide collaborations between plant roots, soil, and microbes. While they together shed light on novel components intervening these associations, a major point of view of how root-microbiome connections are adjusted by natural conditions still requires extending the scope of living organisms

and thought of a more extensive board of ecological conditions, including an assortment of atmosphere and soil properties.

MECHANISM OF BELOWGROUND INTERACTIONS IN THE RHIZOSPHERE: BEYOND PLANT'S INNATE IMMUNE RESPONSE

A number of characteristic traits, such as growth patterns, behavior under stress and its mitigation, etc., have been displayed by the plant species present in an ecosystem. These traits allow the plant species to occupy different niche in space and time; this leads to the reason of having a high diversity of plant species, which can exist in correlation in a provided habitat (Kraft et al., 2015). For interactions of microbes with plants, it is essential to demark the previously formed barriers in plant species including defense responses and signaling cascades (Mhlongo et al., 2018). The defense response of the plant's immune system is based on the recognition of the pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). The first line of defense action is thought to be the PTI that includes the protein recognition receptors (PRRs) present at the surface of the cells. The conserved patterns known as pathogen (microbe)–associated molecular patterns (MAMP) serve as the binding sites for the PRR initiating a signaling cascade mechanism of defense responses, thereby inhibiting the microbe's (pathogen's) growth (Deslandes and Rivas, 2012; Denancé et al., 2013; Gao et al., 2013). However, some pathogens may cause the downregulation of PTI by secreting the effector proteins. This leads to the activation of second lineage of defensive actions, i.e., ETI, where intracellular resistance (R) genes having nucleotide-binding leucine-rich repeats are present. These R genes facilitate the binding of coding proteins to the effector virulence proteins of microbes triggering a signaling mechanism to cause cell death. The cascades PTI and ETI may involve sharing of certain biochemicals; however, they are often viewed as distinct in activities with more conserved evolutionary responses of PTI than that of ETI (Zhang and Zhou, 2010; Dempsey and Klessig, 2012). It has been highlighted that the immune system of the plant involves the strict regulation of coevolving interactive responses with multitude signaling processes among which phytohormones play a significant role inducing both systemic and local effects (Bartoli et al., 2013). The pathways in which the phytohormones play an active role involve induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Pieterse et al., 2012; Fu and Dong, 2013). To achieve an efficient plant and microbe symbiosis, the aforementioned innate responses and predefined restrictions need to be circumvented through chemistry of chemical cross talking between microbes and plants. Hence, the interactions between the plant roots and microbes as well as plant root–root associations must be considered beyond innate defense responses.

The advancements made in the associations of plant and microbes in the rhizosphere have enhanced the demands of developing and commercializing the microbe-based inoculants/formulations. Microbial inoculants are the agricultural amendments that can be applied to the soil or plant for enhanced crop productivity. These inoculants may be the natural diversity of a rhizosphere or synthetic composition of one or more microbes (Johns et al., 2016). It may be facilitated in several ways including introducing new microbial species to the rhizosphere, manipulating the environmental parameters such as moisture, pH, temperature, etc., and growing plants that modify the microbial diversity of soil (Finkel et al., 2017; Pineda et al., 2017).

During inoculation of bacterial formulation in the rhizosphere, sophisticated and complex interactions among plant–microbe and microbe–microbe take place, which are governed by the establishment of chemical communication in rhizosphere. The process of root exudation actively engages itself in the signaling cascades prompted in the rhizosphere due to inoculation. These associations hold a vital importance in achieving resistance to plant pathogens (Bertin et al., 2003), making nutrients available to the plants, facilitation of root–root interactions (Mommer et al., 2016a), and inhabited microbial community regulations (Sasse et al., 2018). However, there is competitive pressure with respect to nutrients selectivity, chemotaxis, and root colonization on the introduced microbial inoculant to make its place in the rhizosphere, along with native microbial communities. The discretion of root exudate compounds in nourishing specific rhizobacterial species has been investigated where key substrate driver was observed to be organic acids that facilitated the chemotaxis by attracting bacterial species to the roots (Zhalnina et al., 2018). Exometabolomics was deployed to delineate the substrates specifically required by bacterial strains grown on root exudates. Root exudates, having specificity to plant genotype or species, display the ability to highlight the communication knowledge between microbes, roots, and plants (Mommer et al., 2016b; Sasse et al., 2018).

Microbial species in an assemblage secrete several signaling molecules influencing the expression of genes of host plant species. Such signaling compounds comprise VOCs, for example, ketones, alcohols, alkanes, terpenoids, etc., which serve as communication channel between microbial communities in rhizosphere (Kanchiswamy et al., 2015). VOCs secreted by bacteria and plants are widely known for promoting plant growth and inducing defense responses, as well as expression of nutrient (ion) transporters (Chung et al., 2016). However, for establishing symbiosis with the plants, rhizomicrobes or microbial inoculants secrete plant beneficial compounds triggering the specific alterations in plant transcriptome. Phytohormones such as auxins, cytokinins, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), gibberellins, etc., apart from produced from plants, are secreted by beneficial microbes (Fahad et al., 2015). PGPRs, defined as the beneficial microorganisms especially bacterial species in the rhizosphere that help in plant growth promotion (PGP) by multiple means either directly or indirectly, can also produce VOCs to which certain plants respond. For instance, the consortium (two or more microbes when displaying synergism in order to improve plant growth) of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a was inoculated to *Arabidopsis*

seeds in Petri dish and enhanced its growth by secreting the volatiles acetoin and butanediol, which were common to both the microbes (Ryu et al., 2003).

MULTITUDE OF FUNCTIONS OF MICROBIAL CONSORTIA IN THE RHIZOSPHERE WITH EMPHASIS ON PHYTOHORMONES, NUTRIENTS, AND MICROBIAL DEFENSE MECHANISMS

Coevolving of plants with microbes follow the symbiotic association in order to colonize the terrestrial ecological systems (Werner et al., 2014). The knowledge of beneficial characteristics of natural PGPRs and their interactions could support the agriculture by decreasing the utilization of chemical-based fertilizers and enhancing the plant productivity. Among several traits displayed by PGPRs, the direct properties include the nutrient assimilation, phytohormone secretion and signaling, and biological nitrogen (N_2) fixation and siderophore production for making iron available to the plants (**Figure 2C**), and indirect ones include pathogen suppression, e.g., by releasing gaseous substances such as hydrogen cyanide (HCN), inducing ISR and SAR and ACC deaminase enzyme production for reducing the concentration of ET in plants.

Phytohormones

Several PGPRs as well as pathogenic bacteria are capable of producing phytohormones such as auxins, cytokinins, and gibberellins, thereby influencing the plant growth by working in conjugation with endogenous formation of these hormones in plants (Jones and Dangl, 2006; Gamalero and Glick, 2011; Spaepen, 2015). Rascovan et al. (2016) noticed a variety of microorganisms in wheat and soybean roots, which included *Pseudomonas*, *Paraburkholderia*, and *Pantoea* with significant plant growth properties such as P solubilization, N_2 fixation, IAA, and ACC deaminase production. Auxins have a significant role in regulation of plant root growth and stress responses (Liu et al., 2014). Lateral root formation and elongation of nodular meristem are essentially performed by auxins (Oldroyd et al., 2011). IAA is produced by both the PGPRs and pathogens in the rhizosphere or soil, and in case of secretion by pathogens, it is associated with virulence factor. For instance, T-DNA transfer by *Agrobacterium tumefaciens* to constitutively encode IAA production causes tumor formation (undifferentiated tissues) in plants (Spaepen and Vanderleyden, 2011).

Ethylene is a volatile hormone that influences the plant growth as evidenced in plants such as bean and oats (Laan, 1934; Sukumar, 2010). The enhancement in ET biosynthesis in *Nicotiana tabacum* can indicate the importance of ET in defense response of plants at the early PTI responses (Sharon et al., 1993). Subsequently, in *Arabidopsis thaliana*, the evidence was provided for involvement of ET signaling in expressing receptor kinases (FLS2) for binding with bacterial flagellin (flg22) to initiate the defense responses (Mersmann et al., 2010). Its association with resistance to stress incidences was also reported (Thao et al., 2015). The defense responses via ET are indicated not only by individual microbes but also through the regulation of microbial community that are influenced by ET (Nascimento et al., 2018). Several studies have followed the mutant generation approach by using *A. thaliana* to determine the potential factors that affect the bacterial community structure (Bodenhausen et al., 2014). The mutants with ET-disabled gene displayed shifts in bacterial communities at genus level; however, it could not be correlated that the enhancement in abundant species is due to the ET levels or its cross talk with other hormones. Further, the experiments of Doornbos et al. (2011) signified that initial composition of bacterial communities has a critical role in regulating ET for their capability to influence other microbial communities. This effect might elicit ET responses in shaping the microbial structure, which then can be manipulated to act against stress responses. The essentiality of JA in defense responses came into light with an infection-mediated wound response (Farmer and Ryan, 1992). Later, it has also been observed to act under necrotrophic plant defense responses (Plett et al., 2014; Wei et al., 2016). Some studies have suggested that root exudates display their involvement in regulation of hormone JA that shapes the microbial communities around the root (Bertin et al., 2003; Sasse et al., 2018). For instance, in a recent study, benzoxazinoids (component of root exudates) have been regulated by JA and interestingly demonstrated the ability to modify the microbial community composition (Hu et al., 2018). This benzoxazinoid when inoculated in the soil exhibited improvement in herbivore resistance with enhancement in JA levels. As it has been known that several root exudates have allelopathic and chemotactic properties, this benzoxazinoid has proven chemotactic traits toward *Pseudomonas putida* that cause elicitation in JA priming and provide tolerance against fungal infection (Neal et al., 2012; Neal and Ton, 2013). However, the correlation between the JA and root exudates' functions in order to select and modify the community structure needs further elucidation.

Another essential phytohormone involved in defense signaling is SA. Unlike JA and ET, SA is considered to be associated with SAR. The signaling of SA-JA-ET phytohormones forms the backbone of defensive response action. Its role in modulating the root microbiota has been derived using *A. thaliana* mutants in which knockout mutants of SA, JA, and ET were targeted (Lebeis et al., 2015). The knocked-out mutants displayed lesser rate of survival, and it was observed that some endophyte species might need SA-linked pathways for colonization. The preference of SA to select microbial communities has been displayed when SA was exogenously supplemented suggesting the active involvement of SA in shaping microbial structure (Lebeis et al., 2015). Several other hormones such as ABA, cytokinin, auxins, brassinosteroids, etc., might show antagonism or synergism with SA, JA, and ET pathways (Naseem and Dandekar, 2012; Denancé et al., 2013; Uhrig et al., 2013). For instance, ABA essentially takes part in modulating defense responses against abiotic stresses. It implicates negative effect to SA-linked defense, whereas it displays both negative and positive correlations with JA signaling pathways and affects ET-related responses to biotic stress (Pieterse et al., 2012; Takatsuji and Jiang, 2014). In a

study by Carvalhais et al. (2014), microbial genera such as *Cellvibrio*, *Limnobacter*, and *Massilia* were preferentially selected by supplementing the pot soil with exogenous ABA; however, its definite role in regulating the microbial communities is still greatly unexplored.

Nutrient Acquisition

The importance of PGPRs in rhizosphere has been marked by their ability to make nutrients such as nitrogen, phosphorous, etc., available to plants and thereby act as biofertilizers. Biofertilizers are the microbial preparations that when applied to the soil, plant, or roots provide or enhance the nutrients and increase the fertility of soil. The most highly studied feature is nitrogen (N_2) fixation by *Rhizobia* species symbiotically (Udvardi and Poole, 2013). The mode of action of rhizobial N_2 fixation involves mutual symbiosis with their leguminous plant host and the nod factors (LCOs), which are derived in response to flavonoids (Kondorosi et al., 1989; Oldroyd, 2013). It comprises chitin molecules with N-acyl moieties having varying length fatty acids, which are responsible for conferring the specificity between host and rhizobium (Oldroyd, 2013). The association between bacterial LCOs and host plant relies on direct detection of bacterial signal molecules by the plants. Lysin motif-containing receptor-like kinases (LysMs) are present on the leguminous plant cells as receptors that form bond with and gives responses to MAMPs including chitin (Antolín-Llovera et al., 2012; Liang et al., 2014). This binding of LysM with nod factors initiates several cascade signals such as cytokinin and calcium accumulation and root hair curls, developing infection thread followed by infection that happens in nodules, the place where N_2 fixation by bacteria occurs in exchange to photosynthetic carbon (Limpens et al., 2015; van Zeijl et al., 2015). In an experiment with non-legume plant *A. thaliana*, exogenous LCO from *Bradyrhizobium japonicum* was provided to the media that significantly increased the root tip numbers, length, and surface area of roots (Khan et al., 2011).

Growth and nutrition of plants are also influenced by rhizobacterial chemical secretions that alter plant physiological responses; however, their molecular mechanisms have not been completely identified, but they overlap with plant defense and symbiosis parameters. In a study by Zhang et al. (2009), accumulation of iron was increased by *B. subtilis* G03 in *A. thaliana* by activating host plant's defense machinery. It was identified that *Arabidopsis* when exposed to bacterial volatiles upregulated the Fe deficiency–induced transcription factor 1 required to induce ferric reductase FRO2 and the iron transporter IRT1 expression by *B. subtilis* volatiles (Zhang et al., 2009). When this bacterium G03 was inoculated to other plants, the iron accumulation was observed to be triggered by enhanced transporter expression. For example, G03 supplementation to *Manihot esculenta* (cassava) stem parts before plantation induced increase in iron content by 400% in leaves (Freitas et al., 2015). In a study by Vishwakarma et al. (2018), the efficacies of *Bacillus paramycoides* KVS27, *Bacillus thuringiensis* KVS25, and *Pseudomonas* species KVS20 were tested, and they have been found to increase the growth of *Brassica juncea* by facilitating P solubilization, N_2 assimilation, IAA, siderophore, and HCN production. It was also examined that there exists a synergism between these strains and that they have cumulatively enhanced the *B. juncea* growth.

Microbial Defense Mechanisms

Microbes display role in both disease occurrence and biocontrol activity. A few microorganisms can cause infection manifestations through the generation of phytotoxic compounds. One such pathogenic microbe is *Pseudomonas syringae,* which is very notable for having diverse hosts such as tomato, tobacco, olive, and green bean. Similar pathogenic bacterium is *Erwinia amylovora*, which is known for causing fire blight disease of fruit-bearing trees and ornament plants. Banana and potato crops also face variety of diseases due to the occurrence of *Xanthomonas*, *R. solanacearum*, and *Xylella fastidiosa* (Mansfield et al., 2012). The seriousness of plant disease relies upon several parameters, viz., size of pathogen population, favorable environment, and susceptible nature of host, as well as biotic conditions involved in collective determination of plant–pathogen associations (Brader et al., 2017). The host might acquire resistance against the pathogenic interventions due to the above and belowground bacterial communities by modifying defense responses of plant (de Vrieze et al., 2018).

However, the pathogenic intrusions and disease can be controlled by various biocontrol activities (Hopkins et al., 2017; Berg and Koskella, 2018). Because use of chemicals imposed many serious concerns in the agricultural productivity, hence employing benign microbial population has gained increasing popularity for economic approach (Rosier et al., 2018). This can be facilitated by the lytic enzymes, generation of antibiotics, and production of siderophores and volatile compounds, which are inhibitory to pathogens (Verma et al., 2018). The biological control by the microbes against pathogenic microbes follows different mechanisms such as antagonism, competition of nutrients and niches, and defense responses. Antagonistic microbes do not allow the other microbes to grow in its vicinity and hence can limit the growth of pathogens. Further, the fast-growing microbes can utilize the nutrients for their growth and deplete for other leading to limited or no growth of the pathogenic microbes. A few microorganisms shield the plant from pathogens by regulating plant hormonal levels and inducing resistance in the plant system. The consistent utilization of agricultural soils can develop pathogenic pressure and form disease-suppressive soil that contains microbes that suppress the disease (Durán et al., 2018). In a study, three essential bacterial taxa that belonged to *Firmicutes*, *Actinobacteria*, and *Acidobacteria* were observed to control the *Fusarium* wilt disease at a huge scale (Trivedi et al., 2017). The significance of bacterial communities of the endosphere was observed to suppress the destructive disease (*Gaeumannomyces graminis*), and further endophytes of *Serratia* and *Enterobacter* were recognized as most encouraging competitors against *G. graminis*. The action of ISR happens through the involvement of phytohormones ET and JA in protecting the plant systemically when exposed to beneficial microbes (**Figure 3**) (Verhagen et al., 2004; Pieterse et al., 2014). The priming process of plants is typically known during ISR in which defense responses against pathogenic microbes are

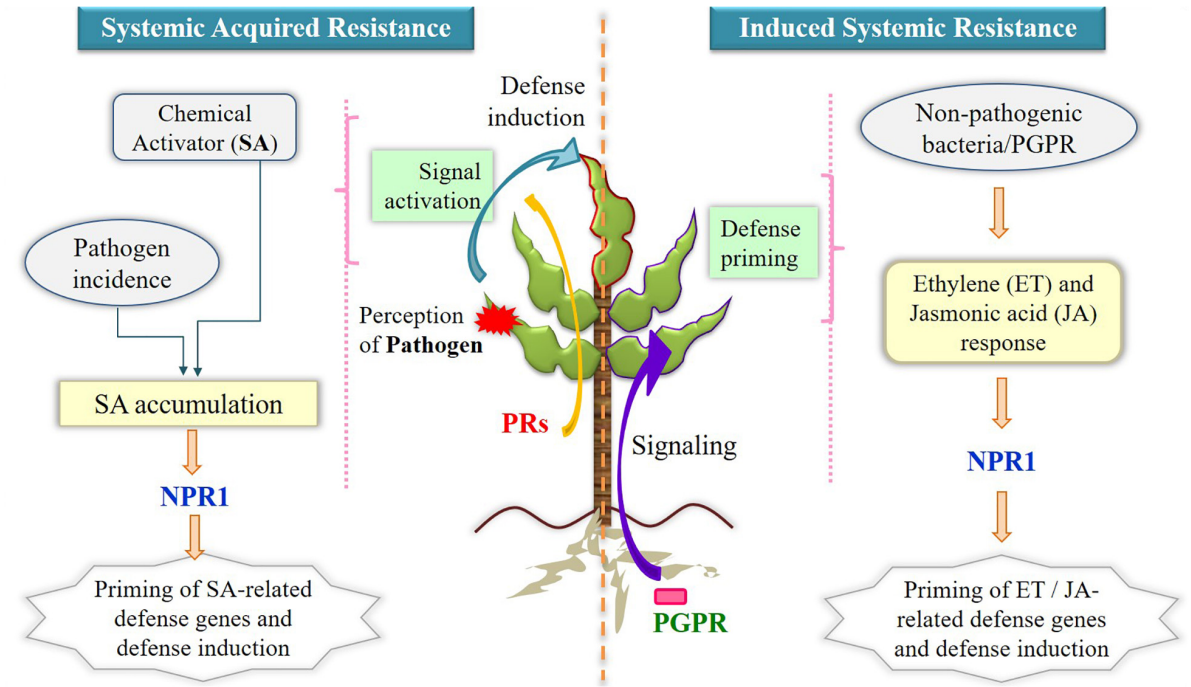

FIGURE 3 | Mechanism of SAR and ISR utilizing phytohormones for inducing defense responses upon microbial incidence. Systemic acquired resistance involves salicylic acid accumulation after perception of pathogen by plants (in red) and expression of pathogenesis-related proteins in resistant tissues (upper leaf with dark red border) for inducing defense actions, whereas in induced systemic resistance, nonpathogenic plant growth–promoting rhizobacteria enable defense responses via ethylene and jasmonic acid priming process. NPR1 is the positive regulator of salicylic acid in SAR and is also needed in downstream processes of ethylene signaling in ISR. SAR, systemic acquired resistance; ISR, induced systemic resistance; SA, salicylic acid; ET, ethylene; JA, jasmonic acid; PRs, pathogenesis related genes; PGPR, plant growth–promoting rhizobacteria; NPR1, non-expresser of PR genes.

activated aboveground very quickly (Conrath et al., 2006), and several growth-promoting rhizobacterial species have displayed plant-priming phenomena (Martinez-Medina et al., 2016). In SAR, MAMP-triggered immunity is induced as a first line of defense as discussed in *Mechanism of Belowground Interactions in the Rhizosphere: Beyond Plant's Innate Immune Response*, and unlike ISR, it utilizes SA to confer the systemic protection to the plants (**Figure 3**) (Fu and Dong, 2013).

To elicit defense responses in plants, bacteria secrete several molecules such as antibiotics, volatiles, quorum-sensing signals, and certain proteins and small compounds (**Figure 2C**). Antibiotics are generally defined as low-molecular-weight, organic molecules with diversified chemical nature formed by microbes in order to limit the growth of other microbes (Thomashow and Weller, 1996). A widely known microbial antibiotic, 2,4-diacetylphloroglucinol (DAPG), promotes the plant growth by suppressing pathogenic bacteria and fungi (Weller et al., 2012). The mode of action of DAPG is to induce the generation of auxins and alteration of root physiology, which further stimulates the plant growth (Brazelton et al., 2008). *Pseudomonas aeruginosa* is widely known to produce DAPG; however, it is also known to generate other class of antibiotic, i.e., phenazines that have been shown to induce the ISR in rice infected with *Magnaporthe oryzae* (Ma et al., 2016). Another important class of antibiotics includes cyclic lipopeptides (cLPs) that have been isolated from *Bacillus* and *Pseudomonas* species to date having unique configurations (Raaijmakers et al., 2010). Among cLPs, *Bacillus* species produce surfactin, fengycin, and iturin, of which surfactins have been considered as potential natural surfactant (Nihorimbere et al., 2012). When surfactin-producing microbe *B. subtilis* 499 was inoculated in tomato and bean plants, the occurrence of disease by *Botrytis cinerea* was significantly suppressed (Ongena et al., 2007). It had induced the lipoxygenase enzyme activity (indicator of ISR induction) in tomato plants infected with *Botrytis* pathogen when inoculated with *Bacillus* species (Ongena et al., 2007). Gram-negative quorum-sensing molecule, *N*-acyl homoserine lactone (AHL), has been observed to upregulate the plant defense responses. Inoculation of *Arabidopsis* by *Sinorhizobium meliloti* (now renamed to *Ensifer meliloti*) producing 3-oxo-C14-HL imparted resistance against *P. syringae* pv. tomato (Zarkani et al., 2013). There is also the activation of systemic tolerance by AHLs observed in fungus *Golovinomyces orontii* and bacterium *P. syringae* pv. tomato DC3000-infected *A. thaliana* (Schikora et al., 2011).

TECHNIQUES FOR MICROBIOME ANALYSIS

To characterize the microbial diversity from a sample, there are number of approaches available. However, the

characterization of whole microbiome and single components with complete details is majorly performed by two next-generation sequencing methods, i.e., amplicon sequencing and metagenomics (**Figure 4**).

Amplicon Sequencing

These strategies depend on the specific binding of the pair of the universal primers to the regions, which are highly conserved within the particular microbial genome of interest. Amplicon sequencing is applied in microbial ecological studies for exploring the microbial communities. It involves the sequencing of subsequent polymerase chain reaction (PCR) products encompassing taxon-specific hypervariable regions (HVRs) (D'Amore et al., 2016). 16S rRNA gene of bacteria are the most widely utilized amplicon targeted for microbiome examination (Kittelmann et al., 2013). Several combinations of primers have been suggested for bacterial 16S rRNA gene for amplifying various HVRs and subsequently generating PCR products varying in their lengths for sequencing platforms (such as Pacific Biosciences vs. Illumina) (D'Amore et al., 2016). The varying sequences of 16S rRNA (for bacteria), 18S rRNA (for fungi) genes, and internal transcribed spacer (ITS) segments (for fungi) along with metagenomic loci possess the information regarding the phylogeny of microorganisms, which can be utilized for inferring and deducing their taxonomy. However, it should be noted that the accuracy of taxonomical identification using marker genes is dependent upon the quality and completeness of the reference databases used. In comparison of 18S rRNA gene, the ITS region was preferred because of the presence of high comprehensive and curated database as well as the higher sequence variability (Schoch et al., 2012). However, it is debatable that the ITS fragments with uneven lengths may enhance preferential PCR amplification of ITS sequences with shorter lengths, which can take to a biased quantification of relative abundances of fungal taxa, and consequently, non-ITS targets can be additionally used in studies of fungi microbiota based on sequencing (De Filippis et al., 2017).

Sometimes, it becomes difficult to distinguish the natural genetic variations from the technical errors during sequencing, which even is less than 0.1% using the Illumina platform (Schirmer et al., 2015). To analyze the microbiome after amplicon-based sequencing, operational taxonomical units (OTUs) clustering is utilized depending on the arbitrary definitive sequence similarity thresholds (for, e.g., 97%). Similar but somewhat variant sequences are assigned to the same taxa by OTU picking giving an assumption for sharing a biological origin. In comparison to OTU-based methodologies, the enhanced specificity and sensitivity are provided by amplicon sequence variants and also diminished the possibility of false identification of OTU sets arriving from wrongly clustered sequences, but they might bear the risk to overestimate the microbial diversities (Kopylova et al., 2016).

Metagenomics

Metagenomics utilizes the entire genome shotgun method to deal with fragmentation and sequencing the complete DNA sequence of a microbial sample rather than 16S rRNA gene fragments or other targeted amplicons. Subsequently, the reads obtained have their origin from bacteria, viruses, archaea, phages, and fungi with other eukaryotes, as well as it can incorporate extrachromosomal fragments, plasmids, and host DNA. In contrast to 16S rRNA gene examination, this strategy requires

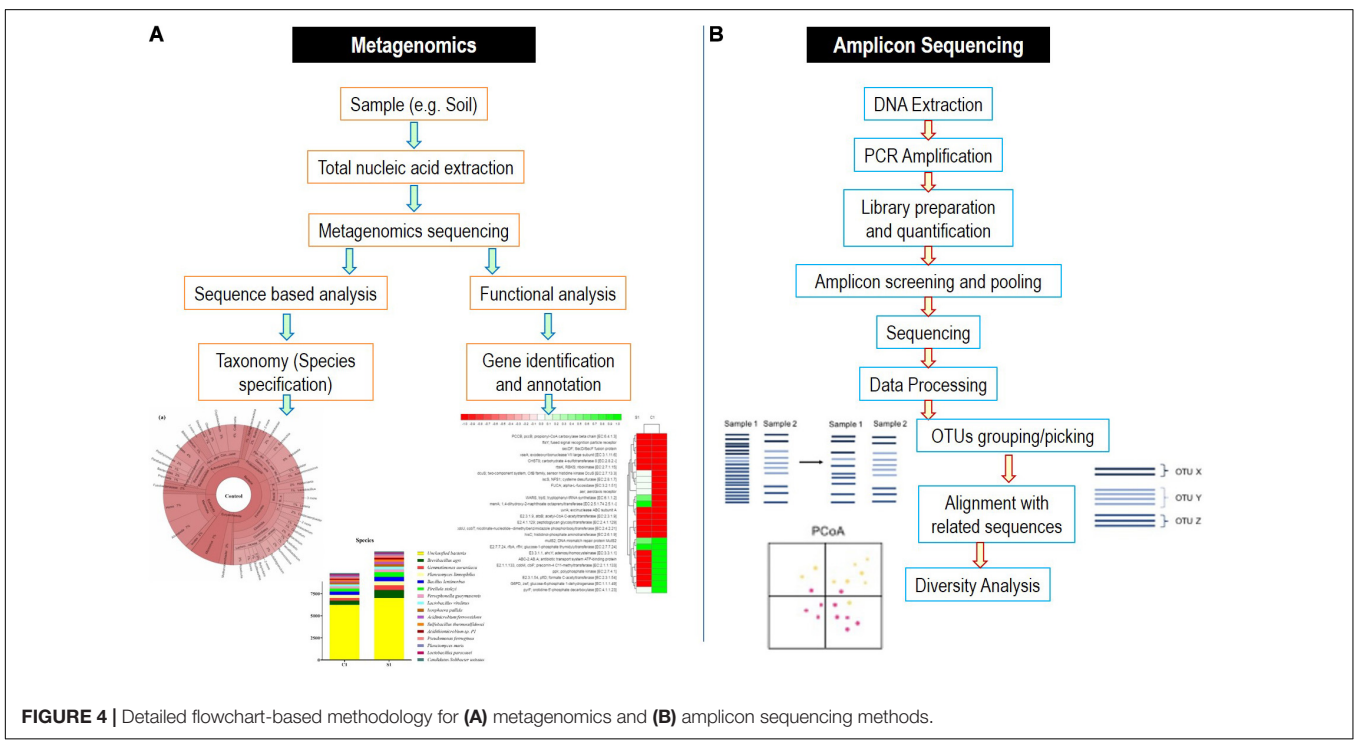

FIGURE 4 | Detailed flowchart-based methodology for **(A)** metagenomics and **(B)** amplicon sequencing methods.

essentially more information to get the depth of sequencing that is necessary to distinguish and characterize uncommon/rare members of microbiome. For robust analysis of the data, several quality control techniques are utilized to trim and filter the metagenomic reads for human, plant, and eukaryotic DNA reads by tools such as KneadDeata, QIIME, RAST, etc. (Nayfach and Pollard, 2016). Web-based tools are nowadays very easily approachable and can provide the measure to compare and map the reads in the references databases. The annotated functions can be identified by various databases such as KEGG orthologs and cluster of orthologous genes.

The metagenomics-based studies improve researcher's ability to characterize microorganisms not only at species level but also even at strain level. This contrasts with 16S rRNA–based NGS methods, which offers only limited characterization resolution because of the high sequence conservation at these taxonomic levels of the amplicons produced (Konstantinidis and Tiedje, 2007). However, additional bioinformatics approach is needed to reconstruct microbial genome from mixtures of small fragments of DNA derived from several microbes and to further enhance sequencing resolution. This is mainly relevant for finding and characterizing microbes at the strain level, where assembly algorithms overcome barriers such as intergenomic repetitive elements and to accurately detect small genetic differences (Ghurye et al., 2016). Lastly, functional level annotation of sequences of genes is allowed in metagenomics and hence has broader explanation of microbial characterization than targeted amplicon sequencing surveys. Generally, two steps of functional annotation are gene prediction and gene annotation. In gene prediction, sequences that may encode proteins are identified by bioinformatics tools. Then, these sequences are matched and annotated with database of protein families (Sharpton, 2014). This information is further used to find new functional gene sequences (Qin et al., 2010). Point to be careful about is that in metagenomics, the prediction of genes does not confirm their actual expression within the initial tested sample. Although amplicon sequencing and metagenomics are next-generation sequencing approaches, they still sometimes pose several limitations during experimentation and analysis (Boers et al., 2019).

CONTRIBUTION OF MICROBIAL INOCULANTS IN AGRICULTURAL SUSTAINABILITY

Albeit less information is available about the specific mechanism of microbial interaction with the plants, accelerating the use of microbes in a targeted way can contribute to sustainability. To enhance the microbial population, extensive research depicted practice of organic farming that enhances occurrence of microbes such as fungal and bacterial load in the soil, commonly known as plant probiotic (Yadav et al., 2017).

The utilization of beneficial microbes has gained the pace against the chemical-based and synthetic pesticides and fertilizers in agriculture industry (Alori et al., 2017). The inoculation of seeds by beneficial microbes reflects their efficiency to colonize the roots when they are placed in soil, as well as help in protection from the pathogens (Ahmad et al., 2018). This process of seed inoculation by microbial consortia possesses advantage of direct delivery of microbes in the rhizosphere where they can establish association with plants (Philippot et al., 2013). Inoculation of microorganisms helps in improving the nutrient availability to the plants, as well as help in effective carbon sequestration belowground (Vishwakarma et al., 2016). In leguminous plants, inoculating the seeds results in high occurrence of rhizobia in the rhizosphere, which further colonizes, forms nodules, and fixes nitrogen in order to achieve maximum yield and productivity (Deaker et al., 2004). *Burkholderia ambifaria* MCI 7 when used for seed treatment has shown growth promotion in maize seedlings, but at the same time, it has shown negative effect on plant growth when applied directly in the soil (Ciccillo et al., 2002).

The rising issues of varying costs and distribution related to the P-based fertilizers led to the enhancement in microbial fertilizers that promote the P acquisition by the plants from soil (Richardson and Simpson, 2011). One of the products commercialized for canola and wheat is JumpStart® (Monsanto BioAg, 2016), which contains *Penicillium bilaii* fungus. It displayed the high yield (66%) in one study (Harvey et al., 2009); however, in some studies, it has been reported to deliver less beneficial properties (Karamanos et al., 2010). The inoculation with fungus on the seeds is facilitated just before the sowing procedure. The species belonging to *Pseudomonas* have shown the plant growth–promoting potential and pathogen suppression; hence, different ways were applied for seed coating by *Pseudomonas* that delivered mixed success levels (O'Callaghan et al., 2006). Two strains of *P. syringae* have been tested under greenhouse conditions in tomato plant in which *P. syringae* pv. *syringae* strain 260-02 promoted the growth of plants and exerted biocontrol of *P. syringae* pv. tomato strain DC3000 against the fungus *B. cinerea* and the virus *Cymbidium* ringspot (Passera et al., 2019). Apart from being a pathogen, *P. syringae* can also be beneficial in some cases. This might be due to its distinct volatile emission profiles and root colonization patterns. In one of the studies, when *P. putida* KT2440 was supplied as root inoculant in maize plants, the induction of ISR was observed against the fungus *Colletotrichum graminicola* that was evident from the significantly decreased leaf necrosis and low fungal load in treated samples (Planchamp et al., 2015). Other bacteria, i.e., *Bacillus* species, have emerged as great candidates for developing stable bioproducts against pathogens, as they are capable of producing heat-resistant and drought-resistant endospores (Yánez-Mendizabal et al., 2012). In tomato plants, coinoculation of *Pseudomonas* and *Bacillus* at various stages of plant growth promoted the yield, growth, and nutritional status of plants (He et al., 2019). Similarly, the coinoculation of *Pseudomonas* and *Rhizobium sullae* enhanced growth and antioxidant levels and reduced cadmium accumulation in *Sulla coronaria* (Chiboub et al., 2019) and that of *Rhizobium* and *Pseudomonas* increased the root and shoot dry weight and overall yield of rice (Deshwal et al., 2011). There are ample studies on inoculation of microbes (both single and consortia) to the plants or seeds in order to promote the growth and development of plants. Some more examples are presented in **Table 1**.

TABLE 1 | Various microbial inoculants in consortia or single application and their effect on plants for growth promotion and defense.

Microbial inoculant	Plant species	Impact	References
Pseudomonas reactans EDP28, *Pantoea alli* ZS 3-6, *Rhizoglomus irregulare*	*Zea mays*	Increase in K^+ content associated by an effective decrease of Na^+ in plant tissues	Moreira et al., 2020
Rhizophagus irregularis, *Pseudomonas jessenii*, *P. synxantha*	*Triticum aestivum*	Enhanced the colonization of PGPR, activities of dehydrogenase and alkaline phosphatase in soil	Varinderpal-Singh et al., 2020
Funneliformis mosseae, *Ensifer meliloti*	*Vitis vinifera*	Increase in volatile organic compounds, monoterpene alcohols associated with plant defense	Velásquez et al., 2020
Thervelics®: a mixture of cells of *Bacillus subtilis* C-3102 and carrier materials	*Oryza sativa* and *Hordeum vulgare*	Production of IAA, protease, siderophores, increase in dry matter production	Jamily et al., 2019
Trichoderma sp. and *Pichia guilliermondi*	Tomato	Better growth of tomato shoot, biomass, and fruit yield	Xia et al., 2019
Yeast Brettanomyces naardensis, Arbuscular mycorrhizal fungi (AMF) Acaulospora bireticulata, Funneliformis sp.	*Helianthus annuus*	Reduced root rot and charcoal rot disease incidence caused by *Macrophomina phaseolina*	Nafady et al., 2019
B. subtilis, *B. megaterium* and *Bacillus* sp.	*Cuminum cyminum*	Enhanced seed yield and essential oil content in plants	Mishra et al., 2019
Funneliformis mosseae and *Pseudomonas fluorescens*	*Zea mays*	Enhancement in vegetative and reproductive traits, uptake of P and N, maize root colonization, and grain yield	Ghorchiani et al., 2018
Pseudomonas putida and *Novosphingobium* sp.	*Citrus macrophylla*	Decreased effects of salt stress by reduced abscisic acid and salicylic acid production	Vives-Peris et al., 2018
Bradyrhizobium sp.	Soybean	Enhanced phosphorus use efficiency and take up of N and P by soybean	Fituma et al., 2018
Pseudomonas syringae pv. *syringae* Pss20 and *Pseudomonas tolaasii* Pt18	Carrot	Increased root formation in carrot and displayed biocontrol activity	Etminani and Harighi, 2018
Cellulosimicrobium funkei KM032184	*Phaseolus vulgaris*	Increase in seed germination, root and shoot length, whole biomass, photosynthetic pigments such as carotenoids, chlorophyll, decreased oxidative damage	Karthik et al., 2016
Pseudomonas fluorescens	*Cucumis sativus*	Better growth of root and shoot, lowered the salt stress	Nadeem et al., 2017
Funneliformis mosseae and *Diversispora versiformis*	*Chrysanthemum morifolium*	Increase in shoot and root development, decrease in salt stress, enhanced N content in roots	Wang et al., 2018
Achromobacter xylosoxidans	*Oryza sativa*	Disease suppression of *Magnaporthe oryzae*	Joe et al., 2012
Bacillus spp., *Pseudomonas aeruginosa*, *Streptomyces* sp., *Paenibacillus polymyxa*	Sunflower	Suppressing the sunflower necrosis virus disease	Srinivasan and Mathivanan, 2011
Bacillus pumilus, *Micrococcus* spp.	*Noccaea caerulescens*	Increased uptake of nickel from soil	Aboudrar et al., 2013

Mycorrhiza describes a symbiotic association between root-colonizing fungi and plants (Sylvia et al., 2005). The mycorrhizal association begins with the exchange of signals between both the partners. The host root releases the signaling molecules known as "branching factors" for initiating extensive hyphal branching for arbuscular mycorrhizal (AM) fungi (Akhtar and Panwar, 2011). AM fungi have long been presumed to generate signal molecules known as "myc factors" that give the molecular and cellular responses to AM fungi for successful root colonization. None of these signals had been isolated and chemically identified until the discovery of 'branching factors" from root secretions of legume *Lotus japonicus*. It was identified as a strigolactone, 5-deoxy-strigol (Akiyama and Hayashi, 2006). It has been widely studied that the plant immunity can be enhanced by the association between the mycorrhizae and plant.

The endophytic fungi are known for existing greatly in the plant's tissues for maintaining health of the plant and possess an essential parameter in plant–microbe associations. The plants and endophytes at the later stage of ecological process become synergistically beneficial. One of the beneficial endophytes is *P. indica* that has been isolated from the roots of plants growing in the desert of Rajasthan, India (Varma et al., 2012). It has been studied widely for their essential properties and tested with many plant species. This fungus enhances the uptake of nutritional elements and facilitates the survival of plants under stressed conditions such as salinity and drought; presents systemic resistance against pathogens, heavy metals, and toxic compounds; and promotes yield and crop productivity (Varma et al., 2012). Many other researchers have observed high biomass delivery and improvement in plant growth when treated with this fungus (Achatz et al., 2010; Gill et al., 2016). More than 150 species of host plants have been tested and observed to beneficially associate with *P. indica* with respect to their benefits in agriculture, medicinal, ornamental, and other plants (Varma et al., 2012). The roots that are colonized by *P. indica* have shown early developmental gene expression indicating more growth at initial stages in treated in comparison to control (Waller et al., 2005). Colonization of exterior root cortex of maize was observed after inoculation of *P. indica* to maize roots, which further significantly increased the growth responses (Kumar et al., 2009). In a study on *Ocimum basilicum* (sweet basil), lead (Pb) uptake in shoots is restricted by combined inoculation of endophytic fungi *Rhizophagus irregularis* and *Serendipita indica*; however, copper (Cu) uptake is limited by *S. indica* only (Sabra et al., 2018). Useful products from *Trichoderma harzianum* are being produced by many countries; for example, in Poland T-22 strain is used

to market a product known as Tianum-P. Many studies have reported the production of useful compounds by *Trichoderma* species and have found that it can produce viriden, isonitryles, gliotoxines, peptaboils, and sesquiterpenes among many other essential compounds (Pylak et al., 2019). A study has shown that *Trichoderma atroviride* G79/11 is able to produce the enzyme cellulase, which makes it suitable candidate for biopreparation of antifungal compounds (Oszust et al., 2017a,b).

Talaromyces is an important fungal genus from the group of heat-resistant fungi (HRFs), among which most common is *Talaromyces flavus* strain. The HRFs have the ability to resist high temperature ranging from 90°C for 6 min to 95°C for 1 min in glucose tartarate–rich medium at pH 5 (Frąc et al., 2015; Panek and Frąc, 2018). It has been reported to produce bioactive compounds such as actofunicone, deoxy-funicone, and vermistatin (Proksa, 2010). These compounds help them in nutrient competition and to grow faster; therefore, this strain has the potential to be used in pathogen biocontrol (Pylak et al., 2019). In production of organic fruits, many bioproducts and biopreparations are being utilized, e.g., Biosept 33 SL and Micosat F. These are dependent on various active ingredients such as plant extracts (e.g., garlic—*Allium sativum*), animal-derived substances (e.g., chitosan), or microbial inoculum (e.g., *Pythium oligandrum*). These biopreparations are appreciated by farmers because of their safety and effectiveness for plants themselves and animals (Reddy et al., 2000; Marjanska-Cichon and Sapieha-Waszkiewicz, 2011).

AGRICULTURAL MANAGEMENT AND STATUS OF MICROBIAL INOCULANTS

Numerous studies have shown that, besides the plant influence, long-term agricultural practices affect the assembly of the rhizosphere microbiota (Chowdhury et al., 2019). It has been observed that recruitment of management process–specific taxa is favored by the plant hosts, which also helps in shifting the nutrient cycling in rhizospheric region (Schmidt et al., 2019). The influence of agricultural management practices and modulated microbiome can subsequently affect the dependent plant characteristics and hence the performance. Apart from microbial inoculations, agricultural practices such as organic farming, crop diversification, and intercropping have been used for sustainability in agriculture. Although there is limitation in the studies that show impact of several practices on plant microbiome, fertilization, or biodiversity protection, it has been shown that utilizing low input farm practices lead to promotion of diversity and abundance of many microbes (Postma-Blaauw et al., 2010). Hence, it is necessary to understand the impact of agricultural practices on plant microbiota to formulate strategies on modulation of microbiome in desired direction.

It has been shown that integrated or organic pest treatment of grapevine may cause different plant and soil microbiota build-up (Campisano et al., 2014). Likewise, studies on viticulture treatment have shown different microbiota build-up in comparison to the biodynamic and organic management practices (Longa et al., 2017). Vineyards were assessed for 10 years under integrated, biodynamic, and organic management practices, and it was found that soil treated with organic management practices had rich bacterial diversity in comparison to integrated management but bacterial community composition found to be similar in both (Hendgen et al., 2018). Further, a study reported that soil under 20 years of organic farming exhibited rich microbial diversity in comparison to conventionally managed soil (Hartmann et al., 2015). In another study, Hartman et al. (2018) analyzed the impact on microbial diversity under conventional and organic farming management types with varying tillage intensities. It was observed that primary soil microbial diversity is influenced by tillage while root microbial diversity such as fungal communities are influenced mainly by management type (conventional and organic) and somewhat due to tillage. Effects of soil management practices depend on, for instance, soil microbiota, soil type, and plant species, and approximately 10% of disparity in microbial diversity can be explained by the farming practices utilized (Hartman et al., 2018). Our understanding on effects of soil management practices on microbial diversity has advanced, but the effects of complex system such as environmental factors are yet to be understood.

Process of Microbial Inoculant (Single/Consortia) Formulation

The identification and characterization of PGPRs and/or consortia involve bottom-up selection procedures, which include collecting the bacterial cultures and investigating the properties in culture-dependent screening methods (Armanhi et al., 2018). The detailed outline of process is given in **Figure 5**. Bacterial stress resistance to desiccation, temperature, or toxic components and promotional activities for plant growth can be assessed for the cultures grown in axenic conditions (Suleman et al., 2018; Compant et al., 2019). These *in vitro* tests can be used as selection criterion to screen the PGP traits (Syranidou et al., 2016; Liu et al., 2017). However, there is no correlation between the efficiency of PGP bacteria and their abundant molecular PGP traits (Tiryaki et al., 2019).

Laboratory screening can give only limited information. In years, the majority of the research were focused on developing strains, understanding mode of action when inoculated to plants, and assessing their effects. And now, research is being focused on conversion of science into technology by producing the inoculants (Yadav and Chandra, 2014). Automation technologies can be adopted for mass and time-efficient production of inoculants such as using sterile liquid inoculants having more microbe load to enhance the shelf life and contamination-free products. According to a report produced by the National Centre of Organic Farming, India has around 225 biofertilizer production units that can produce up to 98,000 Mt per annum through installed capacities (NCOF, 2011, 2012). Initially, the inoculants of *Rhizobium* have gained momentum in commercialization in market followed by *Azotobacter*, *Azospirillum*, phosphate-solubilizing bacteria (PSBs), *Acetobacter*, *Frateuria aurantia* + *Bacillus* species, and the mixtures of *Azotobacter, Azospirillum*, PSB, and *Pseudomonas*

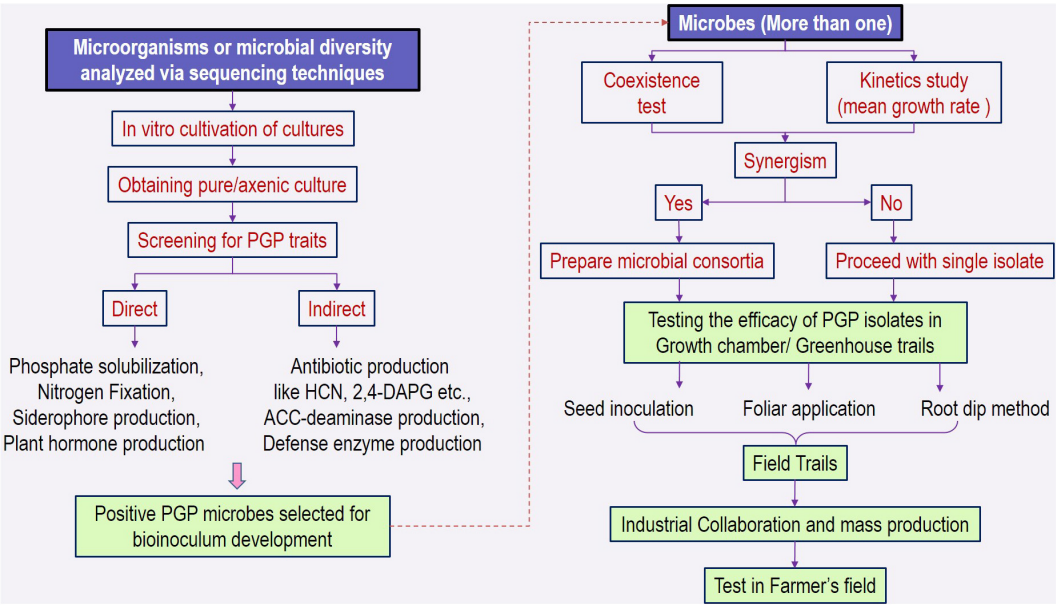

FIGURE 5 | Description of the process involved in screening microbial cultures for PGP traits and development of inoculant. PGP, plant growth–promoting traits; HCN, hydrogen cyanide; 2,4-DAPG, 2,4-diacetylphloroglucinol.

fluorescens. The market is dominated by single-inoculant cultures; however, the trend of employing the consortia is projected to increase within coming years (Yadav and Chandra, 2014). State Governments (in India) supply the majority of such inoculants and biofertilizers to the farmers through various schemes with subsidy varying from 25% to 75%. However, there is still a gap in direct marketing of the biofertilizers via dealers. Moreover, the acceptance rate of biofertilizers by the farmers is still inconsistent for utilization in fields due to temperature-sensitive nature and varying response and the fear that these inoculants are also pests (Sahoo et al., 2013).

FUTURE PROSPECTS, CHALLENGES, AND LIMITATIONS

To ensure long-term viability of microbial cells especially during storage and deliver sufficient viable number of cells to plants grown in fields, the development microbial formulations are needed. Unfortunate scene is that there is lack of suitable formulations for many microbes, in particular, the Gram-negatives (Berninger et al., 2018). Further limitation for viability in formulations is the toleration capacity of bacteria to low-humidity conditions (Köhl et al., 2011). Use of several compounds on the formulations might actually help in improvement of PGP effects. Experiments conducted for addition of LCOs isolated from rhizobia in the formulation or adaptation of growth medium of inoculants help in increasing exopolysaccharides and polyhydroxybutyrate content and increased PGP activities (Oliveira et al., 2017).

It has been observed that the bacterial products/additives do not have clear understanding with respect to their adhesion, but adjustments in droplet size and rheological properties can be achieved by surfactants, which might help in improvement of adhesion to hydrophobic cuticular surfaces (Preininger et al., 2018). Improvement of adhesion of PGPRs to roots has been done by nanoparticles and humid environment provided by encapsulated PGPR macrobeads (Perez et al., 2018; Timmusk et al., 2018). Generally, yield of wheat in field studies is successfully increased by inoculation techniques adopted for inoculating seed, leaf, and soil of same PGPRs (Berger et al., 2018). Interference of seed inoculants with pesticides can be seen, but in such cases, seed inoculant colonizes the plants and activates microbial defense system, which include activation of plant immune response, biofilm production, etc. Development of new methods was done in addition to classical delivery approaches. Mitter et al. (2017) devised the concept of seed microbiome modulation. In this, flower spray inoculation was followed for achieving next-generation seeds colonized with endophytes and modulated seed microbiome. Colonization of germinated plants was done efficiently by inoculant strain, which displayed that the use of alternative approaches may lead to improvement of microbial inoculant performance under field conditions.

Microbial inocula, either single or consortia, have many advantages than limitations. These include their environment-friendly nature; they can restore soil fertility, improve/enhance nutrient availability, protect against biotic and abiotic stresses, increase soil microbial activity, decompose toxic substances, promote colonization of mycorrhizae and other useful microbes, help in recycling soil organic matter, increase plant defense and immunity for suppressing unwanted parasitic and pathogenic attacks, and carry out signal transduction and plant–microbe interactions. Each year, there is nearly 12% increase in

demand for microbial inoculants because of the increasing cost of chemical fertilizers and demand for environment-friendly technologies in society (Calvo et al., 2014). PGPRs such as *Azotobacter*, *Bacillus*, *Azospirillum*, *Pseudomonas*, *Burkholderia*, *Serratia*, and *Rhizobium* species are now being commercially produced at a large scale (Parray et al., 2016), although different countries have their own rules for the use of these microbes based biofertilizers and biopesticides for agricultural practices (Bashan et al., 2014). The main obstacles are consistency, reliability, and shelf life of microbial inoculants under field conditions. Gram-positive bacteria have longer shelf-life in comparison to non–spore-forming gram-negative bacteria. However, studies have reported super-inoculants containing all the required characteristics of a microbial inoculant (Schoebitz et al., 2013). On the other hand, studies have also issued concern about some PGPRs that can be pathogenic to humans, for example, pathogenic *Pseudomonas* species and *Burkholderia cepacia* (Kumar et al., 2013). These species can be harmful to human, despite the PGP activity shown by them, and therefore before their commercial production, they should be addressed properly (Compant et al., 2010). More research is required before incorporating pathogenic PGPRs in sustainable agriculture. Many European and other countries such as the United States are reassessing the biosafety of PGPR-based biofertilizers. Studies have shown the effect of climate change on plant–microbe interactions; however, further studies are needed to know the full capabilities of PGPRs before their acceptance by government regulations, biofertilizer companies, and farmers. There can be the provision to make cost-effective technology of microbial consortium acceptance and utilization by the farmers in the future. There can be government-regulated outlets where biofertilizers/biopesticides with improved shelf life and stability should be provided to the farmers at subsidized rates with an opportunity to replace the old stored batch of inoculum with a fresh batch. The administrative bodies of agriculture-based towns can provide training to farmers highlighting the benefits, proper handling and usage, and their general guidelines. The schemes by the government can be launched to help farmers set up small production units in their area so as to regularize the inoculant production. It will certainly help them in overcoming shelf life, stability, and viable count problems by producing the inoculant as desired for the use.

CONCLUSION

With the increase in world population at alarming rate, there is a need to increase crop production to fulfill the global food requirements and at the same time enhance agricultural sustainability. Plant growth–promoting microbes, which are active constituents of biofertilizers and biopesticides, can be represented as a feasible alternative technology for enhancing plant yield and protecting against pathogens. The microbial inoculums possess the ability to positively impact the agriculture sector; however, plant selectivity along with organic and conventional management procedures also comes into play in shaping the rhizospheric microbiome structure, their concurrence, and subsequent effects. Since the microbial community structure in bulk and rhizosphere region frequently differs in their composition in various plant niches, it becomes necessary to reorganize the priorities of research toward isolating beneficial microbes and understanding the dynamics of their association with plants for enhanced crop productivity, quality, and agroecological sustainability. Despite some limitations of microbial consortia application, the measures to move past these limitations can be taken such as enhancement of shelf-life and viable load at the time of application, as well as developing faith in farmers for consistent utilization of inoculants in their fields. In the future, studies related to large-scale viable production of inoculant can be made using synergistic microbes proven to increase the crop productivity under conventional and organic agricultural practices.

AUTHOR CONTRIBUTIONS

AV and KV designed the structure of the manuscript. CS, NK, SM, and KV wrote the manuscript. CS, NK, KV, and SB prepared the tables, figures and arranged the references. KV and AV critically read and organized the manuscript. All the authors contributed to the article and approved the submitted version.

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Quorum Quenching Activity of the PGPR *Bacillus subtilis* UD1022 Alters Nodulation Efficiency of *Sinorhizobium meliloti* on *Medicago truncatula*

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Plant growth-promoting rhizobacteria (PGPR) have enormous potential for solving some of the myriad challenges facing our global agricultural system. Intense research efforts are rapidly moving the field forward and illuminating the wide diversity of bacteria and their plant beneficial activities. In the development of better crop solutions using these PGPR, producers are including multiple different species of PGPR in their formulations in a "consortia" approach. While the intention is to emulate more natural rhizomicrobiome systems, the aspect of bacterial interactions has not been properly regarded. By using a tri-trophic model of *Medicago truncatula* A17 Jemalong, its nitrogen (N)-fixing symbiont *Sinorhizobium meliloti* Rm8530, and the PGPR *Bacillus subtilis* UD1022, we demonstrate indirect influences between the bacteria affecting their plant growth-promoting activities. Co-cultures of UD1022 with Rm8530 significantly reduced Rm8530 biofilm formation and downregulated quorum sensing (QS) genes responsible for symbiotically active biofilm production. This work also identifies the presence and activity of a quorum quenching lactonase in UD1022 and proposes this as the mechanism for non-synergistic activity of this model "consortium." These interspecies interactions may be common in the rhizosphere and are critical to understand as we seek to develop new sustainable solutions in agriculture.

Keywords: PGPR, symbiosis, consortia, legume, quorum sensing, quorum quenching, nodule, agriculture

INTRODUCTION

Legume crops are an essential component of sustainable agriculture due to their multifaceted benefits to ecology and human health (Stagnari et al., 2017). This is attributable to the mutualism between symbiotic N-fixing bacteria (*Rhizobia*) and their specific legume plant hosts, referred to as "biological nitrogen fixation" (BNF). *Rhizobia* fix atmospheric N in exchange for carbon-rich photosynthates within specialized structures formed on the plant root called nodules (Oldroyd, 2013). Peoples et al. (2009) estimates that 30–40 kg of N is fixed per ton of crop legume dry matter and Herridge et al. (2008) approximates that N-fixation by crop and forage legumes via symbiosis

globally is roughly 50 Tg per year. The ability of *Rhizobia* to fix nitrogen within an agricultural setting is a key factor in de-coupling dependence on synthetic nitrogen application. However, for BNF to effectively replace N-fertilization, a clear understanding of the numerous mechanisms increasing BNF efficiency is required.

The interspecies signaling pathway between legumes and their bacterial symbionts responsible for BNF is well described, especially in the model legume *Medicago truncatula*, which is closely related to the forage crop alfalfa. Symbiosis between *M. truncatula* and *Sinorhizobium meliloti* commences through root exudation of the signaling plant flavonoid luteolin, which acts as a chemoattractant (Hassan and Mathesius, 2012). Luteolin induces transcription and expression of *S. meliloti nod* genes, producing lipo-chitooligosaccharide signals termed Nod factors (NFs) (Kondorosi et al., 1989). Receptors localized at the root hair recognize these NFs, instigating bacterial invasion and nodule organogenesis (Oldroyd et al., 2011; Gourion et al., 2015; Zipfel and Oldroyd, 2017); *S. meliloti* then divide, proliferate, and express N_2-fixing nitrogenase enzyme within the plant-derived nodules (Oldroyd, 2013). Legume symbiosis clearly relies on a finely tuned system of molecular pathways between bacteria and host.

For example, a key factor for the successful initiation of nodulation is the production of exopolysaccharides (EPS) by *S. meliloti* (Marketon et al., 2003). EPS include succinoglycan as well as high- and low-molecular-weight molecules of galactoglucan (EPS II) present in *S. meliloti* biofilms (Rinaudi and Gonzalez, 2009). The EPS II fraction within these biofilms are described as "symbiotically active"; EPS II defective mutants are unable to form pink nodules (González et al., 1996). EPS II production is dependent on the quorum sensing (QS) regulatory network including *expR* (Pellock et al., 2002) and *sinI* genes (Marketon et al., 2003). The *S. meliloti* ExpR/SinI QS system relies on SinI synthase-produced long-chain N-acyl homoserine lactone (AHL) signal molecules (Marketon et al., 2002; Gao et al., 2005). The AHL-bound ExpR protein controls the expression of ∼500 QS genes (Gurich and González, 2009). One of these genes of note, *wggR*, encodes a transcriptional regulator activating the downstream *wge* operons responsible for the biosynthesis and polymerization of EPS II low-molecular-weight galactoglucans, the symbiotically active EPS component of *S. meliloti* biofilm (Gao et al., 2012). This QS pathway is a highly controlled intraspecies mode of communication that is crucial for *S. meliloti* to successfully coordinate activities at a community level. Considering these and the other myriad molecular communications occurring in the rhizosphere, it is imperative to inquire how other bacteria may be influencing these core symbiotic pathways.

Indeed, millions of different species of bacteria inhabit the ecosystem on and around plant roots, termed the "rhizosphere" (Adesemoye et al., 2009; Gamalero and Glick, 2011). Organisms within the rhizosphere improving plant health and resilience directly or indirectly are known as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980; Pii et al., 2015). Many bacteria have been identified as generalist PGPRs for their association with a broad range of plants, and their specific plant beneficial activities have been described. *Bacillus* species are highly researched generalist PGPRs known to promote plant growth through their ability to solubilize nutrients and produce phytohormones, antifungal secondary metabolites, and volatile organic compounds (VOCs) (Aloo et al., 2019). Several *Bacillus* species are also known to express quorum quenching (QQ) enzymes, disrupting QS signaling of other bacteria, including pathogens (Dong et al., 2001; d'Angelo-Picard et al., 2005; Ryan et al., 2009). These activities are promising areas of rhizomicrobiome research as they may have unexpected influences on complex interspecies interactions.

Due to their numerous modes of action, PGPRs such as *Bacillus* have been utilized for crop applications and are a growing proportion of agrochemical company research efforts, where they are broadly termed "biologicals" (Timmusk et al., 2017; Marrone, 2019). Biological global markets are expanding (Arora et al., 2020), and the potential of these products is driving new market formulations incorporating multiple different species of live bacteria in "consortia" (Marrone, 2019). The rationale behind this innovation stems from the knowledge that natural rhizomicrobiomes are occupied by millions of different species of bacteria working in conjunction with one another (Schlatter et al., 2015); restoration of these microbial ecosystems may provide more robust benefits to the plant (Gouda et al., 2018; Sergaki et al., 2018). This concept, described as "synergism", manifests as "additive" plant benefits observed when multiple PGPRs are applied as compared to a single PGPR.

Synergistic plant growth promotion by multiple PGPR species has been observed in certain plant–bacteria–bacteria interspecies systems (Schwartz et al., 2013; Morel et al., 2015; Berendsen et al., 2018) but has also failed to produce in others (Felici et al., 2008; Kang et al., 2014; Maymon et al., 2015). Various co-inoculation combinations of *Bacillus* species and *Rhizobia* on legumes have shown synergistic growth and nodulation outcomes. Most prominently, nodulation was significantly enhanced in soybean (Glycine max L. Merr) systems when *Bradyrhizobium japonicum* was co-inoculated with *B. cereus* UW85 (Halverson and Handelsman, 1991), *B. thuringiensis* NEB17 (Bai et al., 2003), or *B. amyloliquefaciens* strain LL2012 (Masciarelli et al., 2014). *B. subtilis*-specific co-inoculations have been successful when used with *Rhizobium leguminosarum* bv. *viciae* 128C53 (Rlv) onto *Pisum sativum* L. (pea) (Schwartz et al., 2013) and when applied along with *B. japonicum* on soybean (Bai et al., 2003). No direct mechanisms of interaction were queried in these studies. Identifying suitable PGPR consortia requires understanding the multitude of plant beneficial activities that may be altered when the organisms coexist in what is now more commonly being described as the plant holobiont (Zilber-Rosenberg and Rosenberg, 2008). The lack of identified PGPR interspecies interaction mechanisms remains a significant gap in our knowledge, yet poses an opportunity to pursue empirical selections of appropriate PGPRs as we continue to expand our understanding of their plant beneficial activities.

To investigate meaningful legume–PGPR mechanisms, we designed a simplified tri-trophic legume–symbiont–PGPR system consisting *of M. truncatula* A17 Jemalong, its symbiotic mutualist *S. meliloti* strain Rm8530, and the PGPR *B. subtilis*

strain UD1022 (Glazebrook and Walker, 1989; Bishnoi et al., 2015; Rosier, 2016). The organisms in this model were specifically selected to be representative due to their comprehensively described genetics and lifestyles. The primary goal of this work is to identify interspecies interactions between PGPR, which may influence their plant beneficial activities in the legume–*Rhizobia* symbiosis. By using the tri-trophic model as a platform for testing phenotypic outcomes of the "consortium," more fundamental questions regarding the interspecies interactions can be developed. Specifically, does the PGPR and legume symbiont consortia act synergistically to increase *M. truncatula* plant growth, how do the different PGPR species directly or indirectly interact with one another, and do those interactions influence their ability to interact with and confer benefits to the plant? We employed phenotypic and molecular assays to evaluate the legume–*Rhizobia*–PGPR interactions.

MATERIALS AND METHODS

Bacterial Growth

Primary cultures of all bacteria strains were grown and maintained on TYC media [TY media (Beringer, 1974) liquid or agar supplemented with 1 mM $CaCl_2$] with appropriate antibiotics. Subcultures of *S. meliloti* strain Rm8530 and *Bacillus subtilis* strain UD1022 (hereafter "UD1022") prepared for biofilm treatments were sub-cultured into minimal glutamate mannitol (MGM) and low phosphate (0.1 mM), as described in Marketon and Gonzalez (2002). Both UD1022 and Rm8530 strains were grown at 30°C for all experiments. AT medium for culturing pre-induced *A. tumefaciens* KYC55 was prepared as described in Joelsson and Zhu (2005). Strains used in this work are listed in **Table 1**.

Plant Growth and Co-inoculation

Seeds of *M. truncatula* A17 cv Jemalong were acid scarified for 6 min and sterilized with 3% bleach for 3 min. Seeds were imbibed in sterile water at 4°C overnight, rinsed and placed in sterile petri dish, and germinated covered overnight at room temperature (Garcia et al., 2006). Germinated seeds were placed in sterile Magenta® (Magenta Corp.) jars with Lullien's solution (Lullien et al., 1987), sealed with 3M™ MicroPore™ surgical tape, and grown in a controlled environmental chamber at 55% relative humidity and a 14 h, 22°C day/10 h, 18°C night cycle. After 6 days of growth, plants were inoculated with bacteria treatments, with 10 plants per treatment. Rm8530 was grown to OD_{600} = 0.8 and UD1022 was grown at OD_{600} = 1.0. Bacteria were spun down, washed three times in sterile H_2O, and resuspended with 0.5 × Lullein's solution with Rm8530 final OD_{600} = of 0.02 and UD1022 OD_{600} = 0.01 (in Magenta jar). Plants were harvested 7 weeks after inoculation. Experiment was repeated three times.

Cross-Streak for Growth Inhibition Analysis

Rm8530 bacteria were grown to OD_{600} = 0.8 and UD1022 OD_{600} = 1.5. Both cultures were diluted to OD_{600} = 0.5 with sterile H_2O. Bacteria were streaked on TYC agar plates using a sterile loop in a cross pattern.

Biofilm Assays

Preparation of Cell-Free Supernatant (CFS) Derived From UD1022 for Biofilm Assays

UD1022 was inoculated from a single plate colony into 5 ml of TYC and grown overnight (16 h) and then diluted 1:50 in 50 ml of MGM in a sterile 150 ml flask and grown shaking for 8 h to an OD_{600} = 0.8–1.0. Cultures were centrifuged 10 min, 4°C at 4,000 RPM. Culture supernatant was filter-sterilized with 0.22 μm membrane (Steriflip®, EMD Millipore) under gentle vacuum. Supernatant was centrifuged and filter-sterilized once more. A sub-fraction was heat treated in water bath overnight at 65°C.

Preparation of Biofilm Treatments

Biofilm assays were based on methods found in O'Toole et al. (1999) and Rinaudi and Gonzalez (2009). Rm8530 was grown 48 h in TYC to OD_{600} = 1.5–2.0, and then cells were "pre-conditioned" by diluting 1:100 to MGM media and grown shaking 48 h to OD_{600} = 0.8. Stocks of treatments were made by centrifuging and re-suspending cell pellets with fresh MGM, or UD1022 CFS, UD1022 "heat-treated" CFS to a total of 5% by volume in MGM. One hundred microliters of these treatment stocks was then aliquoted to 96-well plates with eight replicate wells per treatment. Plates were sealed with Parafilm® (Bemis Company, Inc.) and placed in a shaker at 30°C and measured at 24, 48, and 72 h. Experiment was repeated three separate times.

Plates were then emptied and gently rinsed three times with sterile water, dried, and stained 20 min with 150 μl of 0.1% crystal violet. Plates were emptied, rinsed gently three times with sterile water. Crystal violet (CV) was solubilized with modified biofilm dissolving solution (MBDS) (Tram et al., 2013). OD_{570} of CV was then measured using Wallac 1420 Plate Reader (PerkinElmer Life and Analytical Science, Wallac Oy, P.O. Box 10, FIN-20101 Tuku, Finland).

Gene Expression Reporter Assays

Reporter lines for Rm8530 were provided by Dr. Max Teplitski of the University of Florida. All cultures grown in liquid TYC broth shaking at 225 RPM at 30°C. Bacteria primary cultures were grown with appropriate antibiotics 48 h to OD_{600} = 2.0–3.0. Cells were further prepared as described in the *Biofilm Assays* section and 29 replicate wells were included per treatment. Every 24 h, total well fluorescence and cell growth were measured using Wallac 1420 Plate Reader (PerkinElmer Life and Analytical Science, Wallac Oy, P.O. Box 10, FIN-20101 Tuku, Finland). Data were reported as fluorescence counts/OD_{570} (Gao et al., 2012). After the 72 h measurement, 96-well plates were processed as described in the *Biofilm Assays* section above to assess qualitative biofilm formation. Gene reporter assays were repeated three times.

Statistical Analysis

For plant growth biological data and biofilm analysis, data normality and homogeneity were reviewed prior to analysis of variance (ANOVA). No data transformations were required. One-way ANOVA was used to test for differences between treatments. When F ratios were significant ($p < 0.05$), treatment

TABLE 1 | Bacterial strains used in this study.

Strain	Genotype	References or Sources
Rm1021	SU47 *str-21 expR102::ISRm2011-1*	Meade et al., 1982
Rm8530	*Sinorhizobium meliloti* Rm1021 *expR*$^+$	Glazebrook and Walker, 1989
Rm8530 SinI-gfp	with integrated pMG309	Gao et al., 2012
Rm8530 WggR-gfp	with integrated pMG310	Gao et al., 2012
KYC55	*Agrobacterium tumefaciens* (pJZ410) (pJZ384) (pJZ372)	Zhu et al., 2003
UD1022	*Bacillus subtilis*	Bishnoi et al., 2015
UD1022 *ytnP*$^-$	UD1022*ytnP*::erm	Dr. Pascale Beauregard

TABLE 2 | Primer sequences used in this study.

Primer	Sequence 5′-3′	Amplicon	Source length
Rm8530 rpoE1-fw	CGAGGAAGAGGTCCTGGAAT	100 bp	Trabelsi et al., 2009
Rm8530 rpoE1-rv	GACGCAGTCCTGCAACAGAT		
Rm8530 SinI F	CCGGAAATCCGTAGTGCGTC	76 bp	Gurich and González, 2009
Rm8530 SinI R	ATGCGCGATCCTGGGAGATT		
Rm8530 WggR F	TCCGTTCGCAGACTTTGGAG	107 bp	This work
Rm8530 WggR R	CGAGCGAATCATCTCCGTCA		

means were compared via Tukey Kramer HSD using SAS-JMP (Cary, NC, United States).

For gene expression reporter results analysis, ANOVA was used to test for treatment differences. Where *F*-ratios were significant ($p < 0.05$), treatment means were compared via Tukey–Kramer test (JMP, SAS Institute Inc, 1989-2019). Non-parametric analyses (Kruskal–Wallis test) were utilized if data failed to meet parametric assumptions. Where *H*-values (Kruskal–Wallis test statistic) were significant ($p < 0.05$), treatment means were compared via Kruskal–Wallis multiple comparison *Z*-value test using NCSS software (Hintze, 2000).

Gene Expression Analysis Using Semi-Quantitative Reverse Transcription PCR (qRT-PCR)

Primer Design for qRT-PCR

Gene sequences were derived from GenBank; *S. meliloti* 1021 sequences were derived from genome (accession: AL591688.1) and mega-plasmids pSymA (accession: AE006469.1). The *sinI* primer pair from Gurich and González (2009) and the *rpo*E1 primer pair from Trabelsi et al. (2009). Primers from this work were designed using GenScript Real-time PCR (TaqMan) Primer Design[1]. Amplicon size was restricted to 150 bp or less. All primer sequences (**Table 2**) were cross-checked on all strain sequences to ensure species specificity.

Experimental Protocol for qRT-PCR

For qRT-PCR analysis, cells were "pre-conditioned" on MGM media as described under the *Biofilm Assays* section. Cells were pelleted and re-suspended in fresh MGM plus the treatment. Co-inoculations were combined as Rm8530 OD_{600} = 0.8 and UD1022 OD_{600} = 0.2. Luteolin treatments contained a final concentration of 5 μM luteolin. Treatments were grown shaking at 30°C, and 1.5 ml samples were collected at time points of 12 and 24 h, centrifuged, decanted, and flash frozen in liquid nitrogen. RNA was isolated using NucleoSpin® RNA from Macherey-Nagel (Düren, Germany). cDNA was generated with 500 ng of RNA using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems[2] and qPCR was performed using PerfeCTa® SYBR® Green SuperMix, ROX, Quanta Biosciences (Gaithersburg, MD), and run on Eppendorf Mastercycler®[3] ep *realplex*[2]. Experiments were repeated three times.

Expression Analysis of qRT-PCR

The relative change in gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method as described in Schmittgen and Livak (2008), which calculates the expression of the gene of interest relative to the internal control in the treated sample compared with the untreated control. The internal control gene for Rm8530 is *rpo*E1. Genes were considered to be differentially expressed if the fold change in expression was ≥2 or ≤ -2.

AHL Biosensor Assays for QQ Analysis

Preparation of the AHL biosensor *Agrobacterium tumefaciens* KYC55 was as described in Joelsson and Zhu (2005) with modifications. KYC55 pre-induced cells were inoculated 1:1,000 into MGM medium for X-Gal soft agar 6-well plates. Pre-induced KYC55 cells were made as described in Joelsson and Zhu (2005). Soft agar plates were treated the same day they were poured. UD1022 was inoculated from a fresh plate streaked from glycerol stock into TYC and grown shaking 30°C for 5 h to OD_{600} = 1.5, then sub-cultured 1:100 to MGM media and grown shaking 30°C for 20 h to OD_{600} = 0.5. Treatments were made using these cultures mixed into sterile

[1]https://www.genscript.com/ssl-bin/app/primer

[2]www.appliedbiosystems.com

[3]www.eppendorf.com

micro-centrifuge tubes with standard C8-AHL and 3-oxo-C16-AHL to a final concentration of 10 μM in a volume of 200 μl. Controls contained standard AHL only. Treatments were incubated shaking 30°C for 24 h. Samples were then centrifuged at 16,000 × *g* for 10 min at 4°C. Supernatants were transferred to new sterile tubes and sterilized open in a biosafety cabinet under UV light for 30 min. Two microliters of treatments was applied to KYC55 X-Gal soft agar six-well plates and allowed to dry. Two treatment replicates were included on two separate six-well plates. AHL biosensor assay was repeated twice.

Sequence Homology and Alignment

The FASTA protein sequence YtnP protein in *B. subtilis* subsp. *subtilis* str. 168 (sequence NP_390867.1) was queried using tblastn search translated nucleotide databases using a protein query for *B. subtilis* UD1022 nucleotide reference sequence (NZ_CP011534.1). The protein sequence for UD10222 YtnP has 281 amino acids and has a molecular weight of 31.8 kDa. The alignment of UD1022 YtnP, AiiA, and other MBL sequences was performed in MEGA (Tamura et al., 2011) by using the software MUSCLE (Edgar, 2004).

Construction of YtnP Mutant

The *ytnP* gene disruption *B. subtilis* subsp. *subtilis* trpC2 *ytnP*::*erm* (Koo et al., 2017) was obtained from the Bacillus Genetic Stock Center and transferred into *B. subtilis* UD1022 by SPP1 phage transduction (Yasbin and Young, 1974).

YtnP Protein Expression and Purification

The *B. subtilis* UD1022 *ytnP* specific sequence was submitted to University of North Carolina School of Medicine Center for Structural Biology (NIH grant P30CA016086) for protein expression and purification. Workers sent the sequence to GenScript for gene synthesis and subcloning into a pET expression vector that contains an N-terminal His tag followed by a TEV site for tag removal during purification [pET-28a(+)-TEV]. The *ytnP*::*E. coli* construct expression was done at UNC using their autoinduction expression system. Purification was performed using a Ni-affinity step, TEV protease tag removal, subtractive Ni-affinity step to separate out the tag, and size-exclusion chromatography to remove potential protein contaminants.

RESULTS

Co-inoculation of UD1022 and Rm8530 Do Not Synergistically Promote Plant Growth

M. truncatula plants were co-inoculated with *B. subtilis* UD1022 and *S. meliloti* Rm8530 6 days after germination and analyzed 7 weeks after inoculation for biomass and nodulation. Though the co-inoculation of Rm8530 and UD1022 resulted in no statistical difference in shoot biomass (**Figure 1A**, $p = 0.06$), there was a slight decrease in observable shoot growth (**Figure 1C**). There was no statistical difference in nodule numbers (**Figure 1B**, $p = 0.59$) between co-inoculated plants from those inoculated with Rm8530 alone. These results indicate that the addition of the PGPR UD1022 to the symbiotic strain Rm8530 did not increase plant health, contrary to results from similar studies (Fox et al., 2011; Morel et al., 2015). We speculated that the lack of growth promotion by the co-inoculation may be due to the antagonistic activity of UD1022 against Rm8530. A standard cross-streak compatibility assay on solid media determined no direct growth inhibitory effect between the two bacteria (**Supplementary Figure S1**).

UD1022 Interacts Indirectly With Rm8530 by Interfering With Rm8530 Biofilm and QS

UD1022 had no observable direct effects on Rm8530 growth; consequently, treatments of UD1022 culture filtrate supernatant (CFS) were tested for indirect influence on the Rm8530 functional phenotype of biofilm production. Biofilm formation by the symbiotic Rm8530 strain is required for efficient nodulation (González et al., 1996) and was evaluated by the semi-quantitative O'Toole assays (O'Toole et al., 2000) in treatments with UD1022 CFS. Biofilm of Rm8530 cultured with 5% by volume of UD1022 CFS was significantly reduced from that of control (**Figure 2**, $p < 0.0001$). Growth of Rm8530 with heat-treated CFS treatment resulted in restoration of control quantities of biofilm (**Figure 2**, $p = 0.86$), suggesting that the active factor of UD1022 CFS may be a heat-unstable molecule such as a protein.

UD1022 Affects Rm8530 QS-Controlled Biofilm Gene Expression

The relative expression of two key Rm8530 QS genes were measured in response to co-culture with UD1022 CFS and in co-culture with live UD1022 cells using qRT-PCR. Rm8530 *sinI* relative gene expression increased by 4-fold and *wggR* relative gene expression decreased by nearly threefold in treatments grown with UD1022 (**Figure 3**). These UD1022 live-cell co-culture qRT-PCR results reflected the same trend of expression as observed in the GFP gene expression reporter assays treated with UD1022 CFS (**Figure 4**): upregulation of *sinI* and downregulation of *wggR*. Treatments with the *M. truncatula*-specific flavonoid luteolin (Peters et al., 1986) were included in the qRT-PCR expression analysis to evaluate possible plant host role in the interaction of the bacteria. Luteolin induces *nod* gene expression in *S. meliloti*, an important initial signaling mechanism to initiate legume–bacteria symbiosis. Rm8530 QS gene expression, as expected was not directly affected by the presence of luteolin alone. However, the presence of luteolin in Rm8530–UD1022 co-culture significantly enhanced the gene expression changes observed in bacteria co-cultures. The increase in *sinI* relative expression doubled to nearly 8-fold and *wggR* decreased expression was extended to 3.4-fold (**Figure 3**). This could indicate that, in the rhizosphere, plant signaling factors such as flavonoids may exacerbate the PGPR interactions causing the changes in QS gene expression.

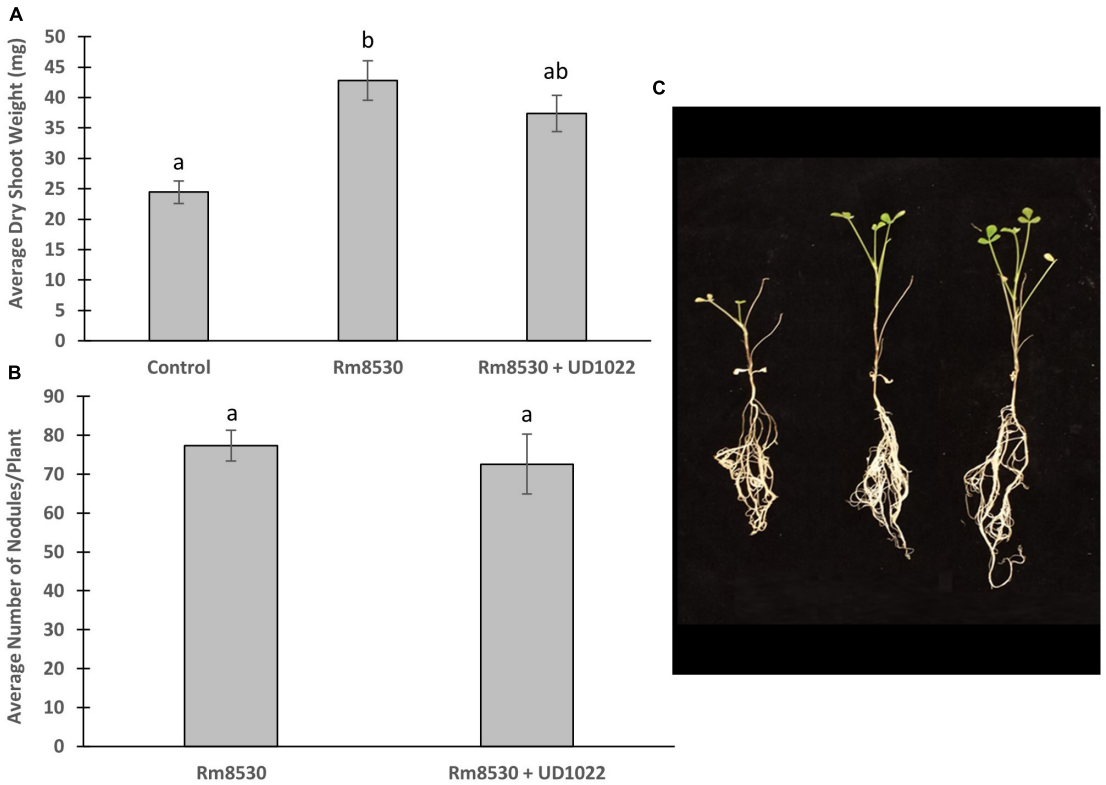

FIGURE 1 | Co-inoculated plant growth and nodulation. **(A)** Average plant dry weight of Rm8530 treated control plants and co-inoculated Rm8530 & UD1022 plants did not differ statistically (*p*-value of 0.06). **(B)** There was no statistical difference between average counts of nodules between treatments (*p*-value of 0.59). **(C)** Overall plant growth of both treatments was greater than control (first plant), but no differences were observed between Rm8530 treatment (second plant) and Rm8530 & UD1022 co-inoculation (third plant).

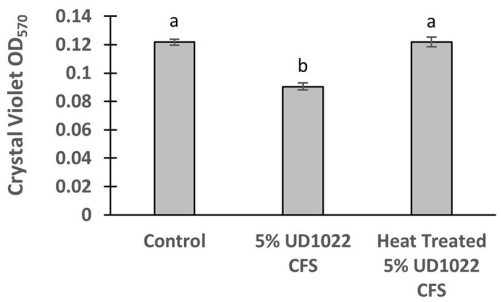

FIGURE 2 | Rm8530 biofilm formation assay. Treatment with 5% UD1022 CFS significantly reduced the formation of biofilm by Rm8530 (*p*-value of <0.0001). Treatment with 'heat treated' UD1022 CFS showed no significant difference compared to the control (*p*-value of 0.86).

UD1022 Is Positive for QQ Activity Against Rm8530

The Rm8530 QS gene expression response patterns coupled with the restoration of WT Rm8530 biofilm formation in UD1022 heat treated CFS treatments suggest that UD1022 may be affecting Rm8530 QS through enzymatic activity of a protein. Interference of QS through interspecific enzymes is termed quorum quenching and can be enacted at several levels of QS regulation, including targeting signal biosynthesis, signal receptors, and direct cleavage of QS signal molecules, including AHLs (Fetzner, 2015). QQ enzymes have been characterized in many soil bacteria including *Agrobacterium* and *Bacillus* genera (Chan et al., 2016).

QQ activity of UD1022 was assessed using the bioreporter strain *Agrobacterium tumefaciens* KYC55 to detect a wide range of AHLs and reports through β-galactosidase activity (Zhu et al., 2003). UD1022 cultures were incubated with 10 μM purified *N*-octanoyl-L-homoserine lactone (C8-AHL) or N-3-oxo-hexadecanoyl-L-homoserine lactone (3-oxo-C16-AHL) (Caymen Chemicals) for 24 h on KYC55 X-Gal plates. Treatments of UD122 with 3-oxo-C16-AHL showed significant reduction in detectable AHL signal as compared to AHL only (**Figures 5A,B**), while C8-AHL showed no discernable difference from the control (**Supplementary Figure S2B**). Thus, UD1022 displays QQ activity, which appears to be geared toward long-chain AHLs.

UD1022 QQ Through the Lactonase YtnP Protein

Several classes of bacterial enzymes QQ through inactivating AHLs, including lactonases and acylases (Chan et al., 2016).

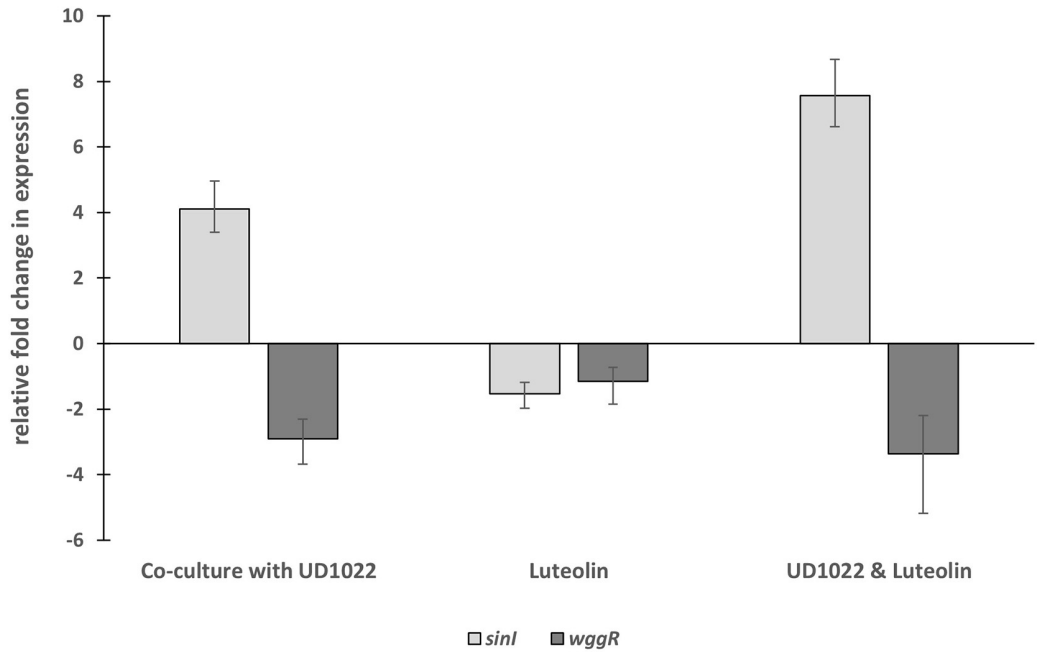

FIGURE 3 | Relative fold changes in expression of Rm8530 *sinI* and *wggR* in co-culture with UD1022. Co-culture with UD1022 increased the relative expression of Rm8530 *sinI* by 4-fold. The presence of luteolin (which represents the condition of Rm8530 upregulating *nod* genes) doubled the effect of UD1022 on Rm8530 *sinI*, increasing expression to nearly 8-fold. Luteolin alone did not meet the threshold of 2-fold change in Rm8530 *sinI* expression. Co-culture with UD1022 decreased the relative expression of Rm8530 *wggR* by 3-fold. The presence of luteolin slightly enhanced the effect of UD1022 on Rm8530 *wggR*, increasing to 3.4-fold. Luteolin alone did not change Rm8530 *wggR* expression. Standard error bars reflect the range of the relative fold change in gene expression in response to the treatment.

A search of the literature for lactonases specifically identified in *B. subtilis* species yielded the putative lactonase YtnP protein in *B. subtilis* NCIB3610 (Schneider et al., 2012). The alignment of YtnP protein sequence with UD1022 returned a 98.44% identity. Using MUSCLE (Edgar, 2004) alignments of the reference protein *B. subtilis* strain 168 YtnP (NP_390867.1) and UD1022 YtnP sequences revealed the hallmark metallohydrolase HXHXDH and HXXGH metal binding motifs as well as a phosphorylated Ser36 residue. To determine if the UD1022 YtnP lactonase protein contributes to the QQ patterns observed, we introduced a *ytnP* deletion cassette in UD1022 ("UD1022 *ytnP*$^{-}$"). In AHL co-incubation assays, UD1022 *ytnP*$^{-}$ treatments with 3-oxo-C16-AHL showed that AHL degradation was less extensive than that of UD1022 WT (**Figure 5C**). It is likely that there are additional QQ active proteins produced by UD1022. Indeed, up to six other probable MBL-like fold sequences having the HXHXDH motif have been identified in UD1022 (data not shown).

The UD1022 specific *ytnP* sequence was submitted to the University of North Carolina School of Medicine Center for Structural Biology (NIH grant P30CA016086) for protein expression and purification. Purified YtnP protein was applied at three different concentrations to 10 μM concentrations of 3-oxo-C16-AHL and C8-AHL. The biosensor reporter showed no degradation of 3-oxo-C16-AHL with treatment of 5 μg/ml YtnP (**Figure 5D**). Long-chain AHL degradation comparable to UD1022 WT live cell treatments was observed with 50 μg/ml YtnP incubation (**Figure 5E**). Treatments of 500 μg/ml YtnP completely abolished detectable levels of 3-oxo C16-AHL (**Figure 5F**). Incubation of UD1022 YtnP with C8-AHL, interestingly, resulted in degradation of the short-chain AHL at 50 and 500 μg/ml (**Supplementary Figures S2E,F**, respectively). This demonstrates unequivocally that UD1022 QQ activity is carried out through the YtnP lactonase protein.

DISCUSSION

Understanding interactions of PGPR in consortia is critical for predicting rhizo-microbiome function in the environment and in agroecosystems. This is especially relevant as biological-based crop solutions become more widely marketed and adopted. Several examples of PGPR co-inoculations using *S. meliloti* resulting in significant improvements of *Medicago* spp. plant growth have been reported. Co-inoculation of *Delftia* spp. JD2, a diazotrophic, IAA-producing PGPR, with *S. meliloti* U143 onto *M. sativa* increased nodulation (Morel et al., 2011) and increased shoot and root dry weights by 13 and 34%, respectively (Morel et al., 2015). Fox et al. (2011) found dual inoculation of *S. meliloti* WSM419 and the PGPR *Pseudomonas fluroescens* WSM3457 onto *M. truncatula* enhanced nodule initiation rates, resulting in increased number of crown nodules and more overall N accumulation. *S. meliloti* B399, a commercial alfalfa inoculant closely related to strain Sm1021, co-inoculated with *Pseudomonas* spp. FM7d nearly doubled shoot dry weight and increased nodule number on

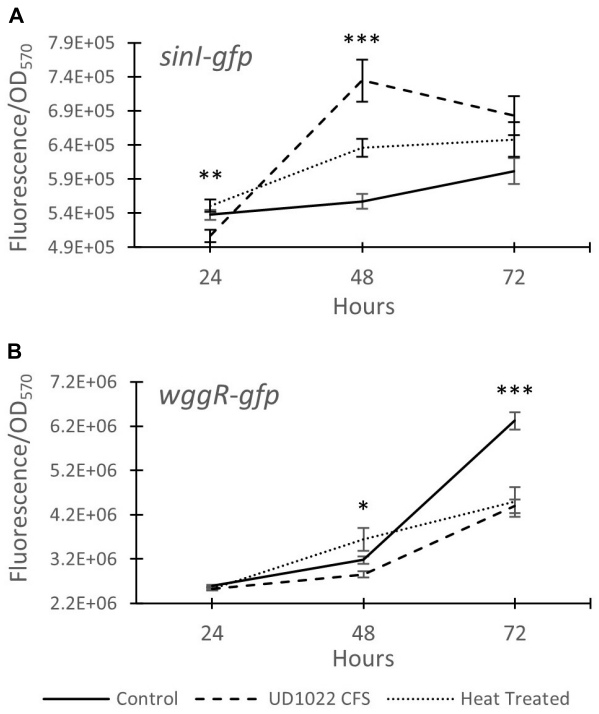

FIGURE 4 | Expression of Rm8530 quorum sensing genes. **(A)** Average GFP activity (fluorescence/OD_{570}) of the *sinI-gfp* fusion reporter. UD1022 treatment was significantly different from control and heat treatments at 24 hours. The differences between treatments at 48 hours is significant at *p*-value of <0.0001. **(B)** Average GFP activity of *wggR-gfp* fusion reporter. The differences between the treatments and the control at 48 hours is significant at *p*-value of 0.04, and 72 hours is significant with *p*-value of <0.0001. Averages for both assays are from 29 technical replicates and the bars at each time point present standard error. *indicates $p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$.

M. sativa L. cv Bárbara SP (INTA Manfredi). Though co-inoculation of B399 with *Bacillus* spp. M7c had significantly higher shoot dry weight, it did not increase nodule number (Guiñazú et al., 2010).

However, not every instance of PGPR dual inoculation with *S. meliloti* has been reported to be beneficial. The dual inoculation of the PGPR *B. simplex* 30N-5 with *S. meliloti* 1021 onto *M. truncatula* resulted in no significant difference in shoot height, plant dry weight or nodule number over that of *S. meliloti* 1021 control (Maymon et al., 2015). This contrasted with their previous work, which showed beneficial growth effects of *B. simplex* 30N-5 when co-inoculated with *Rhizobium leguminosarum* bv. *viciae* 128C53 onto pea (*Pisum sativum*) (Schwartz et al., 2013). Our study using the *expR+ S. meliloti* strain Rm8530 co-inoculated with *B. subtilis* UD1022 also resulted in no significant enhancement of plant growth or nodule number. While other work has yet to query the mechanisms of bacterial interaction, which may account for the non-synergistic plant effects of these rhizobia-PGPR co-inoculations, this work reveals a potential, indirect mechanism of bacterial interaction.

Biofilm formation is important in soil and root-associated bacteria for motility and exchange of signals and metabolites (Angus and Hirsch, 2013; Bogino et al., 2013; Amaya-Gómez et al., 2015). *S. meliloti* biofilms have been shown to play a critical role in motility toward and initiation of nodulation with the *Medicago* spp. plant root (González et al., 1996; Pellock et al., 2000; Hoang et al., 2008). Here, we used Rm8530 biofilm formation as a functional reporter for negative activity by UD1022 and found clear evidence of UD1022 inhibition of Rm8530 biofilm formation. *S. meliloti* biofilm formation is dependent on an intact ExpR/SinI QS system, which is well described for both strains Rm1021 and its ExpR+ relative Rm8530. Importantly, the Rm8530 QS system has been shown to regulate a key symbiotically active component of their biofilms, the low-molecular-weight galactoglucans referred to as EPS II (Rinaudi and Gonzalez, 2009).

Based on the negative effect of UD1022 on Rm8530 biofilm, we hypothesized that UD1022 may be interfering with the QS-controlled molecular regulation of biofilm production. The Rm8530 QS genes *sinI* and *wggR* were selected to test the effect of UD1022 on the QS pathway, including upstream QS signal molecule synthesis (*sinI*) and downstream EPS II polymerization (*wggR*). Using UD1022 CFS treatments on Rm8530-*gfp* expression reporters and subsequent validation with qRT-PCR of live-cell co-cultures, we found that UD1022 significantly activated *sinI* transcription and reduced *wggR* transcription. McIntosh et al. (2009) described that *sinI*-promoter activation occurs at nearly 10-fold lower levels of AHLs than required for its downregulation. WggR activation requires the presence of the transcriptional regulator ExpR and the SinI-specific AHLs $C_{16:1}$-AHL and oxo-$C_{16:1}$-AHL (McIntosh et al., 2009; Gao et al., 2012). The *wggR-gpf* reporter in Rm8530 *sinI* background was more sensitive to $C_{16:1}$-AHL than 3-oxo-$C_{16:1}$-AHL. Expression of *wggR* increased in a dose-dependent manner with close to WT levels at 40–1500 nM $C_{16:1}$-AHL and 200–1,500 nM oxo-$C_{16:1}$-AHL (Gao et al., 2012). Consequently, UD1022 treatment appeared to mimic an expression pattern of the QS genes similar to their response to low AHL signal molecule concentration conditions.

The regulatory network of *S. meliloti* ExpR/SinI is intricately controlled through AHL acyl chain length, acyl chain substitutions, and concentration of AHL molecules (Bartels et al., 2007; Calatrava-Morales et al., 2018). Gene expression for *sinI* synthase is positively regulated by low concentrations of AHLs (1–40 nM) and negatively regulated by high concentrations of AHLs (>40 nM), allowing the ExpR transcriptional regulator of *sinI* to be sensitive to the AHL substrate it is responsible for producing (Baumgardt et al., 2014). The expression of *wggR* is oppositely regulated, requiring upwards of 150 nM $C_{16:1}$ -AHL for increased *wggR* GFP expression reporter activity (Gao et al., 2012). Lower concentration of AHLs in UD1022 treatments would support the patterns of increased *sinI* and decreased *wggR* expression (schematic in **Figure 6**). QQ activities could be promising as a prospective tool to improve plant health and bypass antibiotic resistance in the development of biological products combating plant pathogens (Grandclément et al., 2016; Rodríguez et al., 2020). Many screening techniques have

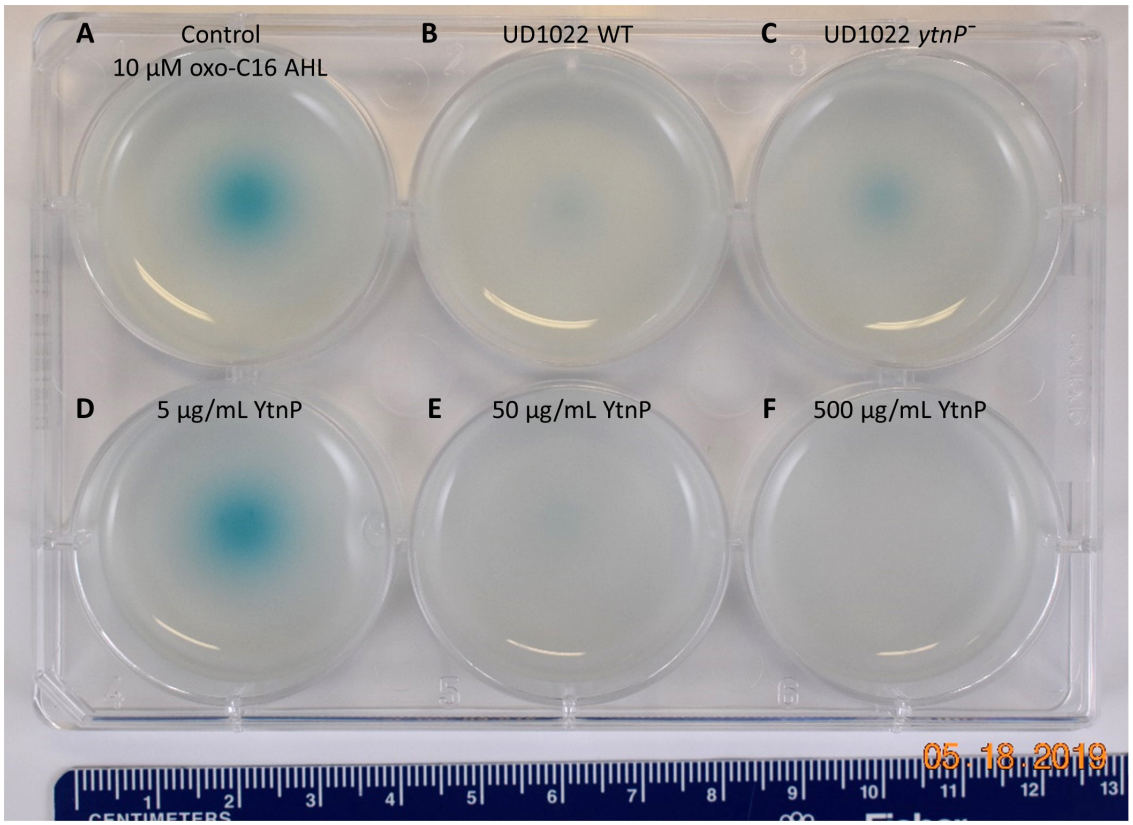

FIGURE 5 | UD1022 quorum quenching biosensor assay plate. The biosensor KYC55-X-gal soft agar plate treated with UD1022-AHL co-cultures. From top left across **(A)** control treatments of standard AHLs with no UD1022. **(B)** QQ activity of UD1022 culture with 3-oxo-C16-AHL **(C)** UD1022 *ytnP*$^{-}$ mutant cultured with AHL **(D)** 5 µg/mL pure UD1022 YtnP protein incubated with AHL, **(E)** 50 µg/mL YtnP protein, **(F)** 500 µg/mL YtnP protein. *Brightness of image increased by 20%, which did not increase pigment intensity or saturation.

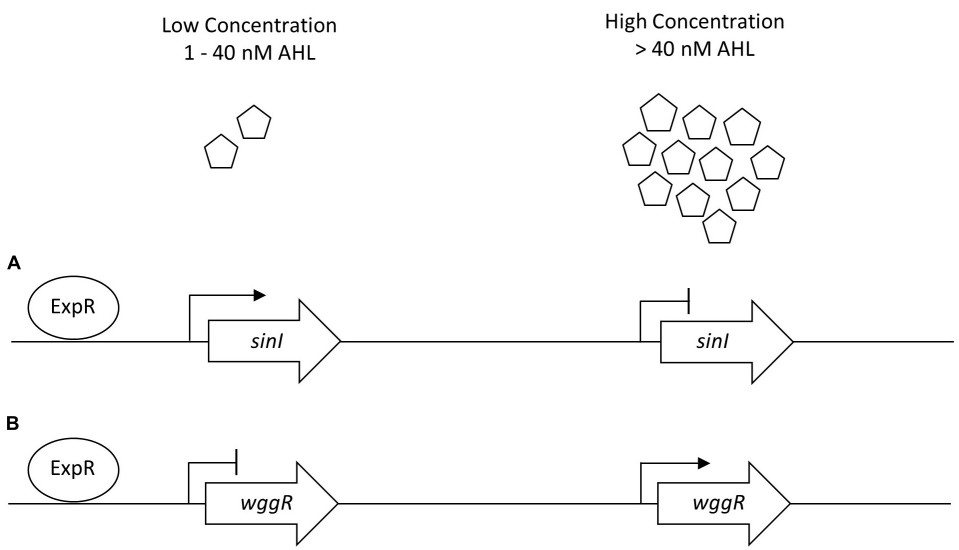

FIGURE 6 | Generalized *sinI*/ExpR quorum sensing transcription model for *sinI* and *wggR*. Expression of genes are controlled on multiple levels including length of AHL chain and AHL concentration. **(A)** Low concentrations of AHL (1–40 nM) upregulate *sinI* expression in a positive feedback regulation. **(B)** Low concentrations of AHLs downregulate *wggR* expression. Adapted from Baumgardt et al. (2014).

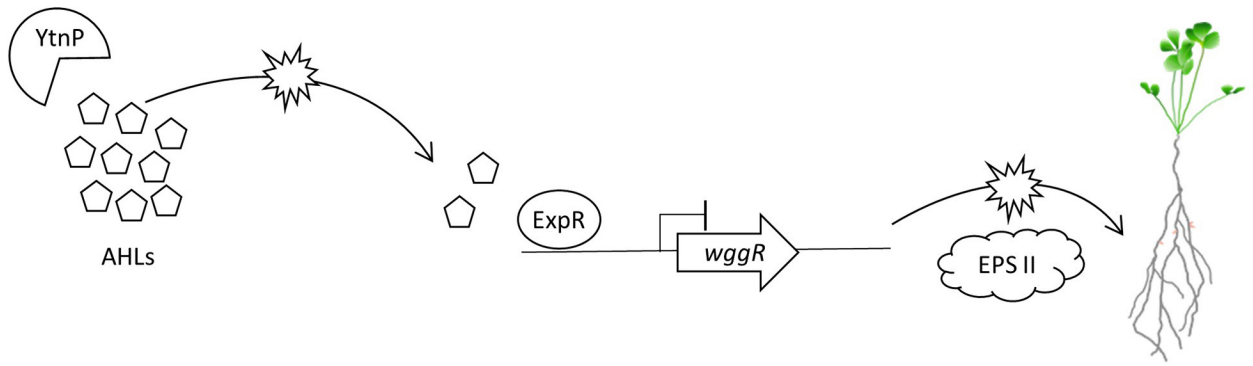

FIGURE 7 | Model of proposed molecular QS and QQ interactions between *M. truncatula* PGPRs. *B. subtilis* UD1022 produces the lactonase YtnP which cleaves *S. meliloti* Rm8530 AHLs. Through quorum quenching, UD1022 YtnP reduces AHL concentrations, inhibiting the upregulation of symbiotically active EPS II genes. This may result in lower nodulation efficiency of Rm8530 in the presence of the PGPR UD1022.

been utilized to identify QQ microbial isolates for this purpose (Tang et al., 2013; Last et al., 2016; Stein and Schikora, 2018). To better understand the capacity of UD1022 for QQ, we used the bioreporter strain *Agrobacterium tumefaciens* KYC55 in soft agar to detect both short- and long-chain AHLs. When co-cultures of UD1022 were incubated with 10 μM purified 3-oxo-C16-AHL for 24 h and applied to the bioreporter, expression of KYC55 β-galactosidase was greatly diminished. This reduction of detectable long-chain AHL demonstrates that UD1022 is capable of QQ activity. Response of the bioreporter to co-cultures of UD1022 with C8-HSL was no different from control treatments.

Many modes of QQ by bacteria have been identified with lactonase hydrolytic enzymes being highly described in *Bacillus* spp. (Kumar et al., 2015). The *B. subtilis* NCIB3610 putative lactonase YtnP protein sequence had high similarity to that found in UD1022 (Schneider et al., 2012). The YtnP lactonase is a metallolactamase and was found to target γ-butyrolactone of *Streptomyces griseus*. The UD1022 YtnP protein possesses the same hallmark metallohydrolase features of the NCIB3610 YtnP, including the HXHXDH motif, indicating that UD1022 YtnP is also a likely a QQ lactonase. The UD1022 *ytnP*$^{-}$ mutant was employed in the AHL biosensor assay to test the role of the specific YtnP QQ activity. Rather than fully abolishing QQ, the partial degradation activity remaining may be due to redundant or multiple lactonase-like genes that continue to be expressed in the single *ytnP* mutant. The purified YtnP protein incubated with long- and short-chain AHLs showed clear and efficient QQ activity. Exogenous application of pure YtnP degraded C8-AHL, which was not observed in UD1022 live-cell assays. The possible substrate specificity of the YtnP lactonase protein may be more attributable to enzyme concentration rather than on acyl-chain length of the AHL. Lactonases characterized to date are described as having broad activity against a range of AHL acyl chain lengths and substitutions, though with variable active site affinities (Bergonzi et al., 2018).

QQ in the rhizosphere likely plays a large part in PGPR interactions and, consequently, in plant health outcomes. The presence of AHL molecules in the rhizosphere have been shown to directly elicit functional and beneficial responses from both non-legumes and legumes (Hartmann et al., 2014; Schikora et al., 2016; Hartmann and Rothballer, 2017). Several studies have employed the use of bacterial QQ lactonases to demonstrate the direct and indirect beneficial activities of AHLs and QS on plants. To identify novel QS-controlled proteins, an *S. meliloti* 1021 construct expressing the QQ lactonase AiiA was found to be significantly deficient in forming nodule initials within the first 12 h after inoculation (Gao et al., 2007). Zarkani et al. (2013) showed that *S. meliloti* producing 3-oxo-C14-AHL increased *Arabidopsis thaliana* resistance to *Pseudomonas syringe* pv *tomato* DC3000, while mutants heterologously expressing the *Agrobacterium tumefaciens* AttM lactonase did not.

The Rm8530 QS system is required to produce symbiotically active EPS II biofilms (Pellock et al., 2000, 2002; Hoang et al., 2004; Gurich and González, 2009). QS is also important in controlling bacterial cell population density, motility toward the plant root, and switching expression pathways from motility to nodulation (Bahlawane et al., 2008; Gao et al., 2012; Calatrava-Morales et al., 2018). The timing and coordination of these activities are intricately controlled through ExpR/SinI QS, and disruptions or interference through QQ has the potential to affect the efficiency and competency of these pathways. The lack of synergistic effects between Rm8530 and UD1022 may be explained, in part, through the QQ activity of UD1022 YtnP lactonase reducing Rm8530 AHL signal molecule concentration, leading to reduced expression of EPS II biosynthesis genes including *wggR*, and ultimately resulting in inhibition of efficient nodule initiation on *M. truncatula* roots (**Figure 7**).

CONCLUSION

We show in our tri-trophic legume–symbiont–PGPR model system that the PGPR *B. subtilis* UD1022 does not synergistically

increase *M. truncatula* plant growth or nodulation by the legume symbiont *S. meliloti* Rm8530. Though there is no direct growth inhibitory effect between the bacterial strains, indirect interactions contribute to the disruption of plant associative activities by the symbiont. UD1022 affects Rm8530 QS controlled biofilm formation through interference with the QS biosynthesis pathway. Further, UD1022 expresses the QQ lactonase YtnP, which cleaves the specific AHLs required to produce symbiotically active EPS II of Rm8530 biofilms. UD1022 likely delays or fails to promote Rm8530 nodulation through the QQ activity of lactonase YtnP and can inhibit synergistic plant growth promotion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AR completed lab work pertaining to microbiology and genetic analysis. AR and HPB conceptualized the idea. PBB designed the experiments for the *Bacillus* mutants. All authors designed the experiments, edited, and contributed to the final manuscript, and approved for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.596299/full#supplementary-material

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Specialized Plant Growth Chamber Designs to Study Complex Rhizosphere Interactions

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The rhizosphere is a dynamic ecosystem shaped by complex interactions between plant roots, soil, microbial communities and other micro- and macro-fauna. Although studied for decades, critical gaps exist in the study of plant roots, the rhizosphere microbiome and the soil system surrounding roots, partly due to the challenges associated with measuring and parsing these spatiotemporal interactions in complex heterogeneous systems such as soil. To overcome the challenges associated with *in situ* study of rhizosphere interactions, specialized plant growth chamber systems have been developed that mimic the natural growth environment. This review discusses the currently available lab-based systems ranging from widely known rhizotrons to other emerging devices designed to allow continuous monitoring and non-destructive sampling of the rhizosphere ecosystems in real-time throughout the developmental stages of a plant. We categorize them based on the major rhizosphere processes it addresses and identify their unique challenges as well as advantages. We find that while some design elements are shared among different systems (e.g., size exclusion membranes), most of the systems are bespoke and speaks to the intricacies and specialization involved in unraveling the details of rhizosphere processes. We also discuss what we describe as the next generation of growth chamber employing the latest technology as well as the current barriers they face. We conclude with a perspective on the current knowledge gaps in the rhizosphere which can be filled by innovative chamber designs.

Keywords: rhizosphere, interactions, plant growth chamber, soil, chamber design

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INTRODUCTION

Roots are not only vital for anchorage and for acquisition of water and nutrients from the soil, but are also engaged in complex physical and chemical interactions with the soil. Plant roots release approximately 11–40% of their photosynthetically fixed carbon, commonly known as root exudates, into the soil (Sasse et al., 2018; Zhalnina et al., 2018a). Root exudates and mucilage act as nutrient sources and as signaling molecules for soil microorganisms, thus shaping the microbial community

in the immediate vicinity of the root system (Venturi and Keel, 2016). In turn, microbial processes promote plant growth by aiding in nutrient acquisition, plant growth hormone production and bio-control of plant pathogens (Afzal et al., 2019). The physicochemical characteristics of the surrounding soil are also affected by interactions between roots and the microbial community. This interplay between the different rhizosphere components is affected by spatio-temporal processes, which culminates in dynamic feedback loops that maintain the complex rhizosphere environment with physical, chemical and biological gradients that are distinct from the bulk soil (Six et al., 2004; Koebernick et al., 2017). Understanding these intricate rhizosphere relationships is vital in devising strategies to increase plant productivity and comprehend localized biogeochemical processes.

In many rhizosphere studies, the use of pots and containers is predominant as it allows the plants to be cultivated under controlled conditions and at low cost. Compared to field studies, growth of plants in defined spaces (e.g., pots) also offers advantages in ease of handling, monitoring and sampling (Neumann et al., 2009). Much of what we know of the rhizosphere microbiome has resulted from such pot-grown plants. However, since the rhizosphere and roots are still out of view in the soil, destructive sampling of the root is required prior to analysis. Destructive sampling may result in the loss of three-dimensional (3D) spatial information on rhizosphere processes over time, which is increasingly being recognized as a critical parameter.

On the other hand, soil free techniques such as hydroponics and aeroponics can provide visual access to the rhizosphere circumventing the need for destructive sampling. Other alternatives are gel-based substrates which can maintain rhizosphere transparency as well as the 3D architecture of roots and have been applied successfully in high throughput imaging, phenotyping and trait mapping platforms (Iyer-Pascuzzi et al., 2010; Topp et al., 2013). Nonetheless, the root phenotype and traits of plants grown under soil-free conditions are known to differ from those of soil-grown plants (Kuijken et al., 2015). These soil substitutes do not also accurately simulate the heterogeneous nature of soil aggregates, thus complicating extrapolations for field relevance. Sophisticated imaging approaches such as magnetic resonance imaging (Metzner et al., 2014; Popova et al., 2016; van Dusschoten et al., 2016) and X-ray computer tomography (Mooney et al., 2012; Helliwell et al., 2013) can be used to analyze root systems in the soil with minimal disturbance but they are low throughput, expensive and may not be easily accessible (Oburger and Schmidt, 2016; Morris et al., 2017). It is apparent that structural changes in design catered to solving specific challenges in the rhizosphere are indeed necessary.

To overcome these challenges relating to the rhizosphere in soil, specialized plant growth chamber systems have been designed, and successful implementation has led to multiple variations of similar designs. These specialized systems often have a visible rhizosphere which enables coupling with other technologies thereby increasing the breadth of experimental techniques applicable to the rhizosphere system. This review discusses representative growth chamber systems designed to study major rhizosphere processes and interactions in soil. Growth platforms resembling conventional containers such as pots and tubes are not covered. Specifically, the reviewed growth systems are selected based on the following criteria: (1) the growth chamber is amenable for use with soil/soil-like substrates (e.g., vermiculite or sand) and therefore, hydroponics, aeroponics and agar/gel-based systems are not discussed except in microfluidic-based platforms, (2) it is built with the intention to maintain growth of the plant and has architectural features distinct from conventional pots, and lastly (3) it is able to be set up in a laboratory; i.e., field measurement systems and observation platforms are not included. For instance, a minirhizotron, consisting of a camera mounted in a glass tube submerged in the soil which provides non-destructive root imaging over time (Taylor et al., 1990) will not be discussed as it is out of the scope of this review. Through our assessment of lab-based chamber systems, we identify unique advantages and challenges associated with each system (**Table 1**). We hope that future fabrication designs can benefit and improve on designs that work well. Lastly, we offer our perspectives on areas in which technological advances are needed to fill current knowledge gaps.

SPECIALIZED CHAMBERS TO STUDY MAJOR RHIZOSPHERE INTERACTIONS

In studying rhizosphere processes, the myriad of complex interactions among members of the rhizosphere are often dissected to two interacting variables such as root-and-soil or root-and-microbes, etc. Each of these interactions inherently operates under distinct parameters and requires specifically designed platforms to effectively answer different research questions. This review is structured in a way that first describes each rhizosphere process briefly and then reports on the specific growth chamber systems designed to facilitate experiments for answering related research questions. The major rhizosphere processes discussed below include root system architecture, physicochemical gradients in the soil, exudation patterns by the roots and interactions between roots and nematodes, fungi or bacteria.

Investigating Root System Architecture

Root system architecture (RSA) encompasses structural features that provide spatial configuration such as root length, width, spread and number (Khan et al., 2016; **Figure 1**) and is an important rhizosphere parameter in regulating soil porosity, and nutrient and water uptake efficiency by plants (Helliwell et al., 2017; Fang et al., 2019). Plants have been observed to "sense" and direct root growth toward nutrient sources in soil, and the RSA of a plant exhibits great malleability in response to environmental stimuli (changes in nutrients, pH, soil moisture, and temperature) which in turn, influences microbial communities (Bao et al., 2014; Saleem et al., 2018). For instance, bean plants grew deeper roots under drought conditions to enhance water foraging capabilities while low phosphate (P) conditions stimulated the formation of dense lateral roots involved in P uptake from upper soil layers (Ho et al., 2005).

TABLE 1 | Key attributes of different growth chambers designed to study rhizosphere processes and interactions.

Growth chamber setup	Basic design principles	Advantages	Disadvantages	Experimental scale	Tested rhizosphere processes	References
Rhizotron/ rhizobox setup	- Chamber built with two sheets often made of PVC or acrylic, of which at least one sheet is transparent and/or removable. - Many chamber designs are based of this basic set up	- Versatile and easy set up. - Allows visualization of the rhizosphere. - Can be coupled to many visualization techniques.	- Information limited to 2D plane. - Loss of information on roots occluded by soil particles.	cm to m	All major rhizosphere processes possible.	Devienne-Barret et al., 2006; Neumann et al., 2009; Bontpart et al., 2020
Rhizobox with side-compartment	- A side chamber is built into a basic rhizobox connected via a controlled aperture. - Rhizosphere visualization is on the side chamber.	- Allows isolation of individual roots via controlled root growth through the aperture. - Easy differentiation of old vs. new roots	- Root growth into side compartment only controlled via timing of aperture opening. - Loss of information on roots occluded by soil particles.	cm	Bacterial interactions	Jaeger et al., 1999; Nuccio et al., 2020
Vertical root mat chambers	- Root growth is restricted from the soil through a size-selected membrane; root hairs and solutes move freely through the membrane. - Can maintain full plant growth or act as secondary container for root only growth	- Allows visualization of the whole root system.	- Unnatural root growth in complete 2D plane	mm to cm	Exudate collection, Nematodal interactions	Oburger et al., 2013; Dinh et al., 2014
Horizontal root mat in rhizobox	- Particularly used in root exudate collection. - Root growth is restricted by membrane at the bottom of rhizobox; root hairs and solutes move freely through the membrane	- Possibility of root exudates collection into soil or liquid substrate. - Possibility of root growth in soil substrate	- Unnatural rhizosphere environments in high density root mat.- Tangled roots and loss of exudate profiles from individual roots	mm to cm	Exudate collection, Physicochemical gradients in the soil	Chaignon et al., 2002; Chaignon and Hinsinger, 2003
Mycorrhizal compartments	- Rhizobox compartments separated by membranes to restrict movement of roots but not hyphae of mycorrhizal fungi or solutes. - An additional wire net may be placed between compartments to create air gap to restrict solute movement	- Long range (cm) foraging capabilities and connectivity of mycorrhizal hyphae	- Visualization of mycorrhizal hyphae not possible	cm	Fungal interactions	Tanaka and Yano, 2005; Kaiser et al., 2015; Wang et al., 2016
Split-root systems	- A physical barrier separates the roots into generally two compartments under different conditions. - Developed roots may be manually split into the compartments or new roots may be directed to grow into the different compartments, often achieved after excising parts of the root	- Enables investigations of the systemic response of plants. - Applicable in non-specialized containers such as pots	- Root damage during split-root transplant.- Cut roots show lower survival rates	cm	Systemic response of plants to rhizosphere processes	Agapit et al., 2020; Saiz-Fernández et al., 2021

(Continued)

TABLE 1 | Continued

Growth chamber setup	Basic design principles	Advantages	Disadvantages	Experimental scale	Tested rhizosphere processes	References
Nylon soil pouches	- Nylon membranes often made into bags/pouches restrict root growth. - Applicable in conventional pots as well as specialized rhizoboxes	- Accessible and easy separation of root-free soil from the rhizosphere.	- Over-estimation of rhizosphere range.	cm	Bacterial interactions	Yevdokimov et al., 2006; Wei et al., 2019
Microfluidic chambers	- 3D fabricated flow-through device with seedling port and microchannel for primary root growth	- Allows analysis of microscale processes with high spatiotemporal resolution. - Precise control of the reproducible conditions utilizing the laminar flow and automated fluidic operations. - Well integrated with conventional imaging techniques.- Rapid prototype testing	- Small size limits choice of plants and testing time frame to young seedling.- Only hydroponics systems to date.	mm	Major rhizosphere processes in hydroponic conditions	Grossmann et al., 2011; Stanley et al., 2016
EcoPODs	- Enclosed pilot scale ecosystem chambers with multiple built-in equipment and sensors	- Manipulation of various aspects of environmental conditions above and below ground of the plant. - Bridges the gap between lab scale studies to field studies	- Not easily accessible. - Significant financial investment involved. - Requires dedicated maintenance	cm to m	All major rhizosphere processes possible	Ke et al., 2020
EcoFABs	- 3D fabricated flow-through devices designed for the development of model rhizosphere ecosystems	- 3D fabrication allows easy adaptation and modification to the system. - Standardized protocols increases reproducibility. - Rapid prototype testing	- Small size limits choice of plants and testing time frame.- Roots limited to a plane	mm to cm	Microbial interactions demonstrated so far	Gao et al., 2018; Zengler et al., 2019

*The corresponding schematic images for the different chambers are illustrated in **Figure 2**.*

Given that most soils are heterogenous, understanding the RSA of plants becomes critical in improving resource use efficiency and agricultural yields (Ingram and Malamy, 2010; Khan et al., 2016). Often, RSA in pot-grown plants is investigated by excising the roots via mechanical means such as root washing or blowing with compressed air (Judd et al., 2015). These methods are, however, time-consuming, cause inevitable damage of fine root hairs and result in loss of spatial and temporal information (Judd et al., 2015).

An appealing alternative for studying RSA is the use of rhizotrons. Rhizotrons were initially constructed as underground facilities designed for viewing and measuring roots in the field (Klepper and Kaspar, 1994). In the lab, the rhizotron implies a chamber constructed using two vertical sheets with at least one or both of the sheets being transparent and/or removable (**Figure 2A**). This allows repeated visual inspections of individual roots; a feature unachievable with destructive sampling. In some cases, the word "rhizobox" is used for a similar set up although this was first introduced in as compartmentalized systems to separate the root and soil compartments (Kuchenbuch and Jungk, 1982). Rhizotrons/rhizoboxes are often constructed with PVC or acrylic materials and come in many sizes to accommodate different plants with soil or soil-less substrates (Neumann et al., 2009). Root growth and morphology in the rhizotron can be tracked by a variety of methods ranging from manual tracing onto a plastic sheet, using handheld or flatbed scanners to fully automated time-lapse imaging camera systems (Mohamed et al., 2017).

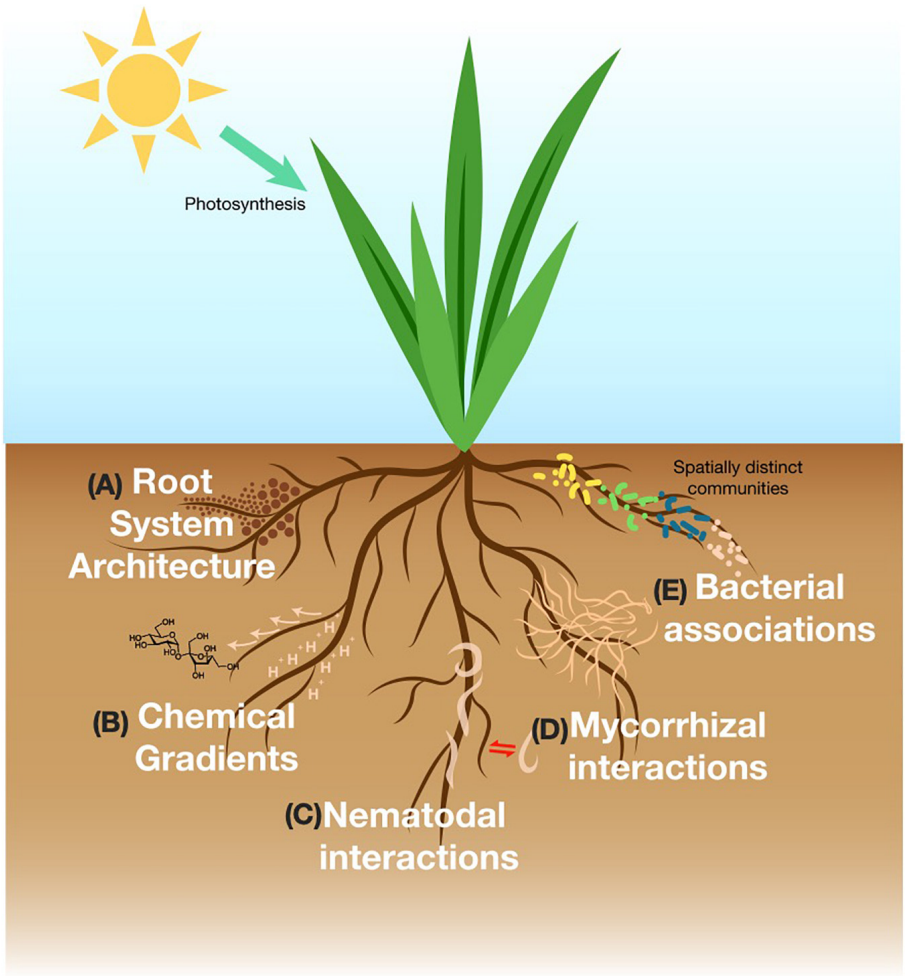

FIGURE 1 | Representative figure of major rhizosphere processes in the soil discussed in this review. **(A)** Root system architecture is concerned with structural features of the root and responds to with environmental stimuli. **(B)** The rhizosphere produces photosynthetically fixed carbon that exudes into the soil and influences soil physicochemical gradients. **(C)** Free-living or parasitic nematodes interact with the rhizosphere via signaling interactions. **(D)** Mycorrhizal fungi create intimate relationships with the roots and engage in nutrient exchange. **(E)** Bacterial composition is distinct upon different parts, age, type of the roots.

Data can be subsequently analyzed with a wide range of software packages (Kuijken et al., 2015). Affordable and robust RSA imaging platforms using rhizotrons have also been developed for increased accessibility in low-income countries (Bontpart et al., 2020).

The versatile construction of a rhizotron design for RSA studies has inspired many variations. For instance, ara-rhizotrons were designed to enable the study of 3D canopy competition with simultaneous root growth observation in an Arabidopsis plant population (Devienne-Barret et al., 2006). The horizontal and radial design of Horhizotron^{TM} and mini-Horhizotron consisting of transparent quadrants attached to a central chamber were developed to study lateral growth of roots in a semi-3D space and to perform post-transplant assessment (Wright and Wright, 2004; Judd et al., 2014). The separated quadrants can also be used with different soil substrates simultaneously to study substrate effects on root growth (Wright and Wright, 2004). A rhizotron fitted with water-tight gasket seals has also been used successfully to investigate the RSA of plants under water-logged conditions (Busch et al., 2006).

Despite the continuous real-time visual read-out, most rhizotron designs suffer from inevitable loss of information from roots occluded by soil particles. The GLO-Roots system overcomes this by imaging from both sides of the rhizotron while using bioluminescent roots to create higher contrast against the soil, enabling quantitative studies on RSA (Rellán-Álvarez et al., 2015). Following advances in engineering and device fabrication, more rhizotron variants adapted to specific plant growth conditions can be envisioned.

Mapping Physicochemical Gradients in the Rhizosphere

In a typical topsoil, approximately half is composed of solid minerals and organic matter while the rest is a fluctuating composition of water and gas filled spaces influenced by

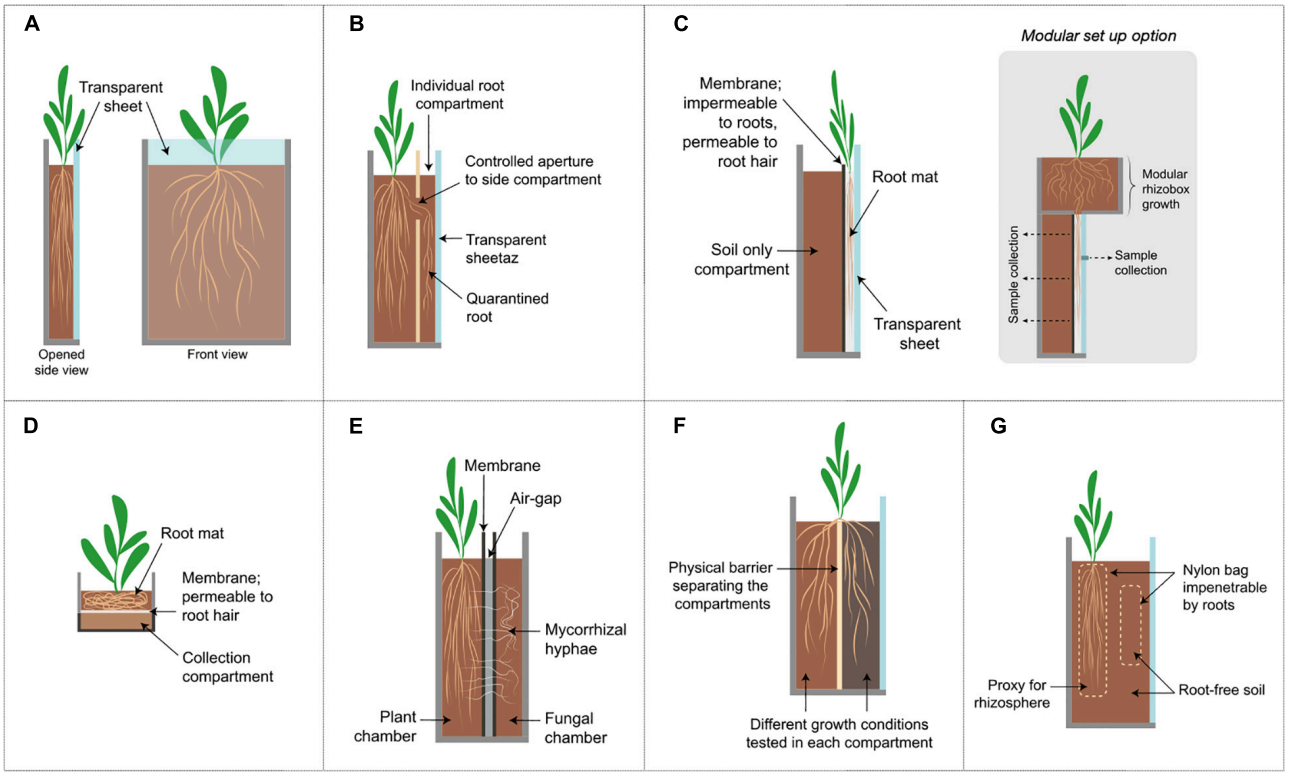

FIGURE 2 | Schematic diagrams of representative growth chamber designs. Further description and characteristics are listed in **Table 1**. **(A)** Rhizotron/Rhizobox set up, **(B)** Rhizobox with side-compartment, **(C)** vertical root mat chambers; a modular option is show where the plant can be pre-grown in a separate compartment and transplanted afterward onto the main examination chamber, inset shows a modular set up option, **(D)** horizontal root mat in rhizobox, **(E)** Mycorrhizal compartments, **(F)** split-root systems shown here in a rhizobox set up; **(G)** Nylon bag to separate root and root-free soil; roots may be restricted in the bag or the soil may be protected from root penetration by the bag.

environmental conditions and uptake/release of solutes from plants (O'Donnell et al., 2007). Changes in gaseous and hydrologic parameters, such as ions, O_2 and moisture among others, create a spatially complex environment that influences microbial communities and overall plant health. These physicochemical fluxes are heterogeneously distributed along roots and vary with root types and zones (Neumann et al., 2009). Often, they exist as gradients in the rhizosphere (Kuzyakov and Razavi, 2019), thus emphasizing the need for non-destructive sampling in order to accurately capture processes occurring at biologically relevant times and scales.

Rhizotron chambers with a visually accessible rhizosphere allows *in situ* and continuous mapping of these gradients in the soil through the use of different types of imaging methods. For instance, photoluminescence-based optical sensors enable *in situ*, repeated detection of small molecule analytes in addition to pH (Blossfeld et al., 2010), O_2 (Frederiksen and Glud, 2006) and NH_4 (Santner et al., 2015). Methods like zymography to detect enzyme activity (Spohn et al., 2013) and diffusive gradients in thin film (Santner et al., 2012; Valentinuzzi et al., 2015) can be used to map solute concentrations in the soil down to sub-mm scales with high spatial resolution more realistically than traditional destructive approaches. For example, transport and distribution of water in the rhizosphere soil has been imaged on both 2D and 3D planes by coupling a rhizotron with neutron radiography and tomography, respectively (Esser et al., 2010) and showed varying moisture gradients along the root system with higher water uptake at the rhizosphere compared to bulk soil. On the other hand, if the rhizotron slabs are thin enough (~4 mm), even simple imaging solutions based on light transmission can be set up to capture water uptake by roots in sand (Garrigues et al., 2006). Despite trade-offs in method sensitivity between these two studies, a rhizotron set up is critical in both designs and illustrates its adaptability to multiple equipment.

Characterizing Root Exudates

Roots exude a substantial amount of photosynthetically fixed organic carbon into the soil consisting of a wide variety of compounds such as sugars, organic acids, and primary and secondary metabolites (Sasse et al., 2018; de la Fuente Cantó et al., 2020). Together with mucilage and border cells (which are mainly expelled from root tips), root exudates provide a major source of nutrients for the rhizosphere microbiome (**Figure 1**). Root exudation is regulated under genetic control (i.e., genotype, root type and developmental stage) (Canarini et al., 2019) as well as in response to environmental conditions in the soil such as nutrient limitations or increase in toxicity (van Dam and Bouwmeester, 2016). Exudate patterns are also

recognized as one of the strongest drivers shaping the rhizosphere microbiome (Dessaux et al., 2016; Zhalnina et al., 2018b; de la Fuente Cantó et al., 2020). As a central player in the rhizosphere ecosystem, it is imperative to understand root exudation patterns to unravel subsequent impacts to the surrounding soil and microbial community.

Improvements in analytical instrumentation have made it possible to move from targeted to untargeted explorations with mass spectrometry to create root exudate fingerprints in its entire complexity (Oburger and Schmidt, 2016). Regardless, the impact of such techniques relies partly on our exudate sampling techniques. Detection of exudates in real-time is difficult due to rapid biotransformation and sorption to the soil matrix. As such, common collection methods rely on root washing in hydroponic systems to overcome complications in the soil matrix and preserve native exudation profiles. However, a comparison between a soil-based collection method and hydroponic methods showed varied responses particularly in amino acid exudation although the underlying cause was not elucidated (Oburger et al., 2013). It is possible that the differing growth conditions between hydroponics and soil, which include differences in gas concentrations, mechanical impedance and microbial spatial composition, can elicit differing root exudation responses to the same environmental stimuli.

Rhizoboxes offer the advantage of localized sampling in soil using sorption media such as paper and membrane filters, compound specific ion exchange binding resin or micro-suction cups placed closed to root zones of interest to collect exudates (Kamh et al., 1999; Neumann et al., 2009, 2014). Moreover, in a rhizobox fitted at the bottom with a porous root-impenetrable membrane, a root mat is allowed to be formed which is then further transferred onto a collection compartment (**Figure 2D**; Chaignon et al., 2002; Chaignon and Hinsinger, 2003). The collection compartment containing soil could then be cut into thin slices (1–3 mm) parallel to the membrane to represent differing distances from the rhizosphere (Neumann et al., 2009). While this approach can be used to investigate exudate release and sorption under soil conditions, the root mat growth generalizes exudate production in terms of the whole root system and occludes spatial exudation patterns. In a hybrid set up by Oburger et al. (2013), the rhizobox is transplanted to a second specialized rhizobox for continued vertical root growth. This specialized rhizobox consists of a nylon membrane (30 μm pore size) close to the transparent side to restrict root growth into the soil except for root hairs (**Figure 2C**). This creates a vertical flat root mat onto which localized exudate samples can be collected. A comparison of this novel set up to conventional collection methods showed that amino acid exudation rates were most varied among the different methods (Oburger et al., 2013), further highlighting the need for specialized chambers.

Nonetheless, successful implementation of these chambers is still limited to fast-growing plants which can form active root mats. The high density of root mats could also lead to unnatural root exudate levels and an overestimation of rhizosphere effects. In addition, care has to be given to the choice of membrane as selective sorption of certain root exudates onto the membrane may also occur (Neumann et al., 2009).

Investigating the Biology and Ecology of Rhizosphere Nematodes

Free-living nematodes are ubiquitous in the soil. They are beneficial to the plants by playing a role in nutrient cycling and in defense against insects and microbial infections through signaling interactions with the roots (Rasmann et al., 2005; Manosalva et al., 2015; **Figure 1**). Conversely, infections by parasitic nematodes in the roots increase the plant's susceptibility to stress and other pathogenic bacteria, fungi, and viruses creating major losses in crop productivity (Powell, 1971; O'Callaghan et al., 2018). With an impending rise in nematode infections due to climate change, understanding nematode behavior and interactions in the rhizosphere becomes important to develop appropriate biocontrol methods to ensure long term food security (O'Callaghan et al., 2018).

Traditional nematode studies are performed in petri dishes with agar or culture media (Dinh et al., 2014; O'Callaghan et al., 2018). However, these substrates do not accurately emulate the physical textures and heterogeneity of soil and create homogenous solute and temperature gradients which could impact nematode behavior and interactions with the roots (Lockery et al., 2008). Indeed, nematode motility speed and dispersal decreased in substrates more closely mimicking sand (Hapca et al., 2007). On the other hand, studying nematode behavior in the soil is a difficult endeavor as its near-transparent body and small size makes it almost indistinguishable from soil particles. Cross-sectioning and staining infected roots make it possible for nematode visualization but they are destructive and provide only static snapshots of cellular changes or nematode behavior during infections (Dinh et al., 2014).

On the other hand, microscopy rhizosphere chambers provide non-invasive detection and observation of nematode activity in the rhizosphere (Froelich et al., 2011; Kooliyottil et al., 2017). The roots in these chambers grow between a glass slide and a nylon membrane (unknown pore size) (**Figure 2C**). The membrane restricts movement of roots except root hairs into the soil while the transparent glass enables microscopy of the roots at high resolution (Froelich et al., 2011). Coupled with fluorescently stained nematodes, microscopy rhizosphere chambers allowed for non-destructive *in situ* observations of nematode infection in its host species over the entire life of the parasite (Dinh et al., 2014; Kooliyottil et al., 2016).

Nonetheless, staining nematodes is an additional challenge as nematode cuticles are impermeable to stains (O'Callaghan et al., 2018). This can, however, be alleviated by using advanced imaging technologies which eliminates the need for staining. A recent study demonstrated live screening of nematode-root interactions in a transparent soil-like substrate through the use of label-free light sheet imaging termed Biospeckle Selective Plane Illumination Microscopy (BSPIM) coupled with Confocal Laser Scanning Microscopy (Downie et al., 2014; O'Callaghan et al., 2018). Using this set up, researchers were able to monitor roots for nematode activity at high resolution and suggest its possible

use in rapid testing of chemical control agents against parasitic nematodes in soil-like conditions (O'Callaghan et al., 2018).

Investigating Soil Fungal Communities

Fungal communities in the rhizosphere are involved in the degradation of organic matter in the soil and subsequent nutrient turnover affecting plant health as well as the microbial community (Buée et al., 2009). Fungal biomass often reaches a third of total microbial biomass carbon (Joergensen, 2000) and almost all terrestrial plants are able to form symbiotic associations with mycorrhizal fungi (Van Der Heijden et al., 2016; **Figure 1**). The majority of these associations are with arbuscular mycorrhiza fungi (AMF) (Smith and Read, 2008) which penetrate into root cortex cells to form highly branched structures (Harrison, 2005). The investment of photosynthetic carbon by plants to AMF is rewarded with increased nutrient availability made possible by the extended hyphal network in the soil. For instance, up to 90% of phosphorus uptake in plants can be contributed by symbiosis with AMF (Ferrol et al., 2019). AMF networks in the soil also influence water retention and soil aggregation further impacting plant growth (Augé, 2004). Moreover, next-generation sequencing technologies and advances in imaging techniques have greatly improved our knowledge on the taxonomical and functional properties of fungal communities in the rhizosphere (Oburger and Schmidt, 2016). However, these methods are optimized for fine scale (millimeter) analysis and are not capable of assessing the foraging capabilities of hyphal networks which can span across centimeter to meter scales.

Toward this end, several researchers have used compartment setups with physical barriers created by 20–37 μm nylon membranes (**Figure 2E**) which restrict movement of roots but not mycorrhizal fungi. This separation creates root-free and plant-free soil compartments connected only by mycorrhizal fungi to examine the transport of various compounds across these compartments. Using this set up, the importance of mycorrhizal fungi in the flow of different elements such as carbon (Kaiser et al., 2015), nitrogen (Tanaka and Yano, 2005) and phosphorus (Wang et al., 2016) between plants, soil and microbes over centimeter distances have been validated. Repeated disruption of the hyphal connections also led to a decreased resistance in plants to drought stress (Zou et al., 2015). The membranes can also be placed horizontally to create different depth gradients to investigate hyphal contributions to water uptake (Ruiz-Lozano and Azcón, 1995). In some studies, an additional 1.5–3 mm air gap is created between two membranes with a wire net to restrict solute movement between two chambers (Tanaka and Yano, 2005; Zhang et al., 2010; Koegel et al., 2013; **Figure 2E**). A common feature of these set ups is the size-exclusion membranes which proved to be critical in distinguishing fungal hyphae processes in the rhizosphere soil.

In addition to AMF interactions, a split root set up, which separates the roots of one plant into halves, can be introduced to investigate the systemic response of plants (**Figure 2F**; Vierheilig et al., 2000). In essence, the split-root system directs the growth of the roots to generally two different growth conditions and enables the investigation of whether a local stimuli (microbial interactions, nutrient limitations, etc.) have a local or global response which can be observed at the root or shoot level (Agapit et al., 2020). Split-root systems are widely studied (Larrainzar et al., 2014; Saiz-Fernández et al., 2021) and have been adapted to rhizoboxes (Zhu and Yao, 2004; Mitchell et al., 2018) as well as to pots and tubes (Kosslak and Bohlool, 1984; Marschner and Baumann, 2003).

Characterizing Bacterial Interactions

In the rhizosphere, plants host a wide diversity of bacteria on the surface of the root (epiphytes) as well as within roots in the vascular tissue (endophytes). Due to its abundance and importance, the bacterial community in the rhizosphere is perhaps the most widely studied among other microbial members in the rhizosphere ecosystem. While the study of endophytic bacteria requires inevitable destructive sampling due to its localization, several non-destructive approaches have been developed to study microbes inhabiting the rhizoplane.

One of the most widely studied plant-microbe interactions in the rhizosphere is that of the symbiotic relationship between legumes and rhizobia (Hirsch et al., 2001). Once a potential nodule forming bacteria is isolated, it is often required to authenticate its nodule forming phenotype by inoculating on host plants. However, conventional methods such as the use of soil pouches do not allow long term incubation, while "Leonard jars," consisting of two stacked glass jars forming the top soil layer and the bottom nutrient solution layer, can be expensive and time consuming (Yates et al., 2016). A recent study challenges this by describing the use of clear plastic CD cases as mini-rhizotrons with potential for use in phenotyping root traits such as legume formation, and demonstrated innovation that democratizes research opportunities in rhizosphere research (Cassidy et al., 2020).

Other microbial interactions in the rhizosphere, however, may not result in visible changes to the root system and often rely on next-generation omics technologies. As such, physical separation of the rhizosphere from the bulk soil becomes paramount in elucidating changes to microbial community and interactions. One approach to this end is the use of nylon bags with differing pore sizes (10–50 μm) (**Figure 2G**). The nylon bag restricts the movement of roots and the soil inside the bag is then regarded as the rhizosphere soil to compare against the surrounding root-free bulk soil (Yevdokimov et al., 2006; Shrestha et al., 2010; Nie et al., 2015). Developing further on this concept, Wei et al. (2019) designed a specialized rhizobox that allowed repeated non-destructive sampling by adding individual nylon bags of root-free soil surrounding the root compartment which are then used as a proxy for the rhizosphere (Wei et al., 2019).

These methods allowed easy distinction of the rhizosphere and the bulk soil but, we now know that the rhizosphere community is not only distinct from the bulk soil but also varies with type, part and age of the root, largely as a consequence of varying root exudation patterns (Sasse et al., 2018). Studying this phenomenon *in situ* in the soil requires separation of desired roots from others without disturbance to plant growth or soil. To address this, researchers have used a modified rhizobox design with a side compartment to regulate root growth and quarantine specific roots from the main plant chamber (**Figure 2B**). This additionally

creates easy distinction between old and new roots and allows testing on specific quarantined roots despite plant age. A study using this set up showed specific microbial chemotaxis toward different exudates (sucrose or tryptophan) on an individual root (Jaeger et al., 1999) whereas another showed spatial and temporal regulation of niche differentiation in microbial rhizosphere guilds (Nuccio et al., 2020). Similar physical perturbations to regulate root growth in response to microbial stimuli have also been applied in the microscale and are explored in the next section.

NEXT GENERATION OF PLANT GROWTH CHAMBERS

Our assessment of the major growth chambers showed that most of the systems applied share similarities in basic structural components such as in the use of two parallel sheets in rhizobox-based devices. While these growth chambers brought many of the rhizosphere processes to light, limitations do exist. One limitation is with the scale of applicability. Most of these growth systems are mesoscale and can easily reproduce pot-scale studies (Devienne-Barret et al., 2006) but may not be easily translatable to interactions occurring at the microscale nor recapitulate processes occurring at field-relevant scale. The next section describes advances in technology resulting in a new wave of unique devices making use of microfluidic processes and fabricated ecosystems which are specifically made to investigate specific rhizosphere processes.

Microfluidic Chambers

A complex web of biochemical processes and interactions occur in microscale dimensions in the rhizosphere. Having the ability to interrogate and manipulate these microscale processes and environmental conditions with high spatiotemporal resolution will elucidate mechanistic understanding of the processes. Microfluidics has proven to be a powerful approach to minimize reagent usage and to automate the often-repetitive steps. The microscale of the channels also allows precise control of reproducible conditions utilizing the laminar flow and automated fluidic operations (**Figures 3A,B**). In addition, the microfluidic devices are well integrated with conventional imaging techniques by using a glass slide or coverslip as a substrate bonded with polydimethylsiloxane (PDMS). These characteristics, as well as the ability to rapidly prototype and reproducibly manufacture using soft lithography technique, have enabled new ways of interrogating and studying the rhizosphere environment in a reproducible manner.

Many of the microfluidic devices used for studying the rhizosphere share a similar design concept (Khan et al., 2019). They have an opening port, sometimes with pipette tips inserted into the PDMS body where the seed of the seedling rests and a microchannel where the primary root grows into. The dimension of the channel depends on the type and age of the plant. For example, an *Arabidopsis thaliana's* seedling is typically grown in a microfluidic device up to 10 days, with chamber dimension around 150 to 200 μm in height, whereas the *Brachypodium distachyon* seedling chamber is 1 mm in height due to its thicker roots (Massalha et al., 2017; Khan et al., 2019). Media and/or inoculation of the microbiome is achieved through additional channels to the main chamber. The PDMS body with the channels is typically bonded on a 50 mm by 75 mm microscope slide, and is made to accommodate multiple plants to increase throughput. Automated control offers the ability for continuous imaging and manipulation of media conditions with high temporal resolution.

One notable example of a microfluidic device for rhizosphere studies is the RootChip, which uses the micro-valves in a PDMS device to control the fluidics (Unger et al., 2000; Grossmann et al., 2011). The first study using the RootChip grew 8 Arabidopsis plants on a single device with micro-valves (Grossmann et al., 2011) but by the second iteration, the throughput has been doubled (Jones et al., 2014; Keinath et al., 2015) indicating rapid technological advances in the field. In addition, all these studies demonstrated spatiotemporal imaging at single-cell resolution and dynamic control of the abiotic environments in the rhizosphere.

Another microfluidics-specific application to rhizosphere study is to use the laminar flow to generate the spatially precise and distinct microenvironment to a section of the root as demonstrated by Meier et al. (2010). A young *Arabidopsis*' seedling was sandwiched and clamped between two layers of PDMS slabs with microchannel features to tightly control synthetic plant hormone flow with 10 to 800 μm resolution to the root tip area, enabling observations of root tissues' response to the hormones. As many root bacteria produce auxin to stimulate the interactions with the root, this study showed the possible mechanism of microbiome inducing the interaction by stimulating root hair growth. Another application of laminar flow utilized the RootChip architecture by adding the two flanking input channels to generate two co-laminar flows in the root chamber, subjecting a root to two different environmental conditions along the axial direction to study root cells adaptation to the microenvironment at a local level (Stanley et al., 2018). These studies revealed locally asymmetrical growth and gene pattern regulations in Arabidopsis root in response to different environmental stimuli.

Microfluidic platforms have also been successfully employed to study the interactions between the root, microbiome and nematodes in real time (Parashar and Pandey, 2011; Massalha et al., 2017; Aufrecht et al., 2018). In the systems, additional vertical side channels are connected perpendicularly to the main microchannel to enable introduction of microorganisms and solutes to the roots in a spatially and temporally defined manner (Parashar and Pandey, 2011; Aufrecht et al., 2018). A recent microfluidic design incorporated a nano-porous interface which confines the root in place while enabling metabolite sampling from different parts of the root (Patabadige et al., 2019). These studies demonstrated the potential of microfluidics in achieving spatiotemporal insights into the complex interaction networks in the rhizosphere.

Despite several advantages of microfluidics in rhizosphere research as described above, some challenges remain. All the microfluidic applications grow plants in hydroponic systems where clear media is necessary for the imaging applications and packing solid substrates in the micro-channels is not

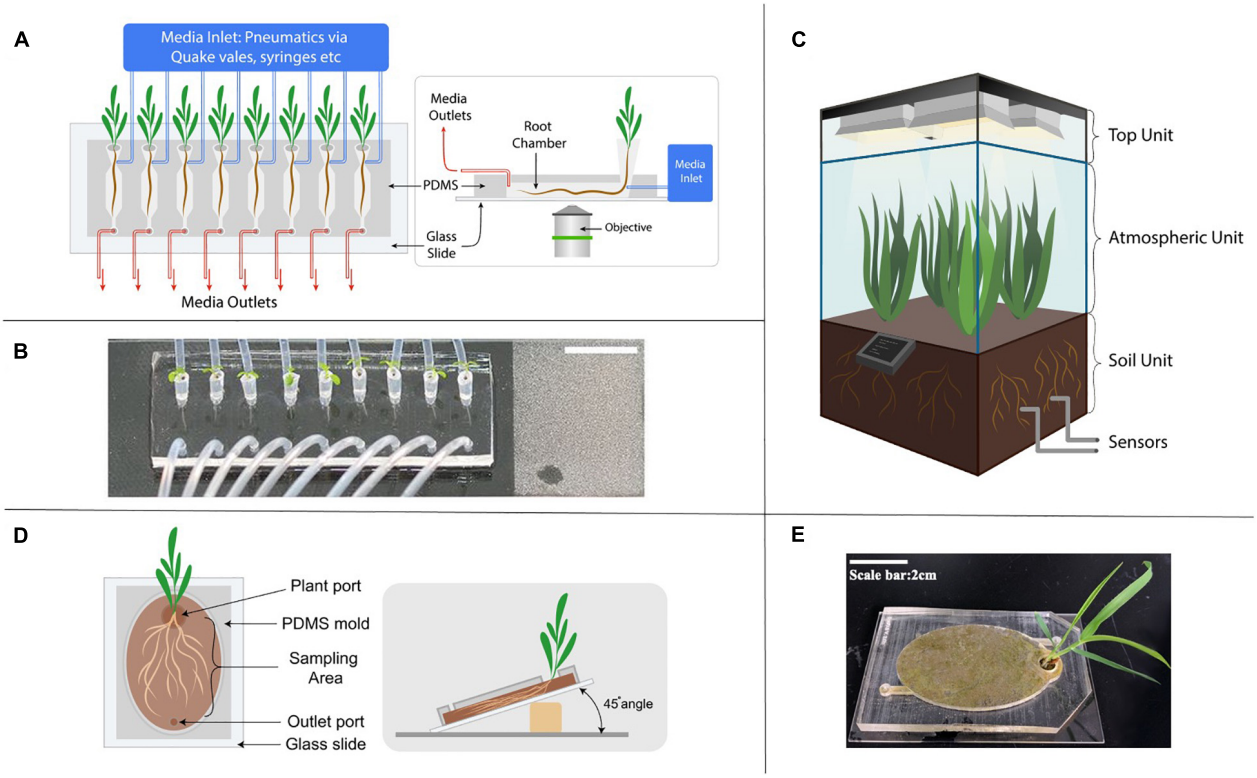

FIGURE 3 | Next generation of growth chamber systems developed to study rhizosphere interactions. **(A)** Schematic diagram of a representative microfluidic device in studying root response to environmental stimuli. The media inlet is controlled by various pneumatic methods such as syringes and automated Quake-valve system. **(B)** An image of a RootChip, demonstrating the increased throughput by the parallel growth of 8 Arabidopsis seedlings on a single device (a figure by Massalha et al., 2017). **(C)** Schematic diagram of an EcoPOD showing three dedicated units with different features; sensors monitor operational parameters closely and the central hub located at the base of the atmospheric unit controls the EcoPOD. **(D)** Schematic diagram of an EcoFAB with soil sealed to a glass slide. **(E)** An image of the EcoFAB bonded to glass slide with *Brachypodium distachyon* grown in soil.

trivial. The microscale of the channels limits the applications of these devices to young seedlings. Thus, interrogating the microscale interactions in bigger, more developed plants is not possible with current microfluidic channel configurations. In addition, technical challenges such as operating the micro-valves and microfabrication present a barrier to device design and construction for non-specialists.

Fabricated Ecosystem Chambers

Fabricated ecosystems aim to capture critical aspects of ecosystem dynamics within highly controlled laboratory environments (Zengler et al., 2019). They hold promise in accelerating the translation of lab-based studies to field applications and advance science from correlative and observational insights to mechanistic understanding. Pilot scale enclosed ecosystem chambers such as EcoPODs, EcoTrons and EcoCELLs have been developed for such a purpose (Griffin et al., 1996; Lawton, 1996; Ke et al., 2020). These state-of-the-art systems offer the ability to manipulate many parameters such as temperature, humidity, gas composition, etc., to mimic field conditions and are equipped with multiple analytical instruments to link below ground rhizosphere processes to above ground observations and vice versa (**Figure 3C**; Griffin et al., 1996; Lawton, 1996; Ke et al., 2020). Currently, however, accessibility to such systems is low as there are only several places in the world which can host such multifaceted facilities due to the requirement of significant financial investments.

Switching back to lab-scale systems, a recent perspective paper calls for the need to standardize devices, microbiomes and laboratory techniques to create model ecosystems (Zengler et al., 2019) to enable elucidation of molecular mechanisms mediating observed plant-microbe interactions e.g., exudate driven bacterial recruitment (Zhalnina et al., 2018a,b). Toward this goal, open source 3D printable chambers, termed Ecosystem Fabrication (EcoFAB) devices, have been released with detailed protocols to provide controlled laboratory habitats aimed at promoting mechanistic studies of plant-microbe interactions (Gao et al., 2018). Similar to a rhizotron setup, these flow-through systems are designed to provide clear visual access to the rhizosphere with flexibility of use with either soil or liquid substrates (**Figures 3D,E**). Certainly, there are many limitations to these devices (discussed in more in **Table 1**) in that they are limited to relatively small plants and limit the 3D architecture of the root system. Still, an advantage with the EcoFAB is that its 3D printable nature allows for adaptations and modifications to be made and shared on public data platforms

such as Github for ease of standardization across different labs and experiments (Sasse et al., 2019). In fact, a recent multi-lab effort showed high reproducibility of root physiological and morphological traits in EcoFAB-grown Brachypodium distachyon plants (Sasse et al., 2019). The development of comparable datasets through the use of standardized systems is crucial to advancing our understanding of complex rhizosphere interactions. Open science programs such as the EcoFAB foster a transparent and collaborative network in an increasingly multi-disciplinary scientific community.

PERSPECTIVES ON CURRENT AND FUTURE GROWTH CHAMBER DESIGNS

Specialized plant chamber systems are necessary for non-destructive visualization of rhizosphere processes and interactions as all destructive sampling approaches tend to overestimate the rhizosphere extent by 3–5 times compared to those based on visualization techniques (Kuzyakov and Razavi, 2019). Nonetheless, plants in such chambers are still grown in defined boundaries and suffer from inherent container impacts. For instance, studies have pointed out that container design (size, density, depth) significantly influences root growth during early developmental stages and leaves lasting impacts on plant health and phenotype (Howell and Harrington, 2004; South et al., 2005; Tsakaldimi et al., 2005; Kostopoulou et al., 2011). The majority of the lab-based chambers are also centimeter scale and are unlikely to replicate exact field conditions in terms of soil structure, water distribution, redox potential or root zone temperatures (Neumann et al., 2009). While comparisons between chamber-grown (e.g., rhizobox) and pot-grown plants show similar outputs (Devienne-Barret et al., 2006), studies comparing plants grown in confined spaces to those directly grown in the field are missing.

A recent review mapped the gradient boundaries for different rhizosphere aspects (physico-chemical gradients, root exudates and microbial communities, etc.) and found that despite the dynamic nature of each trait, the rhizosphere size and shape exist in a quasi-stationary state due to the opposing directions of their formation processes (Kuzyakov and Razavi, 2019). The generalized rhizosphere boundaries were deducted to be within 0.5–4 mm for most rhizosphere processes except for gases (e.g., O_2) which exceeds > 4 mm and interestingly, they are independent of plant type, root type, age or soil (Kuzyakov and Razavi, 2019). Bearing this in mind, our assessment of the different growth chambers revealed possible overestimation of rhizosphere ranges in some chamber set ups. For instance, the use of root-free soil pouches representing rhizosphere soil despite being cm-distance away from the rhizoplane. This prompts the need for careful evaluation of new growth chamber designs to ensure accurate simulation of natural rhizosphere conditions.

To date, many rhizosphere microbiome studies and growth chambers systems focus on the impact of plant developmental stage, genotype and soil type on microbial composition and function (Chaparro et al., 2014; Edwards et al., 2015; Wagner et al., 2016). On the other hand, predation as a driver in the rhizosphere microbiome remains understudied. For instance, protists are abundant in the soil and are active consumers of bacteria and fungi and play a role in nutrient cycling yet remain an overlooked part of the rhizosphere (Gao et al., 2019). Viruses are also pivotal in modulating host communities thereby affecting biogeochemical cycles but their influence in the rhizosphere is poorly studied (Bi et al., 2020). These predator-prey interactions in the rhizosphere deserve in-depth studies which can be facilitated by these specialized growth chambers.

Another area worth investigating in the rhizosphere is in anaerobic microbial ecology. At microbially relevant scales, soils primarily exist as aggregates (<2 mm). Aggregation creates conditions different from bulk soil, particularly in terms of oxygen diffusion and water flow resulting in anoxic spaces within aggregates and influences the microbial community (Wilpiszeski et al., 2019). The rhizosphere is also rich in a wide range of compounds which can serve as alternative electron acceptors such as nitrate, iron, sulfate and humic substances in the absence of oxygen (Lecomte et al., 2018). However, most anaerobic studies in the rhizosphere focus only on aqueous environments such as water-logged paddy soils despite biochemical and metatranscriptomic evidence pointing to the possibility of anaerobic respiration in the rhizosphere (Lecomte et al., 2018). To fully understand biogeochemical cycles in the rhizosphere, it is imperative to investigate rhizosphere processes in the microscale and to include localized redox conditions as one of the influencing parameters. Microfluidic platforms with its fast prototyping capabilities can be helpful in creating growth chambers designed to stimulate these redox changes.

In the study of the rhizosphere microbiome, genetic manipulation strategies are foundational in deep characterization of microbial mechanisms and current manipulation techniques require axenic isolates. However, the uncultivability of a significant portion of soil microorganisms continues to hamper efforts in gaining mechanistic knowledge. Even for culturable isolates, the process of isolation introduces selective pressure and disturbance to the community with inevitable loss of information on spatial interactions. A recent innovation in gene editing technologies using CRISPR-cas systems demonstrated *in situ* editing of genetically tractable bacteria within a complex community (Rubin et al., 2020). Coupled with the use of transparent soil-like substrates (Downie et al., 2014), the application of such a technique for the editing of *in situ* rhizosphere microbiome while preserving spatial and temporal associations would indeed bring invaluable insights. Specialized growth chambers using 3D fabrication and microfluidic technologies are primed to facilitate such innovations.

Finally, this review revealed that while similarities exist among the different growth chamber systems, many of these systems are bespoke. This makes it difficult to replicate experiments and determine reproducibility which are important cornerstones of scientific advancement. The complexity of rhizosphere interactions also warrant that computational models are essential to gain a better understanding of system level processes (Darrah et al., 2006; Zengler et al., 2019). However, predictive modeling requires data from standardized approaches to be comparable between experiments. Thus, future growth chamber systems and

designs are encouraged to follow the open science framework to enable standardization to an extent, such as in the case of EcoFABs (Sasse et al., 2019).

CONCLUSION

Studying the rhizosphere is a challenge due to the complex and dynamic interactions between many of its members, made further complicated by the opaque soil. Specialized plant chambers have been and continue to be an important tool in investigating these rhizosphere spatiotemporal processes in the soil. We identified representative growth systems used to study various rhizosphere interactions and processes such as root system architecture, exudation and microbial communities and found that they share common features but most are custom made to answer specific research questions. A major benefit of these specialized chambers is the ability to visualize the rhizosphere which allows for coupling with various analytical instruments to probe *in situ* processes through non-destructive sampling. Modern developments in growth chamber systems utilizing 3D fabrication and microfluidic technologies are also gaining prominence in understanding microscale interactions. These chambers also present the opportunity for both top down (community engineering and characterization) and bottom up (isolation-based) approaches to investigate rhizosphere communities. However, it should be noted that as these specialized chambers have been developed for model systems, the findings should ultimately be verified at field relevant conditions for truly predictive ecological understanding. Nonetheless, it is clear that the use of specialized chambers would continue to play a central role in our effort to gain a mechanistic understanding of the rhizosphere ecosystem.

AUTHOR CONTRIBUTIONS

RC conceptualized the idea. MY developed and wrote the manuscript. PK and YL contributed to specific sections of the review. All authors contributed to drafting and editing of the manuscript.

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