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Plant root interaction with associated microbiomes to improve plant resiliency and crop biodiversity, volume II

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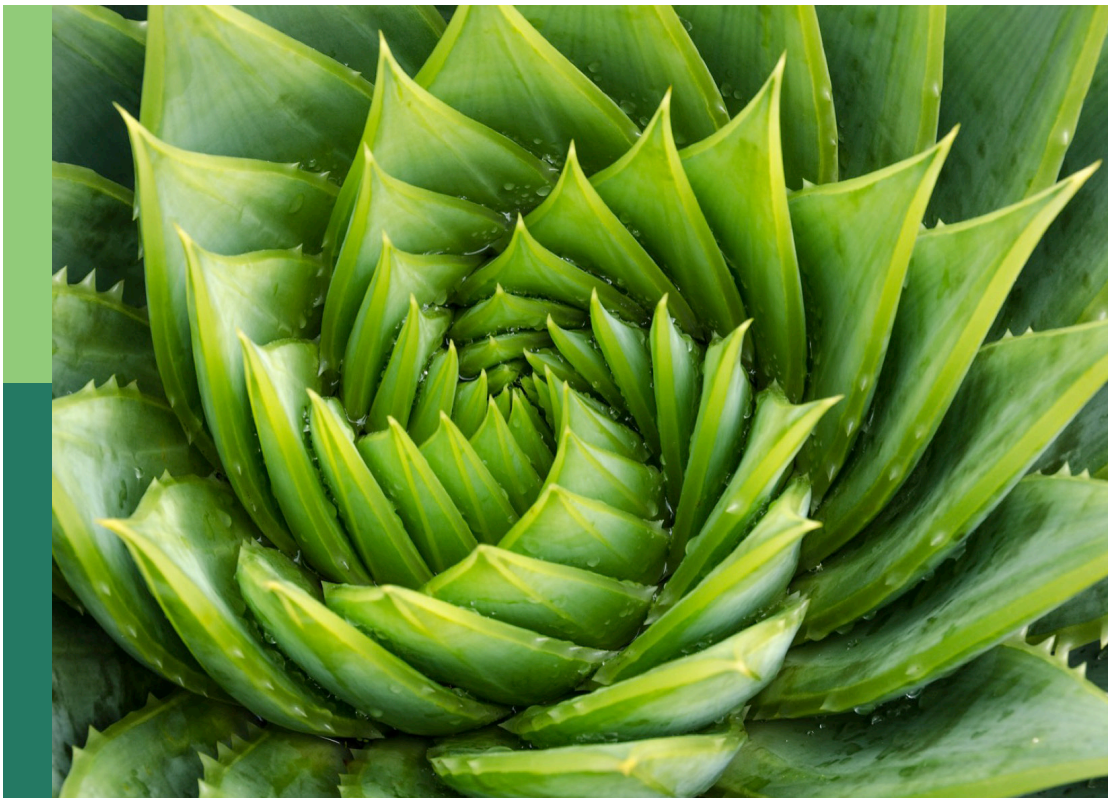

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Plant root interaction with associated microbiomes to improve plant resiliency and crop biodiversity, volume II

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Editorial: Plant root interaction with associated microbiomes to improve plant resiliency and crop biodiversity, volume II

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Editorial on the Research Topic
Plant root interaction with associated microbiomes to improve plant resiliency and crop biodiversity, volume II

Soil is acknowledged as a limited resource, which determines crop productivity and ecosystem sustainability. Microorganisms form an integral part of soil, contributing to soil fertility and plant health. The soil, diverse microbial communities and plants are involved in direct and indirect interactions, which are often difficult to predict, depending on environmental factors (Fierer, 2017). A comprehensive understanding of mechanisms underlying these interactions will enable development and implementation of strategies optimizing crop/soil management to improve crop resilience against biotic and abiotic stresses. The study of plant–soil relations is also considered fundamental to explain the vegetation dynamics that influence ecosystem composition and functioning. This can enhance understanding of ecological evolution and allows better prediction and mitigation of consequences of human-induced global changes, promoting a sustainable provision of ecosystem services (van der Putten et al., 2013).

Soil sickness is a widespread problem associated with replanting fruit crops and monocropping, with a complex etiology, and influenced by soil and climatic conditions (Monaci et al., 2017). In addition to nutrient availability and plant pathogens, recent evidence indicates potential roles played by litter autotoxicity related to the microbial activities and the exposure to fragmented self-DNA (Mazzoleni et al., 2015) in soil sickness. The severity of soil sickness and its symptom expression is significantly affected by complex interaction between soil management, location, root system, soil organic matter content (Polverigiani et al., 2018) in which soil microbiome plays a critical role. Thus, identifying agricultural practices that promote microbial activities become a key approach to overcome soil sickness. Such management practices include accelerating degradation of allelopathic compounds

(Endeshaw et al., 2015), crop rotation, integrating weed management with living mulches (Mia et al., 2021; Neri et al., 2021), and applying different kinds of microbial-based or microbial-derived products (Vassileva et al., 2020).

To advance beyond a simplified view of individual plant-microbe or soil-plant interactions, the plants-soil biota should be considered as a unique "meta-organism", influencing complex interactions that impact plant development and productivity. To facilitate efficient exploitation of beneficial microbial strains in practice, a complex set of data needs to be generated to assess their impact on resident soil microbiome (and its functions), soil physio-chemical properties and plant performance under the influence of agronomical practices (Malusà et al., 2021). This overarching view of plant-soil microbiota interactions in relation to crop performance underpins the Research Topic, which contains 11 contributions of reviews and original research articles.

Large-scale production and formulation of microbial strains, alone or in consortia, are the critical steps in the development of commercial products (Vassilev et al., 2017; Vassilev et al., 2020; Vassileva et al., 2022). Applying products from co-formulation of two or more beneficial microbes with complementary functions resulted in better wheat plant growth/health and more diverse and abundant beneficial endophytic bacterial communities in the root system (Zhang et al.), as also found in other crops. Lignocellulose-decomposing enzymes produced and released by the studied microorganisms were shown to have played a crucial role in the assembly of endophytic bacterial communities and their successful colonization of wheat roots. Stimulation of plant metabolic activities by biofertilizers was reviewed by Chaudhary et al., highlighting the multifunctional potential of such bioinocula in terms of promoting growth and activating plant defense in the presence of biotic and abiotic, particularly climate related, stresses. Inoculation of cuttings of the medicinal plant *Bacopa monnieri* with *Bacillus subtilis* and *Klebsiella aerogenes* individually or in combination induced a significant improvement in the accumulation of bacoside A, a triterpenoid saponin with preventive activity against Alzheimer's disease (Shukla et al.), demonstrating the potential of the *in planta* production of secondary metabolites for medical applications.

Among environmental stresses, acidification is considered to limit soil fertility and health. Li et al. provided a novel insight into the relationship between lime amendment of acidified soil, composition of citrus root-associated microbiota and citrus tolerance to Huanglongbing (a systemic soil-borne disease caused by nonculturable bacteria *Candidatus Liberibacter* spp.). Liming, increased the diversity of root bacterial communities and enriched beneficial microorganisms in roots, two features associated with a lower Huanglongbing disease severity.

Soil fertility and crop productivity are closely related and dependent on soil biodiversity. Alternative strategies to increase crop diversification can supply several ecosystem services related to nutrient cycling, crop productivity and health, all mediated through soil microbiota. Trinchera et al. studied five cropping systems of organic vegetables and showed that multi-cropping increased the total soil microbial mass, promoting specific bacterial and fungal phyla and mycorrhizal colonization. These changes were associated with positive effects on C and P nutrient cycles and pathogen reduction. Multicropping was successfully applied in greenhouse production of *Capsicum annum* intercropped with *Foeniculum vulgare* (Yang et al.). The release of antimicrobial terpene compounds by the fennel roots suppressed *Phytophthora* disease through the induced accumulation of reactive oxygen species in the pathogen.

Biocontrol with specific microbial strains is an efficient tool for achieving sustainable pest control in an environmentally friendly manner. El-Saadony et al. reviewed literatures providing evidence of biocontrol activity of plant growth promoting microorganisms. Exploiting this multifunctional capacity can foster alternative strategies of crop protection, currently still relying on synthetic fungicides, thus responding to the challenge of ensuring healthy and fresh products within a net-zero horticulture (Xu, 2022). Duan et al. isolated a *Bacillus licheniformis* strain from apple roots, characterized its metabolic activity, particularly the degradation of phlorizin [a phenolic compound associated to apple replant disease (ARD)], identified possible mechanisms of action against several soil-borne pathogens, and optimized fermentation process for the large-scale production. Detailed studies about the effect of this strain on plant root development, soil-borne pathogens and soil microbial community led them to propose a framework for managing ARD. In another study (Alwahshi et al.), *Streptomyces violaceoruber* UAE1 isolated from rhizospheric soil of healthy date palms, able to produce 1-aminocyclopropane-1-carboxylic acid deaminase, showed antifungal activities against *Fusarium solani*, the causal agent of the sudden decline syndrome of date palm, highlighting the potential of this strain in disease management.

Belleís-Sancho et al. studied the relationship between gene expression induced by a beta-rhizobial *nifA* mutant (*Paraburkholderia phymatum*, a model organism for studying beta-rhizobia-legume symbiosis) and auxin synthesis during the symbiosis with *Phaseolus vulgaris*. In general, the establishment of this symbiotic interaction relies on a sophisticated molecular and chemical cross-talk between *Rhizobium* and the plant. The gene regulating nitrogenase expression, *nifA*, was shown to control expression of two bacterial genes involved in auxin synthesis, leading to their increased production in nodules occupied by the *nifA* mutant compared to wild-type nodules. It was concluded that the increased abundance of rhizobial auxin in the non-fixing *nifA* mutant strain may have played a role in root infection and early-stage symbiotic interactions.

Safety and quality are important issues for commercialization of biofertilisers and biopesticides. Vassileva et al. reviewed the risks of contamination of microorganism-bearing fertilizers during different production stages, storage, and application in soil. Direct and indirect spread of zooptic and plant pathogens associated with these microbial-based products were discussed with examples of different products, including compost, manure, biosolids, and bioformulated inoculants with plant growth promoting or biocontrol functions.

The work published in this issue confirms the urgent need for studies on soil microorganism-mediated processes responsible for different soil functions and ecosystem services, with the emphasis on their regulating mechanisms. This knowledge can be used to inform advisors and farmers and improve their decision-making in crop

management to improve food security and quality (Borsotto et al., 2022).

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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Conflict of interest

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

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The Phlorizin-Degrading *Bacillus licheniformis* XNRB-3 Mediates Soil Microorganisms to Alleviate Apple Replant Disease

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In this study, an endophytic phlorizin-degrading *Bacillus licheniformis* XNRB-3 was isolated from the root tissue of healthy apple trees, and its control effect on apple replant disease (ARD) and how it alleviates the pathogen pressure via changes in soil microbiomes were studied. The addition of strain XNRB-3 in *Fusarium* infested soils significantly reduced the number of pathogens in the soil, thus resulting in a lower disease incidence, and the relative control effect on *Fusarium oxysporum* reached the highest of 66.11%. The fermentation broth can also protect the roots of the plants from *Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium solani* infection. These antagonistic effects were further validated using an *in vitro* assay in which the pathogen control was related to growth and spore germination inhibition via directly secreted antimicrobial substances and indirectly affecting the growth of pathogens. The secreted antimicrobial substances were identified using gas chromatography-mass spectrometry (GC-MS) technology. Among them, alpha-bisabolol and 2,4-di-tert-butylphenol had significant inhibitory effects on many planted pathogenic fungi. Butanedioic acid, monomethyl ester, and dibutyl phthalate promoted root development of Arabidopsis plants. Strain XNRB-3 has multifarious plant growth promoting traits and antagonistic potential. In pot and field experiments, the addition of strain XNRB-3 significantly promoted the growth of plants, and the activity of enzymes related to disease resistance [superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)] was also significantly enhanced. It also reduced the abundance of four species of *Fusarium* and the content of phenolic acids in the rhizosphere soil, improved soil microbial community structure and nutritional conditions, and increased soil microbial diversity and activity, as well as the soil enzyme activity. The above results indicated that *B. licheniformis* XNRB-3 could be developed into a promising biocontrol and plant-growth-promoting agent.

Keywords: apple replant disease, *Fusarium* spp., biocontrol, biolog, endophytic *Bacillus*, antibiosis

INTRODUCTION

Apple replant disease (ARD) is a soil sickness caused by biotic and abiotic factors. It occurs in major apple cultivation areas worldwide and poses a major threat to the development of the apple industry (Laurent et al., 2010; Tewoldemedhin et al., 2011). This disease is most harmful to young replanted trees, and it can reduce the growth of trees, increase the susceptibility of trees to diseases, discolor roots, result in root tip necrosis, and reduce root biomass, which can ultimately lead to plant death within the first growing season. Furthermore, the yield and quality of fruit trees more than 20 years old is decreased in plants with ARD, and death can occur in severe cases (Mazzola and Manici, 2012; Yim et al., 2020; Balbín-Suárez et al., 2021). An increasing number of studies have shown that biotic factors are the main factors that cause ARD, including nematodes (*Pratylenchus* spp.), fungi (*Rhizoctonia solani*, *Fusarium* spp., and *Cylindrocarpon* spp.), and oomycetes (*Pythium* and *Phytophthora*) (Weerakoon et al., 2012; Hewavitharana and Mazzola, 2016; Sun et al., 2017; Tilston et al., 2020). Abiotic factors may also play an important role in replanting problems, such as soil structure, the accumulation of phenolic compounds or phytotoxins in disease-affected roots, and nutrition (Hofmann et al., 2009; Wang et al., 2018).

Previous studies have suggested that *Fusarium* spp. is one of the main pathogens contributing to the occurrence of ARD in China, South Africa, and Italy (Kelderer et al., 2012; Yin et al., 2017; Wang et al., 2018; Sheng et al., 2020; Duan et al., 2021). *Fusarium* survives in soil in the absence of hosts for up to 10 years as chlamydospores, and, as a consequence, traditional methods of control such as crop rotation are typically ineffective (Liu L. et al., 2018). Yin et al. (2017) found that root exudates can stimulate the germination of *Fusarium* conidia, and the infectious hyphae from the conidia penetrate the roots of the host plant, resulting in plant death. Therefore, conidia are the most important structures mediating the survival of pathogens and initial infection, they are also some of the direct targets for the biological control of *Fusarium*. Currently, chemical fungicides and soil disinfestation with methyl bromide are the main commercially available approaches for controlling ARD (Dandurand et al., 2019). However, methyl bromide is now being phased out from agriculture because of environmental contamination and associated health threats (Raymaekers et al., 2020). The application of biological control agents (BCAs) is considered an eco-friendly and sustainable method for reducing the effects of plant diseases and has become increasingly used in many countries (Zalila-Kolsi et al., 2016; Chowdhury et al., 2020).

Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that colonize plant roots and promote plant growth and development (Kloepper and Beauchamp, 1992). Many diverse bacterial genera, such as *Bacillus*, *Klebsiella*, and *Pseudomonas*, can colonize various plant organs (Reiter and Sessitsch, 2006; Santoyo et al., 2016). These bacteria can stimulate plant growth, increase yield, reduce pathogen infection, and reduce biotic or abiotic plant stress under a wide range of environmental conditions without conferring pathogenicity, making them prime candidate BCAs for their ability to effectively control many different plant diseases in crops (Compant et al., 2010; Kumar et al., 2012; Ji et al., 2014; Liu et al., 2019). Bacteria of the genus *Bacillus* produce heat and desiccation-resistant spores, and are thus more suitable for use in biofungicides (Pérez-García et al., 2011).

To continue, BCAs control plant pathogens and harmful microorganisms through the production of antibiotics, antifungal metabolites, and the degradation of the cell wall by different enzymes (Santoyo et al., 2012; Chowdhury et al., 2015; Eljounaidi et al., 2016; Afzal et al., 2019). In addition to antifungal activity, they can also facilitate plant growth through N_2 fixation; the solubilization of insoluble phosphorus; the production of siderophores, phytohormones (e.g., IAA), and volatile organic compounds (VOCs); and induced systemic resistance (Ahemad and Khan, 2011; Gao et al., 2017). The 2-nonanone and 2-heptanone produced by *B. amyloliquefaciens* L3 isolated from the rhizosphere of watermelon have been shown to have strong antifungal properties against *F. oxysporum* f. sp. *Niveum* (FON), the VOCs acetoin and 2,3-butanediol produced by it promote plant growth (Wu et al., 2019). Marques et al. (2010) found that all the bacterial isolates produce indole acetic acid, hydrogen cyanide, and ammonia when tested *in vitro* for their plant growth-promoting (PGP) abilities. Azabou et al. (2020) isolated a *B. velezensis* OEE1 from root tissue with a strong control effect on *Verticillium* wilt that can significantly reduce the final mean disease severity index (FMS), percentage of dead plants (PDP), and area under disease progress curve (AUDPC). However, there are relatively few biological control agents for *Bacillus* that can be used to control ARD.

In this study, a strain of *B. licheniformis* XNRB-3 that degrades phlorizin was isolated from the root tissue of healthy apple trees in a replanted orchard. The purpose of this study is to (a) characterize the antagonistic activity of the bacterial isolate XNRB-3 against *F. oxysporum*, *F. moniliforme*, *F. proliferatum*, and *F. solani*; (b) evaluate the ability of strain XNRB-3 to degrade phlorizin and promote plant growth; (c) optimize its biocontrol activity against ARD; (d) evaluate the root colonization ability under greenhouse conditions; (e) identify the main antimicrobial compounds involved in its antifungal activity; and (f) verify the effect of XNRB-3 against ARD under outdoor potting and field conditions.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

The culture of plant fungal pathogen (*Fusarium proliferatum*, *Fusarium verticillioides*, *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Alternaria alternata*, *Albifimbria verrucaria*, *Aspergillus flavus*, *Penicillium brasilianum*, and *Phytophthora cactorum*) are used in this study. Details of the fungal plant pathogen cultures are shown in **Supplementary Table 1**. Stock cultures were maintained on potato dextrose agar (PDA) plates at 4°C. Pre cultures were established by transferring a stock agar plug containing mycelia onto fresh PDA plates and incubating for 6 days at 28°C.

Isolation and Screening of Bacteria for Biocontrol Activity

The rhizosphere soil and root system were taken from healthy apple trees in the replanted orchard [replanted for 4–5 years on old apple orchards (more than 25 years old)] in the Northwest Loess region. The repeated cropping of the sampling orchard was serious, and there were dead trees. The basic information of the sampled sites are presented in **Supplementary Table 2**. The isolation of antagonistic bacteria refers to the method of Filippi et al. (2011), Fang et al. (2020), and Duan et al. (2021). Morphologically distinct colonies were replicated, and purified isolates were stored in cryogenic tubes. The bacterial strains screened for antagonistic activity toward plant fungal pathogen using dual culture technique refer to the method of Yu et al. (2011) and Duan et al. (2021).

Identification of Antagonistic Strain

The morphological identification was conducted based on the methods of Zhao et al. (2018). Biochemical identification was based on the methods of Dong and Cai (2001) and Vos et al. (2011). The GEN III MicroPlate^{TM} test panel identification was based on the methods of Bochner (1989).

Genomic DNA was extracted from the obtained isolates using the Bacteria Genomic DNA Extraction Kit (Tiangen Corporation Ltd, Beijing, China) according to the manufacturer's instructions (Sambrook and Russel, 2001). Molecular identification was performed on strain XNRB-3 using 16S ribosomal RNA gene (16S rDNA), DNA gyrase subunit A (*gyrA*), DNA gyrase subunit B (*gyrB*), and RNA polymerase subunit B (*rpoB*) gene sequence analysis. The primers and annealing temperatures are shown in **Supplementary Table 3**. Polymerase chain reaction (PCR) amplification, product purification, and sequencing refer to the method of Duan et al. (2021).

Sequences of 16S rDNA, *gyrA*, *gyrB*, and *rpoB* were aligned using maximum likelihood (ML) methods were performed for the datasets using RAxML-HPC2 on XSEDE (8.2.12) on the CIPRES website[1] (Stamatakis, 2014). Tree diagrams were created in FigureTree v1.4.3 and Adobe Illustrator CS6.

Polymerase Chain Reaction Detection of Antibiotic Biosynthesis Genes

Genes related to the biosynthesis of lipopeptides, dipeptides and polyketides were detected by PCR using the primers listed in **Supplementary Table 4**. Amplification was performed with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems Inc., California, CA, United States). Following amplification, the PCR reaction mix (5 μL) was visualized by gel electrophoresis in a 1.5% agarose gel.

Optimization of Fermentation Conditions

Seed culture of *B. licheniformis* XNRB-3 was prepared by inoculating a single colony into 100 mL of Luria-Bertani (LB) broth (tryptone 10 g, yeast extract 5 g, NaCl 10 g, pH 7.0) and incubated at 37°C overnight with agitation (180 rpm).

[1]http://www.phylo.org

Single Factor Test

Batch fermentation was carried out in 250-mL Erlenmeyer flasks containing 100 mL of fermentation medium (glucose 22 g, tryptone 5.4 g, KH_2PO_4 2.4 g, $CaCl_2$ 0.25 g, $MgSO_4$ 0.5 g, 1 L, pH 7.0) inoculated with 3–10% seed culture, and incubated at 37°C with agitation (180 rpm) for 72 h (Qiao et al., 2012). The OD_{600} absorbance value of the bacterial solution was measured at different incubation times and the serial dilution method was used to count the number of bacteria (**Supplementary Figure 1a**).

The variables used were carbon sources (sucrose, maltose, glucose, lactose, soluble starch), nitrogen sources [yeast extract, peptone, $(NH_4)_2SO_4$, beef extract, NH_4NO_3, urea, and NH_4Cl], inorganic salts (KH_2PO_4, $CaCl_2$, $MgSO_4$, NaCl, $MnSO_4$, and KCl), and culture conditions (filling volume, speed, temperature, and pH) each at different levels, as shown in **Supplementary Table 5**. For the selection of inorganic salts, one inorganic salt is removed at a time, and its influence on the growth of strain XNRB-3 was investigated. According to the results of the inorganic salt removal test, three important inorganic salts were selected, and the orthogonal experiment was designed according to the orthogonal table to determine the best combination of inorganic salts. When the above fermentation conditions were optimized, except for the test single factor as a variable, the other conditions were unchanged. Each treatment contained three repetitions. The 24 h OD_{600} (diluted three times) and the antibacterial rate were determined and combined with the results of single factor experiment, orthogonal experiment (Orthogonality Experiment Assistant V3.1), Plackett–Burman, and Box–Behnken (Design-Expert 8.0.6) in response surface analysis (RSM) to optimize the composition and fermentation conditions of the *B. licheniformis* XNRB-3 sporulation shake flask fermentation medium (Anthony et al., 2009).

Antagonistic Effects of Extracellular Metabolites on *Fusarium*

The cell-free culture filtrate (CFCF) of strain XNRB-3 was obtained according to the method of Abdelmoteleb et al. (2017). Bacteria were grown on the optimized liquid fermentation medium with constant shaking at 191 rpm for 24 h at 33 to obtain fermentation broth (FB), centrifugated at 10,000 rpm for 10 min at 4, and the supernatant (extracellular medium) was passed through a 0.22-μm Nylon66 microporous membrane to obtain the CFCF. The effect of strain XNRB-3 on *Fusarium* spore germination and hyphae refers to the method of Duan et al. (2021).

Plant Growth Promoting Activities

The ability of strain XNRB-3 to promote plant growth was tested with reference to the method in **Supplementary Table 6**. The FB of *B. licheniformis* XNRB-3 was centrifuged at 13,400 *g* for 10 min. The supernatant (extracellular medium) was removed and frozen at 4 for the analysis of the amino acids. Phytohormone extraction and quantitation were conducted using gas chromatography-mass spectrometry/selected ion

monitoring (GC-MS/SIM) (Shimadzu, Japan). The specific method is mentioned in **Supplementary Table 6**.

Determination of Microorganisms Using Phlorizin as Carbon Source

Preparation of 0∼10 mmol L^{-1} Phlorizin Culture Medium

Chemical compounds of phlorizin, cinnamic acid (CA), ferulic acid (FA), benzoic acid (BA), and *p*-hydroxybenzoic acid (PHBA) were Sigma products (Sigma, St. Louis, MO, United States). The mineral salt medium (MSM) [K_2HPO_4 5.8 g, $(NH_4)_2SO_4$ 2.0 g, KH_2PO_4 4.5 g, $CaCl_2$ 0.02 g, $MgCl_2$ 0.16 g, $FeCl_3$ 0.0018 g, $Na_2MoO_4{\cdot}2H_2O$ 0.0024 g, and $MnCl_2{\cdot}2H_2O$ 0.0015 g in 1 L, pH 7.0] containing phlorizin (0∼10 mmol L^{-1}) as the sole carbon source was used to isolate degrading bacteria. Agar plates were prepared by adding agar (18 g L^{-1}) to the MSM, and the MSM solution with phlorizin was used to determine the maximum absorption wavelength and standard curve, and the operation was protected from light as much as possible (Pant et al., 2013).

Tests of Microbial Growth and Phenolic Degradation

The absorbance value of MSM solution with phlorizin (10 mmol L^{-1}) at each wavelength was measured, and it was determined that the maximum absorbance wavelength was 280 nm (**Supplementary Table 7**). The MSM solution with a total of 11 concentrations of 0-10 mmol L^{-1} were prepared. The MSM solution with 0 mmol L^{-1} phlorizin was used as the reference solution for blank calibration and to determine the OD_{280} absorbance value. The absorbance value is taken as the ordinate and the phlorizin concentration as the abscissa to form a standard curve (**Supplementary Figure 4p**).

The tests of microbial growth and phenolic acid degradation in MSM solution refer to the method of Wang et al. (2021), with some modifications. Strain XNRB-3 was inoculated into MSM with 0∼10 mmol L^{-1} phlorizin and cultured at 33°C in the dark to determine the maximum tolerance of phlorizin (**Supplementary Figure 4n**). Strain XNRB-3 was inoculated with 6 mL of liquid fermentation medium in a 15-mL plastic tube with a cover, followed by overnight incubation at 33°C. After centrifugation at 2,500 *g* for 5 min, the supernatant was discarded and the pellet was diluted to an OD_{600} value of 1.0 using ddH_2O. Afterward, 0.1, 0.15, and 0.2 mL of the resuspended isolates were transferred to 10 mL of the MSM solution with phlorizin (3 mmol L^{-1}) and incubated for 60 h in the dark at 33°C with shaking (191 rpm min^{-1}). Then, 1 mL of the bacterial suspension was taken from each treatment, centrifuged at 13,000 *g* for 5 min, the supernatant was taken to measure the OD_{280} value, and it was converted to the concentration of phlorizin according to the standard curve. The degradation rate of phlorizin = (phlorizin concentration in uninoculated culture solution-phlorizin concentration in inoculated culture solution)/phlorizin concentration in uninoculated culture solution × 100%.

To test the isolate's ability to utilize other phenolic acids, strain XNRB-3 was inoculated into the MSM solution with 0.5 g L^{-1} of CA, FA, BA, or PHBA, and their growth was tested according to the previously described method, where 0.2 mL of the suspension was transferred to 10 mL of MSM solution with 0.5 g L^{-1} of CA, FA, BA, or PHBA. After incubation for 12, 24, 36, 48, and 60 h at 33°C with shaking (190 rpm min^{-1}), 1 mL of the bacterial suspension was taken from each treatment and was centrifuged at 13,000 g for 5 min. The pellet was resuspended with 1 mL ddH_2O to detect OD_{600}, and the supernatant was mixed with an equal volume of methanol. Then, 0.1 mL of the mixed solution was added to 1.9 mL of 50% methanol solution and was sterilized by filtering through 0.22-μm pore-size filter membranes prior to High Performance Liquid Chromatography (HPLC) analysis. The control used was MSM solution of phenolic acids but without bacteria, where all assays were performed in triplicate.

The concentrations of phenolic acids were detected based on peak areas using external standards using an UltiMate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA, United States). All separations were performed using Symmetry® C18 column (4.6 mm × 150 mm, 5.0 μm; Waters, Milford, MA, United States). Mobile phase solutions were 0.1% methanoic acid + 2% methanol (A) and acetonitrile (B). The gradient elution composition used was as follows: 0 min, 96% A plus 4% B 10 min; 10% A plus 90% B 16 min; 96% A plus 4% B 30 min; with a flow rate of 1.0 mL min^{-1}, an injection volume of 10 μL, a column temperature of 20°C and Ultraviolet (UV) detection at 280 nm (Wang et al., 2021).

Soil Treatment With Microbes and Analysis of Phenolic Acid Degradation

The soil obtained from a 31-year-old apple orchard in Manzhuang Town, Taian, China (117.081039 longitude, 36.06682 latitude) was dried at 60°C for 6 h and was passed through a 0.84-mm sieve. Here, 30 μg g^{-1} of phlorizin, 10 μg g^{-1} of CA, 90 μg g^{-1} of BA, 100 μg g^{-1} of FA, and 20 μg g^{-1} of PHBA was added to the dried soil. The soil was dried again at 60°C for 2 h. Then, 200 mL with OD_{600} = 1. 0 of bacterial suspension was added to 1 kg of soil; sterile water was used as control. Each treatment consisted of three replicates. After incubation for 3, 6, and 9 days at 25°C/20°C (16 h/8 h, light/dark), ddH_2O was added to keep the soil moist. 60 g of soil was obtained from each pot for HPLC analysis (Zhang Z. Y. et al., 2010).

Extraction of phenolic acids from soil using the method of Yin et al. (2013). The HPLC analysis procedure followed that described by Xiang et al. (2021). An UltiMate 3000 HPLC system (Dionex, United States) was used for quantification with a Symmetry® C18 column (4.6 mm × 250 mm, 5.0 μm; Waters, Milford, MA, United States), and a column temperature of 30°C. Mobile phase A was acetonitrile, and mobile phase B was water (adjusted to pH 2.8 with acetic acid). The flow rate was 1.0 mL min^{-1}, the automatic injection volume was 10 μL, and the detection wavelength was 280 nm. The HPLC solvents were purchased from Burdick & Jackson Inc. (Muskegon, MI, United States). The retention time was used for qualitative analysis, and the peak area external standard method was used for quantification.

Root Colonization Assay

A spectinomycin- and rifampin-resistant mutant of strain XNRB-3 (denoted XNRB-3^R) was obtained by inoculation of strain

XNRB-3 into the LB medium containing gradually increasing concentrations of spectinomycin and rifampin (20, 50, 75, 100, 150, 200, 250, and 300 mg mL^{-1}; **Supplementary Figure 4o**) refer to the method of Yu et al. (2011). *Malus hupehensis* Rehd. seedlings were selected as the test material.

The ability of *B. licheniformis* XNRB-3 to colonize roots was determined according to the method of Sanei and Razavi (2011), with some modifications. The *M. hupehensis* Rehd. seedlings were carefully uprooted from the substrate, their roots thoroughly washed in tap water without intentional wounding, and dipped in a bacterial (XNRB-3^R) suspension [1×10^8 colony-forming units (CFU) mL^{-1}] for 10 min. For the control treatment, plants were treated similarly except that the roots were dipped in 10 mM $MgSO_4.7H_2O$. Plants were then transplanted (one per pot) into pots (AC140: the outer diameter is 12.5 cm, the inner diameter is 11 cm, and the height is 9.5 cm) filled with an autoclaved (121°C, 1 h, twice on consecutive days) soil mixture (180 mL vermiculite first, then 540 mL of soil), and finally covered with 180 mL of sterile vermiculite. There were four replicated plants for each treatment in a randomized complete block design. The experiment was repeated three times. Plants were incubated under greenhouse conditions. The air temperature during the experiment fluctuated between 18 and 33°C. Plants were watered as needed. To determine colonization of root tissue by the bacteria, plants were uprooted delicately from the pots and the root systems were thoroughly washed under running tap water, dried with sterile filter paper, and cut into 1-cm-long pieces. For each plant, samples of 2 g of root pieces were surface-deinfested in 1% NaOCl for 3 min, washed three times in sterile distilled water, and ground in 10 mL of 10 mM $MgSO_4.7H_2O$ using an autoclaved pestle and mortar. Serial dilutions of the macerates were plated onto LB agar (LB supplemented with spectinomycin and rifampin at 300 mg mL^{-1}) and incubated at 37°C for 48 h. Then, bacterial colonies were counted, and the bacterial populations were expressed as CFU g^{-1} of fresh root tissue.

The Protective Effect of Strain XNRB-3 on Plant Roots

The protective function of strain XNRB-3 on the roots of *M. hupehensis* Rehd. seedlings was verified by Periodic Acid Schiff (PAS) staining according to the method of Shao et al. (2020) and Duan et al. (2021).

Biological Control of the Strain XNRB-3

The ability of strain XNRB-3 to control ARD was investigated in a *Fusarium*—infested sterilized soil following the method described by Wu et al. (2019), with some modifications. Treatments included a sterilized soil inoculated with sterile distilled water as a negative control, a sterilized soil inoculated with *Fusarium* as a positive control, and sterilized soil inoculated with *Fusarium* and the strain XNRB-3. Each treatment included 15 *M. hupehensis* Rehd. seedlings. The spore suspension of *Fusarium* was first drenched into the sterilized soil, followed by a suspension of strain XNRB-3. The final concentration of *Fusarium* (10^5 spores g^{-1}) and strain XNRB-3 (10^8 CFU g^{-1}) was included in the growth substrates. Plant seedlings were then transplanted in the substrate trays (AC140), and then grown at 16 h light/8 h dark at 28°C. Plants were watered as required for plant growth and disease development. Each pot contained one *M. hupehensis* seedling, and all pots were arranged randomly with 15 replicates per treatment (Cachinero et al., 2002). The experiment was repeated three times. Disease severity was estimated over the course of 5 weeks starting 1 week after inoculation. The scoring criteria and calculation formula are presented in **Supplementary Table 8**.

The PDP was measured to estimate wilt severity and the ability of plants in different treatment groups to recover from the disease. To fulfill Koch's postulates, isolates from discolored fibrous roots were also obtained from all dead plants at the end of the experiment. Isolation and identification of each organism were performed to the genus level. The growth substrate samples near to plant root rhizosphere were collected weekly after being transplanted and stored at –20°C. The population of *Fusarium* in the growth substrate samples were determined by real-time PCR.

Separation and Identification of Extracellular Metabolites

Inhibition of Mycelial Growth by Cell-Free Culture Filtrate

The CFCF was used to assess influence of extracellular metabolites on *Fusarium* radial growth as described by Azabou et al. (2020), with some modifications. The bacterial culture was grown in a shaker incubator at 100 rpm for 72 h. The culture was then centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was collected and filtrated through 0.22-μm membrane filters. The CFCF was added to a warm PDA medium (55°C) to the final concentrations (from 5 to 75%). The PDA plates without culture filtrate were used as controls. Fungal mycelial plugs 5 mm in diameter were placed centrally in the amended media and incubated at 25°C until negative control growth covered the whole surface of the plate. The inhibition of the pathogen growth was estimated following the formula described by Erdogan and Benlioglu (2010): $PI = (D - d)/D \times 100\%$, where *PI*, inhibition of pathogen growth (%); *D*, diameter of pathogen growth in control plates (mm); and *d*, diameter of pathogen growth for the tests (mm).

Identification of Extracellular Metabolites by Gas Chromatography-Mass Spectrometry

The fermentation supernatant of strain XNRB-3 was extracted with ethyl acetate and then concentrated under reduced pressure with Rotary Evaporator N-1300D-WB (Tokyo, Japan) to obtain a crude extract. A small amount of methanol was added to dissolve the extracts and then passed through a Nylon66 0.22-μm filter membrane. The compounds in the extract were identified by GC-MS followed by an NIST17 database search. The GC-MS conditions were as described by Duan et al. (2021). The peak area normalization method was used to calculate the relative content of each component.

Verification of Synthetic Compounds Against Plant Fungal Pathogen

Among the identified extracellular metabolites, 16 standard compounds were purchased from the reagent company (**Supplementary Table 9**). The antifungal activity of the standard

compounds was assessed using the I-plate system described by Yuan et al. (2012). The I-plates combined with the addition of methanol or distilled water were used as the control. The colony diameter of the plant fungal pathogen was recorded after incubating for 7 days. The experiment was repeated three times.

Plant Growth Promotion Activities of Synthetic Compounds

The growth promotion activities of the 16 compounds were measured by the modified method as described by Duan et al. (2021). Briefly, the synthetic compounds were diluted separately in ethanol, and 20 μL of the resulting suspension was applied to a sterile filter paper disk on the other side of the I-plate. A total of 10, 100, 500, and 1,000 μg doses of each synthetic compound were tested. Each treatment was repeated three times. The fresh weight of the *Arabidopsis thaliana* Col-0 seedlings was measured after 10 days.

Carrier Characteristics

In the present study, 15 carriers purchased from different Chinese commercial enterprises through Taobao were used as formulation carriers for strain XNRB-3. The selected properties of these carrier candidates are listed in **Supplementary Table 10**. The carriers were dried to a moisture content of 5% in an oven (Shandong, China) at 80°C for 24 h, finely ground in a hammer mill to pass through a 1-mm screen, and stored at room temperature for further study.

The effect of the carrier as a substrate on the survival of strain XNRB-3 was evaluated using plastic bottles equipped with a 0.22-μm filter membrane in the cap to allow for air exchange, which was in reference to the method of Wei et al. (2015), with some modifications. Then, 20 mL of fermentation broth of strain XNRB-3 was mixed with 100 g of each carrier (sterilization at 115°C for 30 min). Carriers treated with 20 mL of aseptic fermentation broth were used as the controls. Each treatment included three replications (three bottles). In addition, these plastic bottles were covered with black plastic bags to avoid the influence of light on the survival of strain XNRB-3. The bottles were stored at 25°C and periodically sampled at 0, 10, 20, 30, 60, 90, 120, and 180 days post-inoculation. The population of strain XNRB-3 in each carrier at each sampling time point was determined by the plate count method using LB agar.

Four key factors including inoculation amount, pH, temperature, and rotating speed were selected to carry out the Box–Behnken test design with four factors and three levels. When the above fermentation conditions were optimized, except for the test single factor as a variable, the other conditions were unchanged, and the population number of strain XNRB-3 was determined for 180 d. Using Box–Behnken (Design-Expert 8.0.6) in the RSM to optimize the solid fermentation conditions of strain XNRB-3 (Anthony et al., 2009).

Pot Experiment

The BIO product was produced by Chuangdi Microbial Resources Co., Ltd. (Dezhou, China). The mixture of cow manure compost and wheat straw (1:2, w/w) was aerobically fermented with strain XNRB-3 for 6 days at 40°C, and the bacterial density was 5.0×10^9 CFU g^{-1}. All operations are carried out under optimized fermentation conditions. The content of organic matter was 46.21%, total nitrogen was 2.36%, P_2O_5 was 1.49%, and K_2O was 3.03%.

In May 2017, the pot experiment was conducted at the National Apple Engineering Experiment Center of the Horticultural Science and Engineering College of Shandong Agricultural University and the State Key Laboratory of Crop Biology (117.156540 longitude, 36.164443 latitude). The collection and treatment of the experimental soil was conducted as described by Duan et al. (2021). The physicochemical properties of the tested soil were presented in **Supplementary Table 11**. There were four treatments: (1) 31-year-old orchard soil (CK1), (2) 31-year-old orchard soil fumigated with methyl bromide (CK2), (3) bacterial fertilizer carrier treatment (T1), and (4) XNRB-3 bacterial fertilizer treatment (T2). The addition amount of strain XNRB-3 and fertilizer carrier accounts for about 1% of the weight of the soil. The plants received unified watering and manure management and sample collection and management according to that of Duan et al. (2021).

Field Experimental Trials

To test the potential of strain XNRB-3 bacterial fertilizer to control ARD under field conditions, the field test was carried out in Wangtou Village, Laizhou City (Shandong China, Long: 119.814701, Lat: 37.095159). Physicochemical properties of the tested soil are presented in **Supplementary Table 11**. After the apple orchard was rebuilt, severe ARD occurred, the growth of fruit trees was weak, and the survival rate was less than 50%. In March 2020, 28-year-old trees were removed from the orchard, and the replanted orchard was simultaneously established. The apple seedlings used in the experiment were 2-year-old grafted seedlings. The rootstock and spike combination was from Yanfu 3/T337. The grafted seedlings had a stem thickness of about 10 mm and a fixed stem of 1.4 m. They were purchased from Laizhou Nature Horticultural Technology Co., Ltd. (Shandong, China). The row spacing of the plants is 1.5 m × 4 m, and the tree shape was pruned to a spindle shape. The production of strain XNRB-3 bacterial fertilizer was as described above.

The experiment consisted of four treatments: 28-year-old orchard soil (CK1), 28-year-old orchard soil fumigated with methyl bromide (CK2), bacterial fertilizer carrier treatment (T1), and XNRB-3 bacterial fertilizer treatment (T2). A planting hole of 80 cm^3 was dug according to the row spacing, and the bacterial manure carrier and XNRB-3 bacterial manure were mixed with soil and backfill. The amount of application for each young tree was controlled at 1 kg, and 20 trees were treated for each. A new application was carried out at the beginning of the second spring. All indexes were measured on 15 July and 20 October in 2020 and 2021. The sample collection and management were conducted in accordance with that done by Sheng et al. (2020).

Measurement Indices

Physical and Chemical Properties of Soil

The physical and chemical properties and nutritional characteristics of the soil were measured as described by Xiang et al. (2021) and Zhang et al. (2021). The nitrogen in the soil was determined by the Kjeldahl method (Bremner, 1960), and the pH and electrolytic conductivity were measured

using a sample to water ratio of 1:2.5 (w/v) with a PHS-3E digital pH meter (LEICI, Shanghai, China) and DDSJ-318 conductivity meter (Lei Magnetic, Shanghai, China). The soil particle size distribution (the percentage of clay, silt, and sand) was determined by the hydrometer method (Avery, 1973).

Microbial Culture Methods

The populations of soil microbes (bacteria, fungi, and actinomycetes) were assessed using the dilution method of plate counting as described by Duan et al. (2021).

Biomass and Related Parameters

The biomass (height, new shoot growth, and ground diameter) was measured as described by Duan et al. (2021). The plant root analysis adopts the Microtek ScanMaker i800 Plus scanner (Shanghai Zhongjing Technology Co., Ltd., Shanghai, China) and OXY-LAB oxygen electrode system (Hansatech Ltd., Hansatech, United Kingdom) in reference to the method used by Gao et al. (2010) and Duan et al. (2021).

Enzyme Activity

Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity, and malondialdehyde (MDA) content were determined as described in Li et al. (2015). Soil urease, acid phosphatase (ACP), catalase, and sucrase activities were assayed as described in Liu J. et al. (2018).

DNA Extraction and Real-Time Quantitative Analysis

Sieved fresh soil (5.0 g) was used for DNA extraction with the DNeasy PowerMax Soil Kit (Qiagen, Hilden, Germany). The specific steps refer to the method of Duan et al. (2021). The concentration of plasmid DNA was measured and converted to copy the concentration using the following equation as described by Whelan et al. (2003): DNA (copy) = [6.02 $\times$ 10^{23} (copies moL^{-1}) $\times$ DNA amount (g)]/[DNA length (bp) $\times$ 660 (g moL^{-1} bp^{-1})]. The primers and annealing temperatures are presented in **Supplementary Table 3**. Sterile water was used as a negative control to replace the template. All real-time PCR reactions were done in technical triplicates such that each treatment was analyzed nine times.

Terminal-Restriction Fragment Length Polymorphism Analysis

The DNA was amplified using the universal primers 27F-FAM/1492R and ITS1F-FAM/ITS4R (**Supplementary Table 3**) that target the bacterial of the 16S rRNA gene and the fungal ITS region between the 18S and 28S rRNA regions, respectively. The specific steps refer to the method of Quéric and Soltwedel (2012), Xu et al. (2019), and Duan et al. (2021). The purified PCR product (500 ng) was digested by the restriction endonuclease *Msp*I (Takara Shuzo Co., Kyoto, Japan) for 16S rRNA gene amplicons and *Hin*fI (Takara Shuzo Co., Kyoto, Japan) for ITS amplicons in two separate reactions according to their respective protocols and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China) (Zhang et al., 2018). The Terminal-Restriction Fragment Length Polymorphism (T-RFLP) data analysis was conducted in reference to Duan et al. (2021).

Biolog Plate Analysis

Biolog-ECO plates (Biolog Inc., Hayward, CA, United States) and a plate reader (Multiskan MK3 ELISA) were used to determine the community-level physiological profiles. The assessment method was referenced from previous studies (Classen et al., 2003; Ge et al., 2018), with some modifications. Briefly, 3 g of incubated soil was mixed in 27 mL of a sterile NaCl solution (0.85%) and then oscillated at 220 rpm for 30 min. After the suspensions settled for 10 min, 3 mL aliquots of the suspensions were added to 27 mL of a sterile NaCl solution (0.85%) as tenfold serial dilutions until a final 1:1000 dilution was reached; 150 μL of the supernatant was then added to each well of the Biolog-ECO plates. Microplates were incubated at 25°C and 85% $\pm$ 5% humidity in a dark room. The rate of utilization was indicated by the reduction of tetrazolium violet, a redox indicator dye, which changes from colorless to purple. Data were recorded at 590 nm every 24 h up to 168 h. The OD values at the 96 h incubation were used for subsequent statistical analyses (Dang et al., 2015). All treatments had three triplicates.

During data treatment, OD readings from the control wells were subtracted from the OD of the treatment wells. Wells with negative OD well responses were coded as zeroes. Average well color development (AWCD) and average absorbance of each category were calculated in line with the recommendations of Garland (1996). Indices of Shannon-Wiener diversity index, Shannon evenness index, McIntosh index, and Simpson diversity index were used to evaluate the richness and dominance of species in the soil microbial community (Dang et al., 2015). According to the method described by Ge et al. (2018), PCA was used to analyze the data of Biolog-ECO microplates.

(1) AWCD = [Σ(Ci - R)]/n. *Ci* is the absorbance value of each reaction well at 590 nm, *R* is the absorbance value of the control well, and *n* is the number of wells.
(2) Shannon-Wiener diversity index (*H'*) reflects the species richness. H' = - ΣPi. ln(Pi). The *Pi* represents the ratio of the absorbance value in the ith (1 to 31) well to the total absorbance values of all wells.
(3) Shannon evenness index (*E*): E = H'/Hmax = H'/lnS, where *S* represents the total number of utilized carbon sources (31 carbon sources), the number of wells that vary in color.
(4) Simpson diversity index (*D*) reflects the most common species of the community, and was often used to assess the dominance degree of microbial community. D = 1- Σ $(Pi)^2$
(5) McIntosh index was used to measure the homogeneity degree of the community.

U = $\sqrt{\Sigma ni^{2\cdot}}$ ni was the relative absorbance of the ith hole (*Ci-R*).

Statistical Analysis

All statistical analyses were performed with the IBM SPSS 20.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, United States). Different lowercase letters represent significant differences between treatments (one-way ANOVA, $p < 0.05$)

according to Duncan's multiple range test. The figures were plotted with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, United States) and GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, United States). The R statistical platform (v.4.1.1) was used for principal coordinates (PCoA) and cluster analysis (Duan et al., 2021).

RESULTS

Isolation and Identification of Bacteria for Biocontrol Activity

The bacterium XNRB-3 was isolated from the root system of a healthy apple tree in a replanted orchard in Jincheng City, Shanxi Province. This bacterium had the strongest inhibitory effect on plant pathogenic fungi, with an inhibition rate of more than 70%. *F. oxysporum* and *Alternaria alternata* had the strongest inhibition rates, reaching 84.52 and 84.59%, respectively (**Figure 1c**).

Strain XNRB-3 was cultured on an LB liquid medium at 37°C for 24 h and formed biofilms. According to *Bergey's Manual of Systematic Bacteriology* (2nd edition) and the *Common Bacterial Identification Manual*, strain XNRB-3 matched the physiological and biochemical properties and morphological characteristics of *B. licheniformis* (**Supplementary Figure 2** and **Supplementary Table 12**). Tests of the carbon source utilization and chemical sensitivity revealed that strain XNRB-3 can utilize 32 carbon sources and was sensitive to pH 6, 1% NaCl, 4% NaCl, and 8% NaCl conditions. The values of probability (PROB), similarity (SIM), and distance (DIST) were 0.689, 0.689, and 4.698, respectively, and strain XNRB-3 belonged to *B. licheniformis*, which was identified based on carbon utilization (**Supplementary Tables 13, 14**).

Approximately 555–938 bases were sequenced for *rpoB* and *gyrA*, 1,455 bases for 16S rDNA, and 1,136 bases for *gyrB*. The ML analysis of identities based on the four gene sequence alignments revealed that strain XNRB-3 had the highest homology with *B. licheniformis* (**Supplementary Figure 3**). In sum, strain XNRB-3 was identified as *B. licheniformis*.

Plant Growth-Promoting Activities

The endophytic bacterium XNRB-3 possessed multiple PGP properties such as phosphate and potassium solubilization; nitrogen fixation; IAA, GA, and ABA production; ACC deaminase, ammonia, and amylase production; siderophore, cell wall-degrading enzyme (cellulase, pectinase, β1,3-glucanase, chitosanase, and protease), and antifungal activity against phytopathogens (**Supplementary Tables 6, 15** and **Supplementary Figures 4a–m**).

An HPCE method was used to identify 20 Phenyl isothiocyanate (PITC)-derivatized amino acids, 17 of which were separated (**Supplementary Table 16**), including aspartic acid (Asp), threonine (Thr), serine (Ser), glutamate (Glu), proline (Pro), glycine (Gly), alanine (Ala), cystine (Cys), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), and arginine (Arg).

Identification of Antibiotic Biosynthesis Genes

To determine the mechanisms underlying the effects of strain XNRB-3, PCR was used to screen strain XNRB-3 for genes involved in the biosynthesis of antibiotics. Amplicons of the expected sizes detected included *yndJ* (involved in biosynthesis of Yndj protein), *qk* (involved in subtilisin synthesis), *bamC* (involved in bacillomycin synthesis), *ituD* (involved in iturin A synthesis), *fen* and *fenD* (involved in fengycin synthesis), and *srf* and *srfAB* (involved in surfactin synthesis) (**Supplementary Figure 5**).

Optimization of Liquid Fermentation Conditions

The results of single-factor tests showed that the main carbon source affecting the growth of strain XNRB-3 was sucrose, the main nitrogen source was beef extract, and the inorganic salts were $MgSO_4$, KH_2PO_4, and KCl (**Supplementary Figures 1d,g,h**). According to the orthogonal test results (**Supplementary Table 17**), the organic salt that had the strongest effect on the growth of strain XNRB-3 was $MgSO_4$, followed by KH_2PO_4 and KCl. According to the K value, the optimal combination was F4I3E3 ($MgSO_4$ 1.0 g L^{-1}, K_2HPO_4 1.5 g L^{-1}, and KCl 1.0 g L^{-1}). According to the results of the single-factor experiment (**Supplementary Figure 1**), the Plackett–Burman experiment was performed using the experimental factors and levels in **Supplementary Table 18**, and Minitab 17 Software was used for multiple regression analysis. The optimal equation with OD_{600} as the response value was $OD_{600} = 0.4472 + 0.004009A - 0.004149B - 0.01018C - 0.01204D + 0.00649E - 0.01527F + 0.00465G + 0.001711H + 0.000272I$. According to the size of the *P*-value, the key factor affecting OD_{600} was A (temperature), followed by B (rotating speed), G (sucrose), H (beef extract), F ($MgSO_4$), I (KH_2PO_4), D (pH), C (liquid volume), and E (KCl) (**Supplementary Tables 19, 20**). The effect of A–H was highly significant ($p < 0.01$), the effect of F was significant ($p < 0.05$), and the effect of I–E was not significant. A Box–Behnken test was conducted with four factors (sucrose, beef extract, temperature, and rotating speed) and three levels (**Supplementary Table 21**). Design Expert 8.0.6 software was used to perform regression analysis and obtain the multiple quadratic regression equations: $Y = 22.054 - 0.1142A - 0.0875B + 0.4358C - 0.5942D - 0.1175AB + 1.6175AC + 0.1075AD - 0.4825BC - 0.0975BD - 0.6425CD - 0.8941A^2 - 0.4016B^2 - 1.7641C^2 - 2.1366D^2$, where *Y* is the inhibition zone diameter (mm), *A* is sucrose, *B* is beef extract, *C* is temperature, and *D* is rotating speed. An analysis of variance and a significance test of the regression model were conducted (**Supplementary Table 22** and **Table 1**), the *F*-value of the regression model was 94.09 ($p < 0.0001$), indicating that the regression model was robust. The coefficient of determination of the model was $R^2 = 0.9895$ and $R^2_{Adj} = 0.9790$, indicating a good fit. Therefore, the regression model could be used to analyze and predict the abundance of the strain XNRB-3. In the regression model, the primary term C, D and the secondary term AC, CD significantly affected the

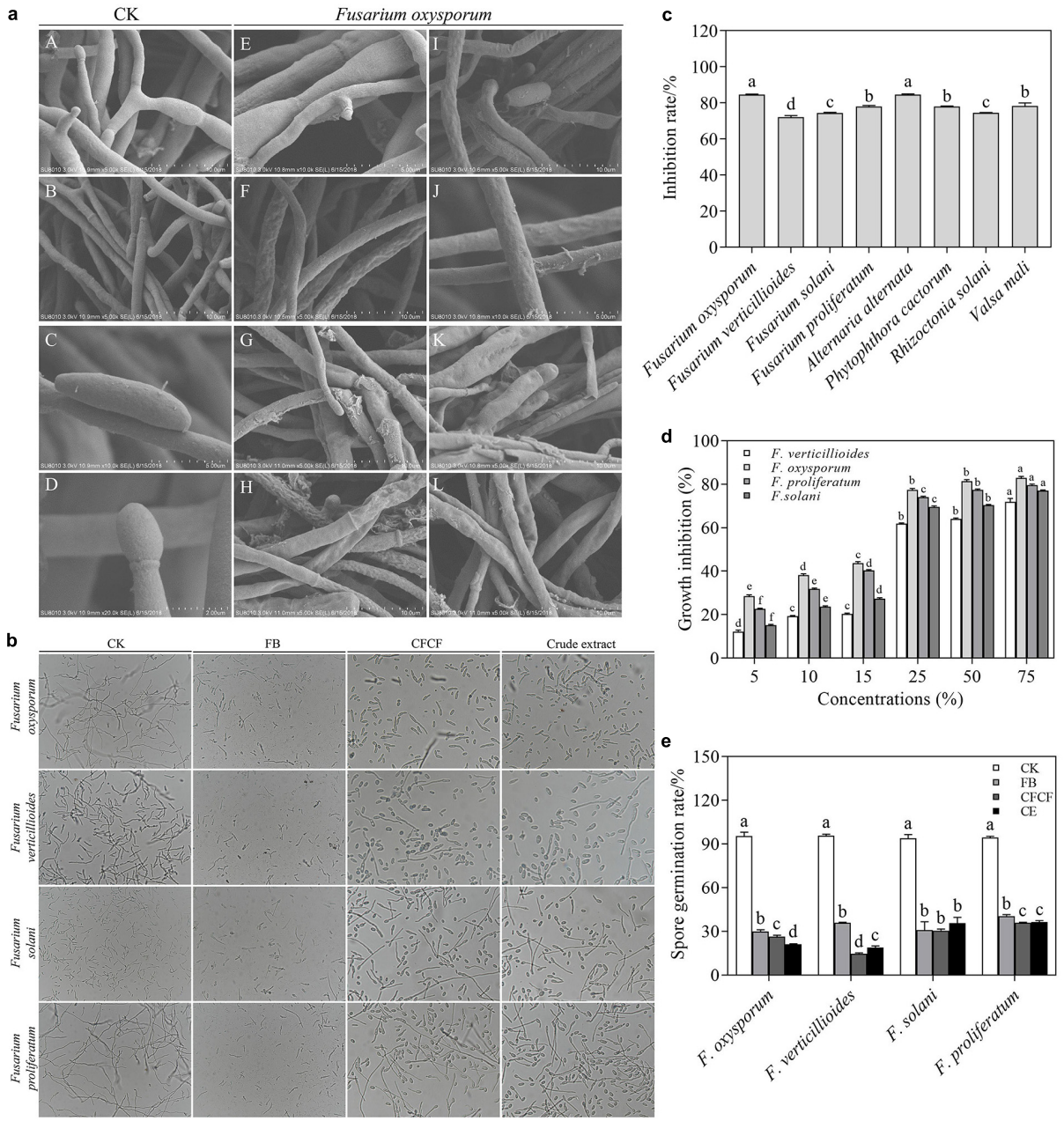

FIGURE 1 | Antifungal activity of strain XNRB-3 against plant fungal pathogen. **(a)** The mycelia and spore morphology of *Fusarium oxysporum* under the scanning electron microscope. (A–D) Was the normal mycelium and spore, (E–L) was the mycelium treated with fermentation broth. **(b)** Effects of different treatments on spore germination of *Fusarium*. CK, *Fusarium* spore suspension was mixed with sterile water at 1:1; FB, *Fusarium* spore suspension was mixed with fermentation broth at 1:1; CFCF, *Fusarium* spore suspension was mixed with cell-free culture filtrate at 1:1. Crude extract (CE), *Fusarium* spore suspension was mixed with extracellular metabolites at 1:1. **(c)** Inhibition rate of strain XNRB-3 against plant fungal pathogen. **(d)** Effects of different concentrations of CFCF on the *Fusarium* mycelial growth. **(e)** Germination rate of *Fusarium* spores after different treatments. Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

diameter of the inhibition zone ($p < 0.0001$); the significance of A, B, C, and D was the same based on the results of the Plackett–Burman test.

The interactions between temperature and sucrose, temperature and beef extract, and rotating speed and beef extract were the most significant; the interactions between rotating speed and sucrose, temperature and rotating speed, and the sucrose and beef extract on the diameter of the inhibition zone were relatively weak (**Supplementary Figure 6**). The optimal fermentation conditions for strain XNRB-3 predicted by the regression model were sucrose 21.03 g, beef extract 8.54 g, temperature 32.73°C, and rotating speed 191.46 rpm.

TABLE 1 | ANOVA for response surface quadratic model.

Source	Degree of freedom	Sum of squares	Mean squares	*F* value	*P*-value (Prob > F)	Significance
Model	14	62.98152943	4.498680673	94.09309895	<0.0001	**
A-Sucrose	1	0.156408333	0.156408333	3.271391293	0.092019216	
B-Beef extract	1	0.091875	0.091875	1.921630828	0.187359213	
C-Temperature	1	2.279408333	2.279408333	47.67544296	<0.0001	**
D-Rotating speed	1	4.236408333	4.236408333	88.60748683	<0.0001	**
AB	1	0.055225	0.055225	1.155070068	0.300664777	
AC	1	10.465225	10.465225	218.8876079	<0.0001	**
AD	1	0.046225	0.046225	0.96682868	0.342160859	
BC	1	0.931225	0.931225	19.47723176	0.000589533	*
BD	1	0.038025	0.038025	0.795319861	0.387567486	
CD	1	1.651225	1.651225	34.53654273	<0.0001	**
A^2	1	5.185200045	5.185200045	108.4521388	<0.0001	**
B^2	1	1.046070315	1.046070315	21.87930303	0.000356012	*
C^2	1	20.18588113	20.18588113	422.2020295	<0.0001	**
D^2	1	29.61073518	29.61073518	619.3295407	<0.0001	**
Residual	14	0.669353333	0.047810952			
Lack of Fit	10	0.472033333	0.047203333	0.956888979	0.568501936	
Pure Error	4	0.19732	0.04933			
Cor Total	28	63.65088276				

R^2 = 98.95%, R^2_{Adj} = 97.90%.
** and ** Represented significant difference at $p < 0.05$ and $p < 0.01$, respectively.*

The maximum theoretical value of the predicted inhibition zone diameter was 22.17 mm. To further verify the predicted value, three parallel experiments were performed using the optimized fermentation conditions. The diameter of the inhibition zone was 22.03 ± 0.24 mm, and the error from the theoretically predicted value (22.17 mm) was only 0.63%, indicating that the predicted value was a good fit with the measured value. Thus, the optimized model was reliable.

In summation, the optimal fermentation conditions for strain XNRB-3 were sucrose 21.03 g, beef extract 8.54 g, $MgSO_4$ 1.0 g, K_2HPO_4 1.5 g, KCl 1.0, 1 L, pH 7.5, temperature 32.73°C, rotating speed 191.46 rpm, inoculation amount 5%, and filling volume 40%. Under optimal conditions, the OD_{600} values (after dilution) of the strain XNRB-3 solution at different incubation times were measured and the growth curves were drawn (**Supplementary Figure 1b**).

Optimization of Solid Fermentation Conditions

A long and stable shelf life is one of the most important commercial characteristics of biocontrol products. Therefore, the population dynamics (an index of shelf life) of strain XNRB-3 using different candidate carriers were characterized in the LB medium. At the end of the storage period, the viable numbers of strain XNRB-3 in cow dung compost (CDC) and wheat straw were greater than 8.78 and 8.74 log CFU g^{-1} dry carrier, respectively. CDC: wheat straw maintained the largest populations of strain XNRB-3 at a ratio of 1:2 (**Figures 2M–O**). Design Expert 8.0.6 software was used to perform regression analysis of the test data of **Supplementary Tables 23, 24**, and the multiple quadratic regression equation obtained was as follows: Y = 8.716-0.43A + 1.2608B + 0.6767C + 0.2692D – 0.52AB – 0.155AC + 0.145AD + 0.6125BC + 0.465BD – 0.1475CD – $0.9405A^2$ – $2.0218B^2$ – $0.7355C^2$ – $0.4243D^2$, where *Y* is population (E + 08), *A* is the inoculation amount; *B* is pH, *C* is temperature, and *D* is rotating speed.

The regression model was further analyzed by variance analysis and significance tests (**Table 2**). The *F* value of the regression model was 102.13 ($p < 0.0001$), indicating that the regression model was robust. The coefficient of determination of the model was R^2 = 0.9903 and R^2_{Adj} = 0.9806, indicating a good fit. Therefore, the regression model could be used to analyze and predict the abundance of *B. licheniformis* XNRB-3. In the regression model, the effects of the first term A, B, C and the second term BC on the diameter of the inhibition zone were significant ($p < 0.0001$). The interactions between pH and inoculation amount, temperature and pH, and rotating speed and pH had the most significant effect on the population of strain XNRB-3 (**Figures 2A–L**). The optimal solid fermentation conditions for *B. licheniformis* XNRB-3 were predicted by the regression model: inoculation amount 20.25%, pH 8.25, temperature 39.23°C, and rotating speed 199.75 rpm. The predicted maximum theoretical value of the population of strain XNRB-3 was 8.91E + 08. To verify the predicted value, three parallel experiments were performed using the optimized fermentation conditions. The diameter of the inhibition zone was (22.03 ± 0.24) mm and the error from the theoretically predicted value (8.91E + 08) was only 0.86%, indicating that there was a good fit between the predicted value and the measured value and that the optimized model was robust.

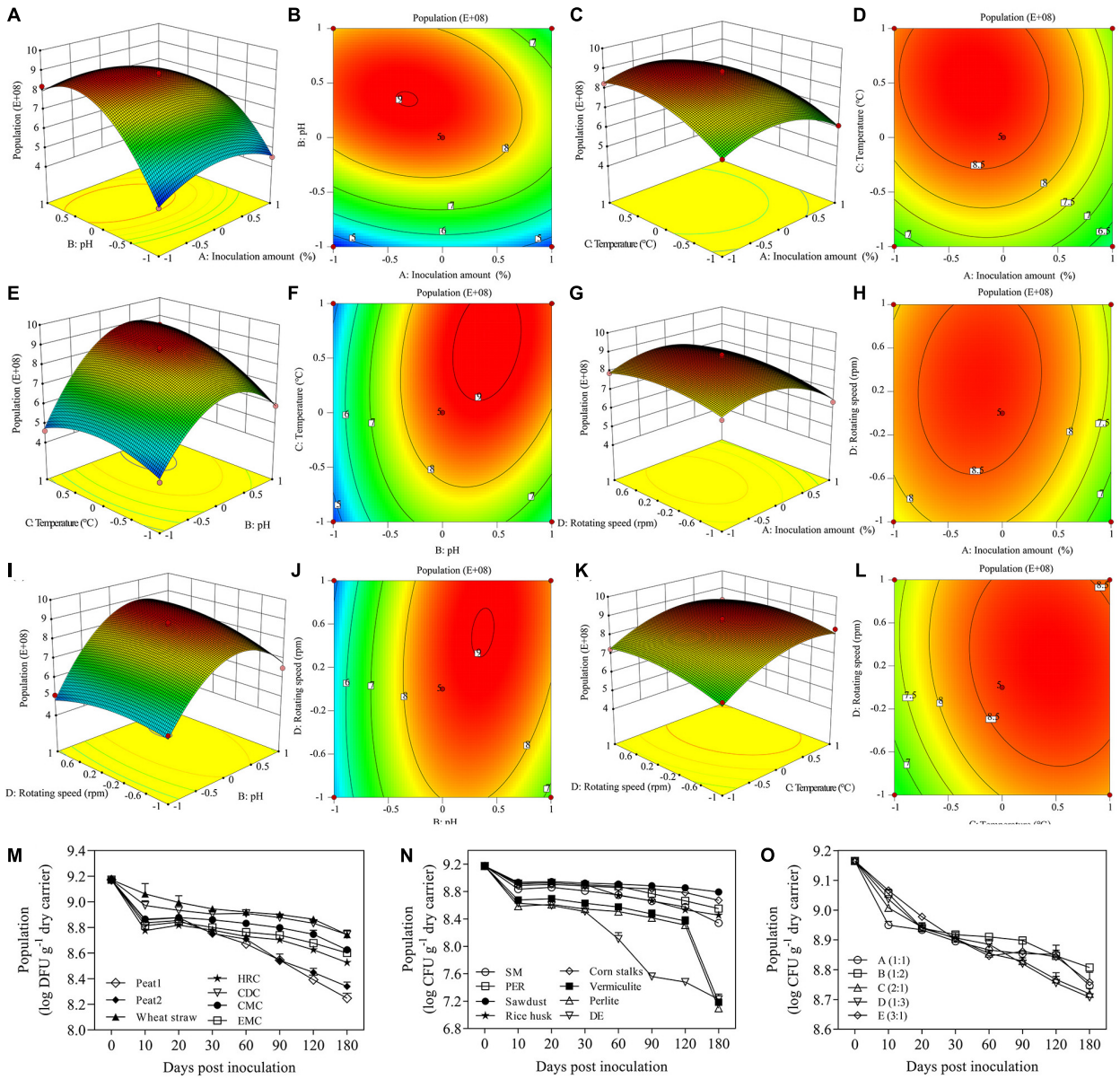

FIGURE 2 | Response surface analysis three-dimensional and contour plot for population **(A–L)**. Shelf life of *B. licheniformis* XNRB-3 in different carrier formulations **(M,N)**. Plate counts on V8-salt medium from Earthworm manure compost (EMC), *Pleurotus eryngii* residue (PER), Chicken manure compost (CMC), Herb residue compost (HRC), cow dung compost (CDC), Diatomite earths (DE), Soybean meal(SM), peat1, and peat2 carriers inoculated with aseptic fermentation broth or *B. licheniformis* XNRB-3 were determined on different days post-inoculation. **(O)** Shelf life of *B. licheniformis* XNRB-3 under different carrier ratios. A: CDC: wheat straw = 1:1, B: CDC: wheat straw = 1:2, C: CDC: wheat straw = 2:1, D: CDC: wheat straw = 1:3, E: CDC: wheat straw = 3:1.

In sum, the optimal solid fermentation component of *B. licheniformis* XNRB-3 was cow dung: wheat straw = 1:2, and the optimal conditions were inoculation amount 20.25%, pH 8.25, temperature 39.23°C, and rotating speed 199.75 rpm.

Determination of Antifungal Activity

Microscopic observations of hyphal and spore morphology revealed that the control *Fusarium* mycelium was uniform in thickness and slender with fewer branches, spore structure was complete, and growth was strong (**Figure 1a**A–D). The mycelia treated with the CFCF were irregularly reticulated, uneven in thickness, shrunken (**Figure 1a**G–L), thinned (**Figure 1a**I,K), the cells had ruptured, and cell contents had overflowed (**Figure 1a**G,H,I,K), the spore cell wall was broken and deformed (**Figure 1a**E,H,I,K). Furthermore, FB, CFCF, and crude extract significantly inhibited the spore germination of *Fusarium* spores, and the spore germination rate decreased by more than 60% (**Figures 1b,e**). The antifungal metabolites produced by strain XNRB-3 were extracted from 3-day-old CFCF. As the concentration of extracellular metabolites increased, the

inhibitory effect on the growth of *Fusarium* became more pronounced. At higher concentrations (25, 50, and 75%), the inhibition rate reached more than 60% (**Figure 1d**).

Identification of Antifungal Compounds

The results of the experiment conducted for the present study showed that a 25% concentration of strain XNRB-3 CFCF showed high activity against fungal pathogens. After GC-MS chromatographic detection and analysis, the main antibacterial substances with Area% > 0.62 and retention index RI > 700 were selected (**Supplementary Figure 7** and **Supplementary Table 25**). Most substances identified from the components were organic acids and esters, alcohols, ketones, alkanes, and phenols (**Figure 3**). Among the 42 identified compounds, 16 pure compounds were purchased for individual testing of antifungal properties (**Supplementary Figure 8**). The nine pure compounds showed antifungal activity against pathogenic fungi (**Supplementary Table 26**). Among them, 2,4-di-tert-butylphenol and alpha-bisabolol had the strongest inhibitory effect on plant pathogenic fungi, especially *Fusarium*. At a concentration of 1000 $\mu g\ L^{-1}$, the inhibition rate was higher than 50% (**Figures 4a,b**). Of the nine pure compounds, the fresh weight and root growth of Arabidopsis plants compared with the control treatment (water and ethanol) were significantly enhanced by butanedioic acid, monomethyl ester (500 μg), and dibutyl phthalate (500 μg) (**Figures 5a–d**). Compared with ethanol treatment, the fresh weight, root length, number of primary roots, and number of secondary roots of Arabidopsis seedlings were increased by approximately 2.56, 1.42, 0.91, and 5.5 times, respectively, when treated with dibutyl phthalate; by 2.01, 4.08, 0.33, and 4.5 times, respectively, when treated with butanedioic acid and monomethyl ester; and by 1.89, 0.89, 0.71, and 10.5 times, respectively, when treated with 3-nonen-2-one. The above findings indicate that the main antifungal compounds produced by strain XNRB-3 play important roles in disease control and growth.

Phlorizin Degradation Ability of Strain XNRB-3

The phlorizin utilization efficiency by strain XNRB-3 in the MSM solution was high (**Supplementary Figure 4q**). After culture for 60 h, the degradation rate was 60.75, 64.79, and 68.83% when the amount of inoculum added was 1, 1.5, 2%, respectively. The ability of strain XNRB-3 to degrade phlorizin increased as the amount of inoculum added increased. Strain XNRB-3 could effectively utilize CA, BA, FA, and PHBA (**Supplementary Table 27**), and the degradation rates ranged from 45.65% to 69.20%. Strain XNRB-3 could also efficiently degrade phlorizin, CA, BA, FA, and PHBA in soil (**Table 3**). The content of phlorizin in the soil decreased to 2.9292 $\mu g\ g^{-1}$ at 9 days after inoculation. The content of BA and PHBA in the soil decreased to 18.4953 and 5.2882 $\mu g\ g^{-1}$, respectively. The FA and CA concentrations in the soil decreased to 13.3669 and 0.3785 $\mu g\ g^{-1}$ at 9 days after treatment, respectively, which was only 13.31 and 3.63% of the control concentration.

The Protective Effect of Strain XNRB-3 on Plant Roots

Strain XNRB-3 can form a thick biofilm in a static medium. After 14 days of planting, the population of strain XNRB-3 colonized on root tissue was approximately 7.30×10^6 CFU

TABLE 2 | ANOVA for response surface quadratic model.

Source	Degree of freedom	Sum of squares	Mean squares	*F* value	*P*-value (Prob > F)	Significance
Model	60.51220155	14	4.322300111	102.1311598	<0.0001	**
A-Inoculation amount	2.2188	1	2.2188	52.4277842	<0.0001	**
B-pH	19.07640833	1	19.07640833	450.7543805	<0.0001	**
C-Temperature	5.494533333	1	5.494533333	129.8297313	<0.0001	**
D-Rotating speed	0.869408333	1	0.869408333	20.54315508	0.0005	*
AB	1.0816	1	1.0816	25.55700892	0.0002	*
AC	0.0961	1	0.0961	2.270736462	0.1541	
AD	0.0841	1	0.0841	1.987189765	0.1805	
BC	1.500625	1	1.500625	35.45810513	<0.0001	**
BD	0.8649	1	0.8649	20.43662816	0.0005	*
CD	0.087025	1	0.087025	2.056304273	0.1735	
A^2	5.737558378	1	5.737558378	135.5721437	<0.0001	**
B^2	26.51333878	1	26.51333878	626.4808023	<0.0001	**
C^2	3.508931351	1	3.508931351	82.91215777	<0.0001	**
D^2	1.167490135	1	1.167490135	27.58649759	0.0001	*
Residual	0.592495	14	0.042321071			
Lack of Fit	0.490975	10	0.0490975	1.934495666	0.2745	
Pure Error	0.10152	4	0.02538			
Cor Total	61.10469655	28				

R^2 = 99.03%, R^2_{Adj} = 98.06%.

** and ** Represented significant difference at $p < 0.05$ and $p < 0.01$, respectively.*

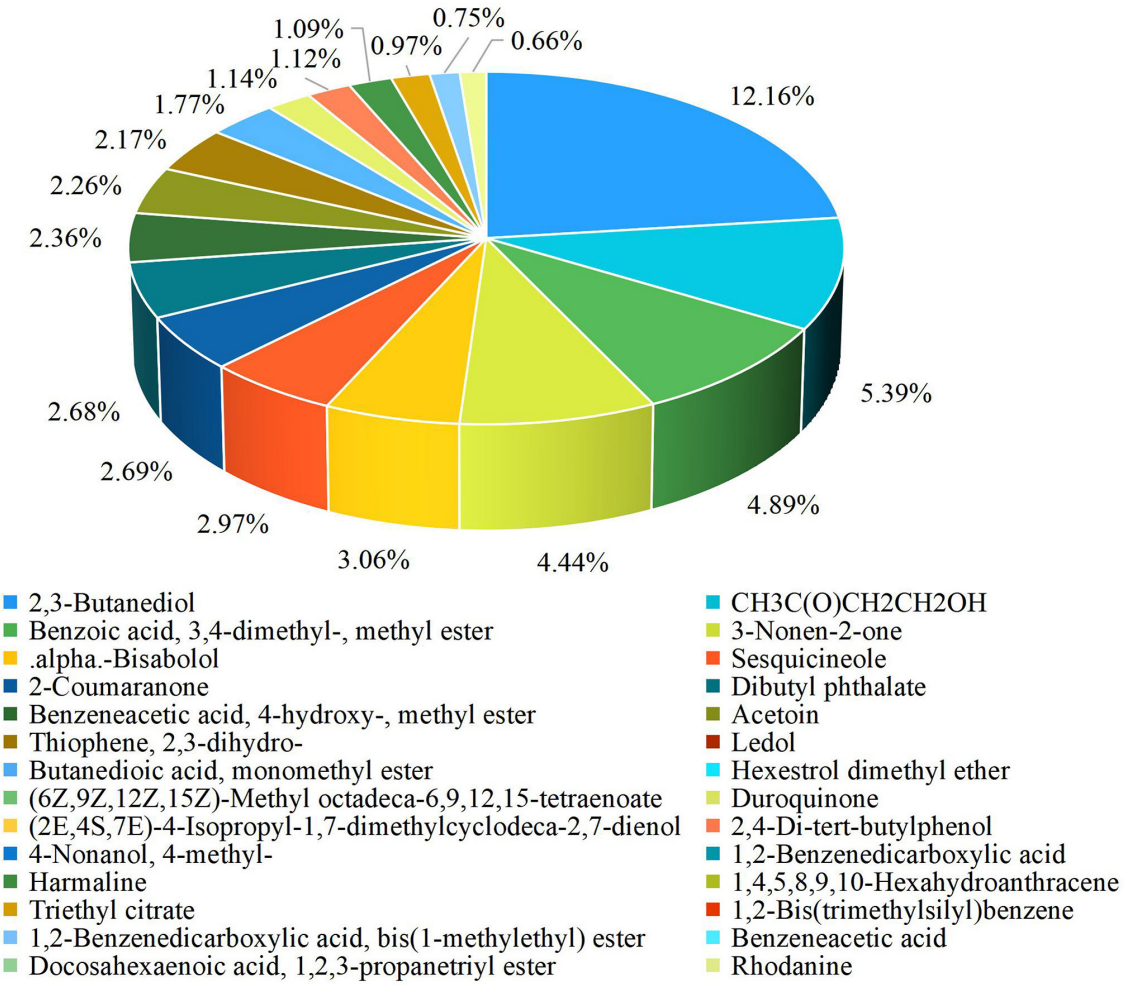

FIGURE 3 | GC-MS identification result of extracellular metabolites.

g^{-1}. The population sharply decreased to 6.47×10^4 CFU g^{-1} after 35 days (**Supplementary Figure 9b**). The conidia and hyphae of *Fusarium* treated with strain XNRB-3 only attached to root epidermal cells (**Supplementary Figures 10A–D**). *Fusarium* hyphae invaded the cortical cells through the intercellular layer and then entered the vascular column, and a large amount of cell content (sticky substances and starch granules) was also observed in the cortical cells and vascular column (**Supplementary Figures 9a, 10**).

Disease Severity Assessment

In the greenhouse test, the symptoms of Fusarium wilt appeared 7 days after the plant seedlings were transplanted into the infected soil. The incidence of *Fusarium* wilt increased rapidly over the next 28 days, and the disease severity index increased progressively over the experimental period and ultimately reached 4.00 ± 0.00; a reduction in disease progress was noted in plants treated with strain XNRB-3 (**Table 4**). In the fifth week, the disease index and relative control effect of plant seedlings inoculated with strain XNRB-3 were both stable at 33 and 50%, respectively. During the test period, plants inoculated with sterile distilled water remained healthy. The disease symptoms were the result of artificial infection with *Fusarium*, which was successfully re-isolated from inoculated plants at the end of the experiment. According to RT-PCR, the size of the *Fusarium* populations of the two treatments significantly increased after 21 days of planting in the infected soil. Compared with the CK treatment (5.50×10^5 copies g^{-1} soil), the size of the *Fusarium* population treated with strain XNRB-3 (2.37×10^5 copies g^{-1} soil) was significantly reduced (**Supplementary Figure 9c**). These results were consistent with the incidence of the aforementioned diseases.

The same results were obtained in the outdoor pot experiment. The biomass of apple plants treated with strain XNRB-3 (T2) in September was significantly higher than that of CK1. The plant height, ground diameter, fresh weight, and dry weight were increased by 88.07, 61.28, 181.35, and 140.44%, respectively, which were second only to the methyl bromide fumigation treatment (**Supplementary Figure 11**). Treatment with strain XNRB-3 also significantly enhanced the root growth of apple

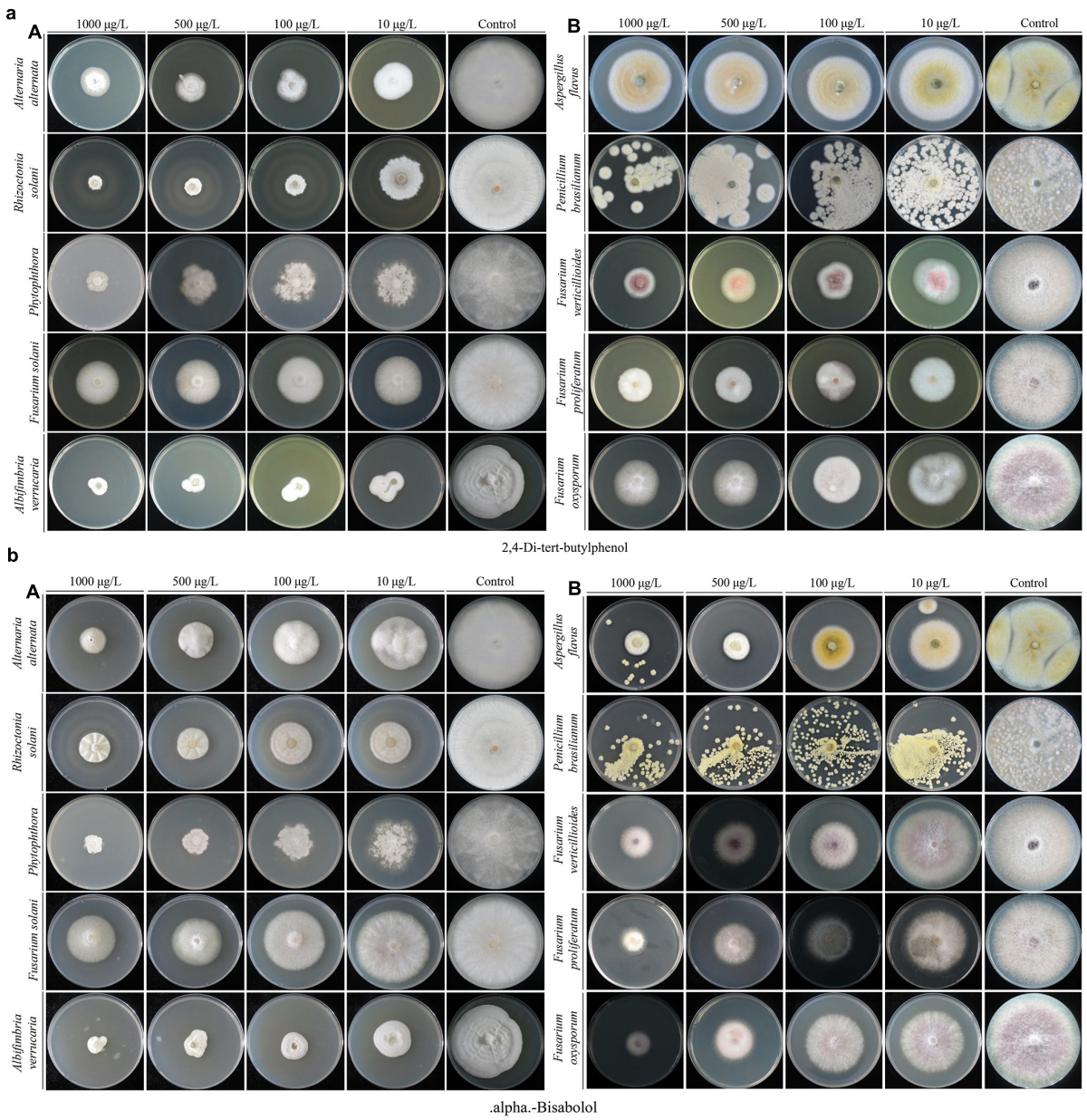

FIGURE 4 | Growth inhibition of plant fungal pathogens on the PDA medium after treated with different gradient 2,4-Di-tert-butylphenol **(a)** and alpha-Bisabolol **(b)**.

plants (**Figures 5e,i,j,l,m**). In September, the plants had grown considerably, and the root length, surface area, number of tips, and number of forks were significantly lower in CK1 than in CK2 and T2. The length, surface area, number of tips, and number of forks were 1.73, 2.62, 5.67, and 2.65 times higher in T2 than in CK1. The root respiration rate and the SOD, POD, and CAT activity increased from July to September in T2 and CK2 (**Figures 5e–h,k,n**). In September, the root respiration rate in T2 was 1037.98 μmol $O_2 \cdot g^{-1}$ FW$\cdot min^{-1}$, which was 1.49 and 1.32 times higher compared with that observed in CK1 and T1. The activity of SOD, POD, and CAT was 1.50, 2.93, and 1.72 times higher in T2 than in CK1. The MDA content showed the opposite trend: the MDA content was 29.96, 60.35, and 78.13% lower in July, August, and September, respectively, in T2 compared with CK1.

Effect of Strain XNRB-3 on the Biomass of Young Apple Trees

The XNRB-3 bacterial fertilizer treatment (T2) significantly promoted the growth of young apple trees, and this difference became significant in October 2021 (**Figures 6A,B**). In July and October in 2020 and 2021, the biomass indicators were significantly lower in CK1 and T1 than in CK2 and T2. In October

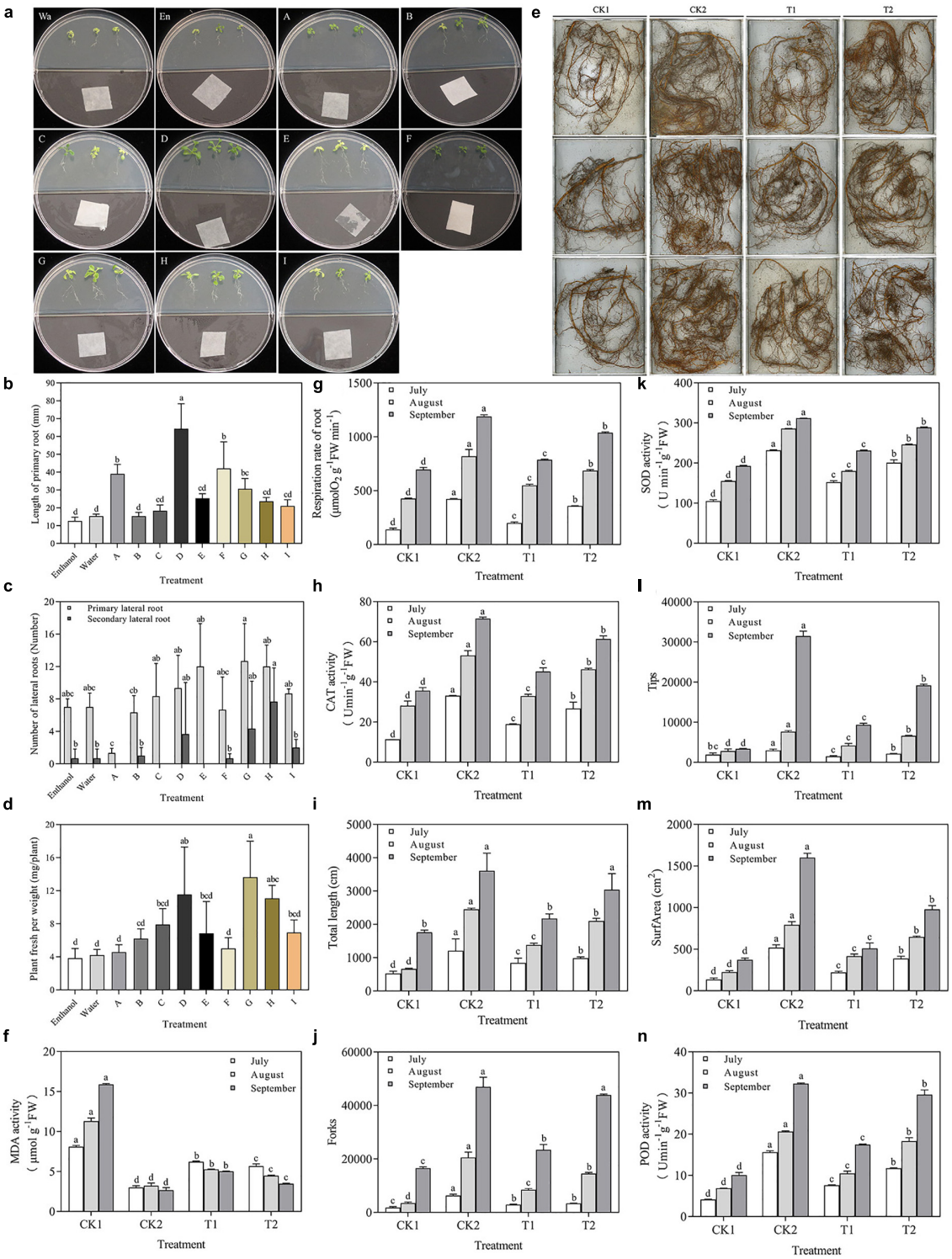

FIGURE 5 | (A) Growth promotion of *Arabidopsis thaliana* Col-0 with exposure to pure compounds. **(a)** Wa, Water, En, Enthanol, (A) 2,3-Butanediol (100 μg), (B) 1,2-Benzenedicarboxylic acid, bis(1-methylethyl) ester (100 μg), (C) 2,4-Di-tert-butylphenol (500 μg), (D) Butanedioic acid, monomethyl ester (500 μg), (E) alpha-Bisabolol (500 μg), (F) Acetoin (500 μg), (G) Dibutyl phthalate (500 μg), (H) 3-Nonen-2-one (500 μg), (I) Benzoic acid, 3,4- dimethyl-, methyl ester (100 μg). **(b)** Plant fresh per weight, **(c)** length of primary root, **(d)** Number of lateral roots. **(e)** Effect of different treatments on the root architecture of *Malus hupehensis* Rehd. seedlings. A Root system scan obtained by Microtek ScanMaker i800 Plus. **(f–n)** Physiological indicators of root system, including MDA activity, root respiration rate, CAT activity, total root length, the number of root bifurcation (forks), SOD activity, root tips, root surface area, and POD activity. Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

2021, the plant height, ground diameter, number of branches, and average branch length increased by 38.10, 62.79, 49.17, and 176.00% in T2 relative to CK1, respectively, and the plant height, ground diameter, number of branches, and average branch length were 1.27, 1.25, 1.37, 1.77, 2.45, and 2.21-fold higher in T2 than in T1, respectively (**Figures 6C–F**).

Effect of Strain XNRB-3 on the Soil Environment

In October of 2020 and 2021, the soil phenolic acid content was highest in CK1 and T1 and lowest in CK2 and T2 (**Figures 7A,B**). The total soil phenolic acid content was significantly lower in T2 than in CK1. In October 2020, the soil content of cinnamic acid, phlorizin, benzoic acid, ferulic acid, and *p*-hydroxybenzoic acid was 54.38, 74.02, 77.29, 54.70, and 71.83% lower, respectively, in T2 than in CK1. In October 2021, the soil content of cinnamic acid, phlorizin, benzoic acid, ferulic acid, and *p*-hydroxybenzoic acid was 87.23, 91.95, 89.80, 84.11, and 91.98% lower, respectively, in T2 than in CK1.

The activity of urease, phosphatase, sucrase, and catalase increased steadily in T1 and T2 in July and October in 2020 and 2021 relative to CK1. The soil enzyme activity decreased in the first year of the fumigation treatment; it then continued to increase, increasing most significantly in T2 (**Figures 7G–J**). In October 2020 and 2021, the urease activity was 1.65-fold and 1.64-fold higher, the phosphatase activity was 1.56-fold and 1.93-fold higher, the sucrase activity was 2.03-fold and 2.34-fold higher, and the catalase activity was 1.69-fold and 1.87-fold higher in T2 than in CK1, respectively. After 2 years of applying strain XNRB-3, the physical and chemical properties of plant rhizosphere soil were significantly improved. Compared with CK1, organic matter, total nitrogen, total phosphorus, total potassium, available potassium, available phosphorus, NH_4^+-N, nitrate nitrogen, and soil pH increased by 228.54, 245.25, 242.29, 30.53, 327.89, 128.64, 56.05, 239.61, and 7.43%, respectively, in T1. Various nutrient indexes of the soil were also increased in T1, indicating that the addition of organic amendments could enhance the nutrient conditions of the soil (**Supplementary Figure 12**).

In July and October of 2020 and 2021, the number of soil bacteria in the XNRB-3 bacterial fertilizer treatment (T2) increased significantly, and compared with CK1, the number of soil bacteria was increased by 107.21, 45.13, 100.79, and 113.08% in T2, respectively (**Figure 7C**). There were significant differences in the number of soil fungi between different treatments. Compared with CK1, the number of soil fungi in CK2, T1, and T2 was significantly reduced, the number of soil fungi in T1 was significantly higher than that in T2, and the effect of T2 was similar to the fumigation treatment. In October 2020, 2021, the number of soil fungi in CK2 and T2 was reduced by 58.55, 50.26%, and 62.74, 52.83% compared with CK1, respectively (**Figure 7D**). The number of actinomycetes and the ratio of bacteria/fungi in the soil were significantly higher in T2 than in CK1 (**Figures 7E,F**).

The qPCR results showed that the abundance of *Fusarium* was significantly reduced in July and October of 2020 and 2021 in CK2 and T2 compared with CK1 and T1 (**Figures 7K–N**). The abundance of *F. proliferatum*, *F. solani, F. verticillioides*, and *F. oxysporum* was 50.43, 40.74, 25.96, and 22.20% lower in T2 relative to T1 in October 2020, and 60.43, 57.97, 28.69, and 40.31% lower in T2 relative to T1 in October 2021, respectively.

Effect of Strain XNRB-3 on the Soil Microbial Community

In the present study, AWCD was used as an indicator of soil microbial activity. The variation in AWCD with incubation time is shown in **Figures 8I,L**. Here, AWCD increased as the incubation time extended for all treatments in October 2020 and 2021. The soil with strain XNRB-3 (T2) had a higher AWCD value than soil in other treatments, signifying that the addition of strain XNRB-3 increased the activity of microorganisms. After 120 h, the AWCD values of T1 and CK1 were similar, and the AWCD value was lower in CK2, indicating that fumigation can inhibit the activity of microorganisms for a longer period.

Biolog-ECO plates have six categories of carbon sources: carbohydrates, carboxylic acids, amino acids, polymers, phenolic compounds, and amines (Guo et al., 2015). In October 2020 (**Figures 8I–K**), the OD value of three types of carbon sources (polymers, miscellaneous, carboxylic acids, and amino acids) increased in T2 relative to CK1, but did not reach significant levels except for miscellaneous. In contrast, amines/amines and carbohydrates were reduced in T2, but did not reach

TABLE 3 | Phenolic acids degradation of strain XNRB-3 in soil.

Treatment	Time	CA	Phlorizin	BA	FA	PHBA
CK	0 Day	10.4321 ± 0.1703^{a}	30.0569 ± 0.0228^{a}	90.4921 ± 0.0536^{a}	100.4403 ± 0.1695^{a}	20.6373 ± 0.1954^{a}
	3 Days	6.6133 ± 0.0349^{b}	23.8619 ± 0.5910^{c}	83.8100 ± 0.2810^{b}	83.8655 ± 0.7399^{b}	18.3768 ± 0.2350^{b}
	6 Days	5.7945 ± 0.0349^{c}	24.9330 ± 0.0137^{b}	77.0167 ± 0.4360^{c}	71.1995 ± 0.3209^{c}	12.2645 ± 0.1751^{c}
	9 Days	5.6424 ± 0.3036^{cd}	24.1495 ± 0.5873^{bc}	67.5674 ± 0.6518^{d}	62.6178 ± 0.2685^{d}	10.3960 ± 0.2109^{d}
Strain XNRB-3	0 Day	10.4321 ± 0.1703^{a}	30.0569 ± 0.0228^{a}	90.4921 ± 0.0536^{a}	100.4403 ± 0.1695^{a}	20.6373 ± 0.1954^{a}
	3 Days	5.2219 ± 0.1101^{e}	14.4323 ± 0.2139^{d}	44.6154 ± 0.0853^{e}	$48.1822 \pm 0.6503_{e}$	12.5532 ± 0.0382^{c}
	6 Days	1.6232 ± 0.0377^{f}	9.4797 ± 0.1108^{e}	35.6996 ± 0.1247^{f}	31.8586 ± 0.0451^{f}	8.9623 ± 0.0035^{e}
	9 Days	0.3785 ± 0.0045^{g}	2.9292 ± 0.0120^{f}	18.4953 ± 0.0370^{g}	13.3669 ± 0.0380^{g}	5.2882 ± 0.0404^{f}

The soil samples were added with phlorizin, cinnamic acid (CA), ferulic acid (FA), benzoic acid (BA), and P-hydroxybenzoic acid (PHBA) ($\mu g\ g^{-1}$). Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

TABLE 4 | Percentage of dead plants, FMS, and AUDPC of *Malus hupehensis* Rehd. seedlings after inoculation strain XNRB-3.

Treatment		Investigation time	DI (%)	Relative control effect (%)	Incidence (%)	PDP (%)	FMS	AUDPC
Negative control		Weeks 1–5	0.00 ± 0.00	–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Positive control	*Fusarium proliferatum*	Week 1	34.45 ± 3.64^{a}	–	64.44 ± 8.01^{a}	0.00 ± 0.00^{c}	2.15 ± 0.05^{a}	30.14 ± 3.19^{a}
		Week 2	67.22 ± 0.55b	–	100 ± 0.00^{a}	55.55 ± 2.22^{a}	2.69 ± 0.02^{a}	88.96 ± 3.67^{a}
		Week 3	87.78 ± 1.47a	–	100 ± 0.00^{a}	68.89 ± 2.22^{b}	3.51 ± 0.06^{a}	135.62 ± 1.46^{a}
		Week 4	100 ± 0.00a	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	149.97 ± 1.35^{a}
		Week 5	100 ± 0.00a	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	284.96 ± 1.62^{a}
	Fusarium verticillioides	Week 1	33.89 ± 0.65a	–	68.89 ± 2.22^{a}	0.00 ± 0.00	1.97 ± 0.03^{a}	29.65 ± 0.49^{a}
		Week 2	70.00 ± 1.67^{ab}	–	100 ± 0.00^{a}	48.89 ± 2.22^{ab}	2.80 ± 0.07^{a}	90.90 ± 1.94^{a}
		Week 3	87.78 ± 0.55^{a}	–	100 ± 0.00^{a}	68.89 ± 2.22^{b}	3.51 ± 0.02^{a}	138.06 ± 1.75^{a}
		Week 4	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	151.18 ± 1.06^{a}
		Week 5	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	286.42 ± 1.27^{a}
	Fusarium oxysporum	Week 1	30.56 ± 1.11^{a}	–	64.44 ± 5.88^{a}	0.00 ± 0.00	1.92 ± 0.12^{a}	26.74 ± 0.97^{a}
		Week 2	70.00 ± 1.92^{ab}	–	100 ± 0.00^{a}	48.89 ± 2.22^{ab}	2.80 ± 0.08^{a}	87.99 ± 2.57^{a}
		Week 3	91.67 ± 0.96^{a}	–	100 ± 0.00^{a}	80.00 ± 0.00^{a}	3.67 ± 0.04^{a}	141.46 ± 1.46^{a}
		Week 4	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	154.59 ± 0.84^{a}
		Week 5	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	290.50 ± 1.01^{a}
	Fusarium solani	Week 1	35.00 ± 0.00^{a}	–	64.44 ± 4.44^{a}	0.00 ± 0.00	2.19 ± 0.14^{a}	30.63 ± 0.00^{a}
		Week 2	72.22 ± 2.00^{a}	–	100 ± 0.00^{a}	42.22 ± 5.88^{b}	2.89 ± 0.08^{a}	93.82 ± 1.75^{a}
		Week 3	88.33 ± 1.67^{a}	–	100 ± 0.00^{a}	71.11 ± 2.22^{b}	3.53 ± 0.07^{a}	140.49 ± 3.19^{a}
		Week 4	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	152.64 ± 2.32^{a}
		Week 5	100 ± 0.00^{a}	–	100 ± 0.00^{a}	100 ± 0.00^{a}	4.00 ± 0.00^{a}	288.17 ± 2.78^{a}
Strain XNRB-3	*Fusarium proliferatum*	Week 1	1.11 ± 0.56^{b}	96.39 ± 1.81^{ab}	4.44 ± 2.22^{c}	0.00 ± 0.00	0.67 ± 0.33^{b}	0.97 ± 0.49^{b}
		Week 2	8.33 ± 0.00^{cd}	87.60 ± 0.10^{b}	26.67 ± 3.85^{c}	0.00 ± 0.00^{c}	1.31 ± 0.19^{b}	8.26 ± 0.49^{b}
		Week 3	12.22 ± 1.11^{b}	86.11 ± 1.07^{a}	33.33 ± 3.85^{b}	0.00 ± 0.00^{c}	1.48 ± 0.08^{bc}	17.99 ± 0.97^{b}
		Week 4	23.89 ± 0.56^{d}	76.11 ± 0.56^{a}	42.22 ± 8.01^{b}	6.67 ± 0.00^{b}	2.46 ± 0.52^{b}	24.79 ± 0.73^{b}
		Week 5	35.56 ± 0.56^{d}	64.44 ± 0.56^{a}	68.89 ± 2.22^{b}	6.67 ± 0.00^{c}	2.07 ± 0.03^{d}	60.96 ± 0.77^{d}
	Fusarium verticillioides	Week 1	4.44 ± 0.56^{b}	86.83 ± 1.83^{c}	17.78 ± 2.22^{b}	0.00 ± 0.00	0.67 ± 0.33^{b}	3.89 ± 0.49^{b}
		Week 2	7.22 ± 0.55^{d}	89.71 ± 0.53^{a}	24.45 ± 2.22^{c}	0.00 ± 0.00^{c}	1.19 ± 0.10^{b}	10.21 ± 0.00^{b}
		Week 3	14.44 ± 3.09^{b}	83.59 ± 3.44^{a}	40.00 ± 6.67^{b}	0.00 ± 0.00^{c}	1.42 ± 0.09^{c}	18.96 ± 3.03^{b}
		Week 4	30.56 ± 0.56^{b}	69.44 ± 0.56^{b}	53.33 ± 3.85^{b}	0.00 ± 0.00^{c}	2.31 ± 0.14^{b}	29.17 ± 2.95^{b}
		Week 5	48.33 ± 0.96^{b}	51.67 ± 0.96^{c}	66.67 ± 3.85^{b}	20.00 ± 0.00^{b}	2.91 ± 0.11^{b}	76.42 ± 3.66^{b}
	Fusarium oxysporum	Week 1	2.78 ± 0.55^{b}	90.82 ± 1.99^{bc}	11.11 ± 2.22^{bc}	0.00 ± 0.00	0.67 ± 0.33^{b}	2.43 ± 0.49^{b}
		Week 2	6.67 ± 0.00^{d}	90.46 ± 0.26^{a}	22.22 ± 2.22^{c}	0.00 ± 0.00^{c}	1.22 ± 0.11^{b}	8.26 ± 0.49^{b}
		Week 3	12.78 ± 0.55^{b}	86.07 ± 0.49^{a}	31.11 ± 2.22^{b}	0.00 ± 0.00^{c}	1.65 ± 0.05^{b}	17.01 ± 0.49^{b}
		Week 4	26.11 ± 0.56^{c}	73.89 ± 0.56^{a}	44.44 ± 5.88^{b}	6.67 ± 0.00^{b}	2.45 ± 0.38^{b}	25.52 ± 0.42^{b}
		Week 5	33.89 ± 1.11^{d}	66.11 ± 1.11^{a}	62.22 ± 4.44^{b}	6.67 ± 0.00^{c}	2.19 ± 0.09^{d}	62.13 ± 0.00^{d}

(Continued)

TABLE 4 | (Continued)

Treatment	Investigation time	DI (%)	Relative control effect (%)	Incidence (%)	PDP (%)	FMS	AUDPC
Fusarium solani	Week 1	0.56 ± 0.56^{b}	98.41 ± 1.59^{a}	2.22 ± 2.22^{c}	0.00 ± 0.00	0.33 ± 0.33^{b}	0.94 ± 1.94^{b}
	Week 2	11.11 ± 0.56^{c}	84.56 ± 1.11^{c}	37.78 ± 2.22^{b}	0.00 ± 0.00^{c}	1.18 ± 0.01^{b}	10.21 ± 0.84^{b}
	Week 3	13.89 ± 1.11^{b}	84.24 ± 1.43^{a}	40.00 ± 0.00^{b}	0.00 ± 0.00^{c}	1.39 ± 0.11^{c}	21.88 ± 0.84^{b}
	Week 4	25.00 ± 0.96^{cd}	75.00 ± 0.96^{a}	46.67 ± 3.85^{b}	0.00 ± 0.00^{c}	2.18 ± 0.25^{b}	27.95 ± 1.06^{b}
	Week 5	41.11 ± 1.47^{c}	58.89 ± 1.47^{b}	64.44 ± 5.88^{b}	6.67 ± 0.00^{c}	2.58 ± 0.15^{c}	68.25 ± 1.82^{c}

Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

DI, disease intensity; Incidence, percentage of diseased plants; PDP, percentage of dead plants; FMS, final mean severity of symptoms; AUDPC, area under the disease progress curve.

significant levels. The effect of strain XNRB-3 treatment (T2) on the utilization rate of the four substrate groups (polymers, miscellaneous, carboxylic acids, and amino acids) was stronger compared with the other treatments. The utilization rate of these four substrate groups was 6.11, 0.97, 15.41, and 10.24% higher in T2 than in T1, respectively. In October 2021 (**Figures 8M,N**), the changes in the utilization rate of microbial substrates under different soil treatments varied starting in 2020. Compared with CK1, the OD values of the two types of carbon sources (carbohydrates and carboxylic acids) increased significantly in T2, and the other four carbon sources (polymers, miscellaneous, amines/amines, and amino acids) also increased but did not reach significance levels. With the exception of miscellaneous, the OD values of amines/amines, carboxylic acids, carbohydrates, polymers, and amino acids increased by 40.79, 42.17, 24.45, 10.56, and 7.91% in 2021, respectively, compared with 2020. These findings indicate that the soil microorganisms treated by strain XNRB-3 mainly use polymers, carboxylic acids, and amino acids. The utilization rate of the six types of carbon sources in CK2 was significantly lower compared with the other treatments, denoting that fumigation greatly affected the microbial community in the soil.

A principal component analysis and cluster analysis demonstrated that the soil microbial community structure in T2 and CK2 significantly differed from that in CK1 (**Figures 8A–H**). The soil bacterial community structure in T2 differed significantly from that in other treatments (**Figures 8G,H**), and the soil fungal and bacterial communities of T1 and CK1 were similar (**Figures 8C,D,G,H**). The Margalef, McIntosh, Brillouin, Simpson, and Shannon indexes reflect the richness and diversity of soil microbial communities (**Supplementary Tables 28, 29**). The abundance of soil fungi and bacterial communities was significantly increased after adding strain XNRB-3. The diversity of soil fungi communities was significantly increased, the dominance index and carbon source utilization were significantly reduced, and the bacterial community showed the opposite pattern. The functional diversity of the microbial community was analyzed based on the data of 96-h Biolog Eco plates in 2020 and 2021, the functional diversity of the microbial community in T1 and T2 showed similar changes. The diversity of soil microbial communities was significantly increased in T1 and T2 than in CK1, which was consistent with the results of the T-RFLP analysis.

These results indicated that XNRB-3 bacterial fertilizer can also improve the soil microbial environment along with its biocontrol activity.

DISCUSSION

In recent years, the introduction of beneficial microorganisms into soil has been shown to be an attractive alternative for controlling plant diseases, especially endophytic strains with antibacterial effects or strains that promote plant growth; however, these microorganisms have been rarely used to control ARD (Nan et al., 2011; Deketelaere et al., 2017). Cao et al. (2011) found that *B. subtilis* SQR 9 isolated from a healthy cucumber

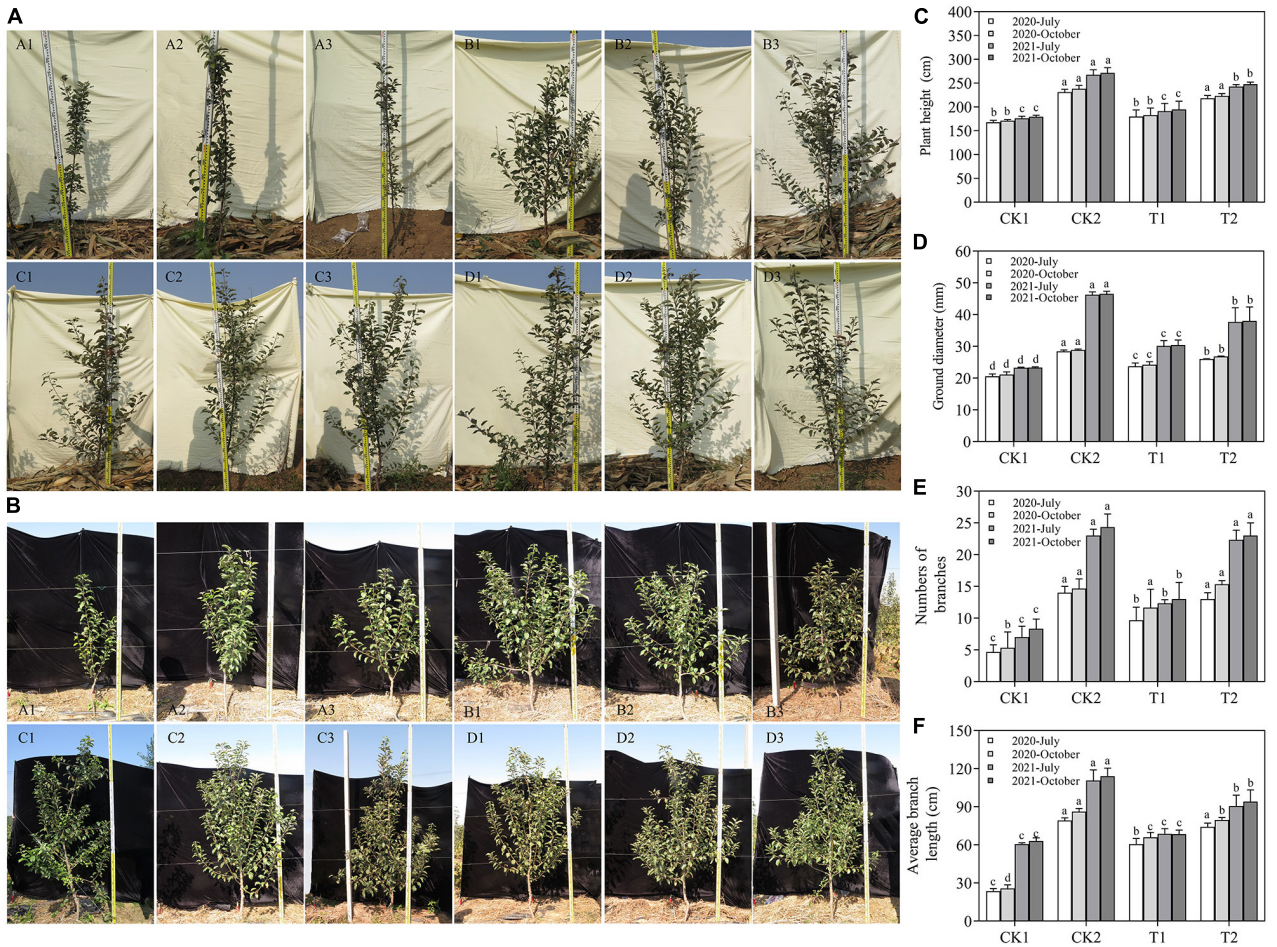

FIGURE 6 | The biomass of young apple trees in different treatments, including plant height **(C)**, ground diameter **(D)**, numbers of branches **(E)**, and average branch length **(F)**. **(A)** The growth of young apple trees in different treatments in October 2020. **(B)** The growth of young apple trees in different treatments in October 2021. (A1–A3) CK1 28-year-old orchard soil, (C1–C3) CK2 Methyl bromide fumigation, (B1–B3) T1 Fertilizer carrier, (D1–D3) T2 XNRB-3 bacterial fertilizer. Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

root in a field with a high incidence of *Fusarium* wilt disease can control cucumber wilt by colonizing plant roots. In the present study, an endogenous *B. licheniformis* XNRB-3 was isolated from the root tissues of healthy fruit trees in orchards where the incidence of ARD was high. Furthermore, this strain could stably colonize the roots of apple seedlings, showing high phlorizin-degrading activity and multiple PGP properties. It could also significantly inhibit the mycelial growth and spore germination of *Fusarium* by producing antifungal compounds. The inhibition of spore germination is essential for the development of fungal disease during the early stage (Miyara et al., 2010). This revealed that the strain XNRB-3 has the potential to be used as a biological control agent to control ARD.

Previous studies indicate that many beneficial endophytes *Bacillus* (*B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis*), exhibit several disease suppression mechanisms and promote plant growth, including preventing vascular pathogens from colonizing ecological niches, biocontrol attributes [the production of siderophores, hydrolytic enzymes, hydrogen cyanide (HCN), and antibiotics], broad-spectrum antibiotics, PGP traits (IAA production, phosphate solubilization, potassium solubilization, nitrogen fixation, and ACC deaminase), and promoting induced systemic resistance (Penrose and Glick, 2003; Ahmad et al., 2008; Senthilkumar et al., 2009; Kumar et al., 2012; Vacheron et al., 2013; Eljounaidi et al., 2016; Dowarah et al., 2021). The endogenous *B. licheniformis* XNRB-3 isolated in this study also has several of the aforementioned PGP properties and antagonistic traits. For example, IAA, CTK, and GA can significantly increase the root length, root tip, and branch number of apple seedlings, the production of IAA can also promote the establishment of a symbiotic relationship between plants and arbuscular mycorrhizal (AM) fungi to improve their adaptability to the external environment (Wahyudi et al., 2011; Liao et al., 2015); nitrogen fixation, ACC deaminase, and ammonia production can induce plant resistance and promote plant growth (Penrose and Glick, 2003; Senthilkumar et al., 2009); and phosphate and potassium solubilization can effectively increase the absorption of phosphorus and potassium

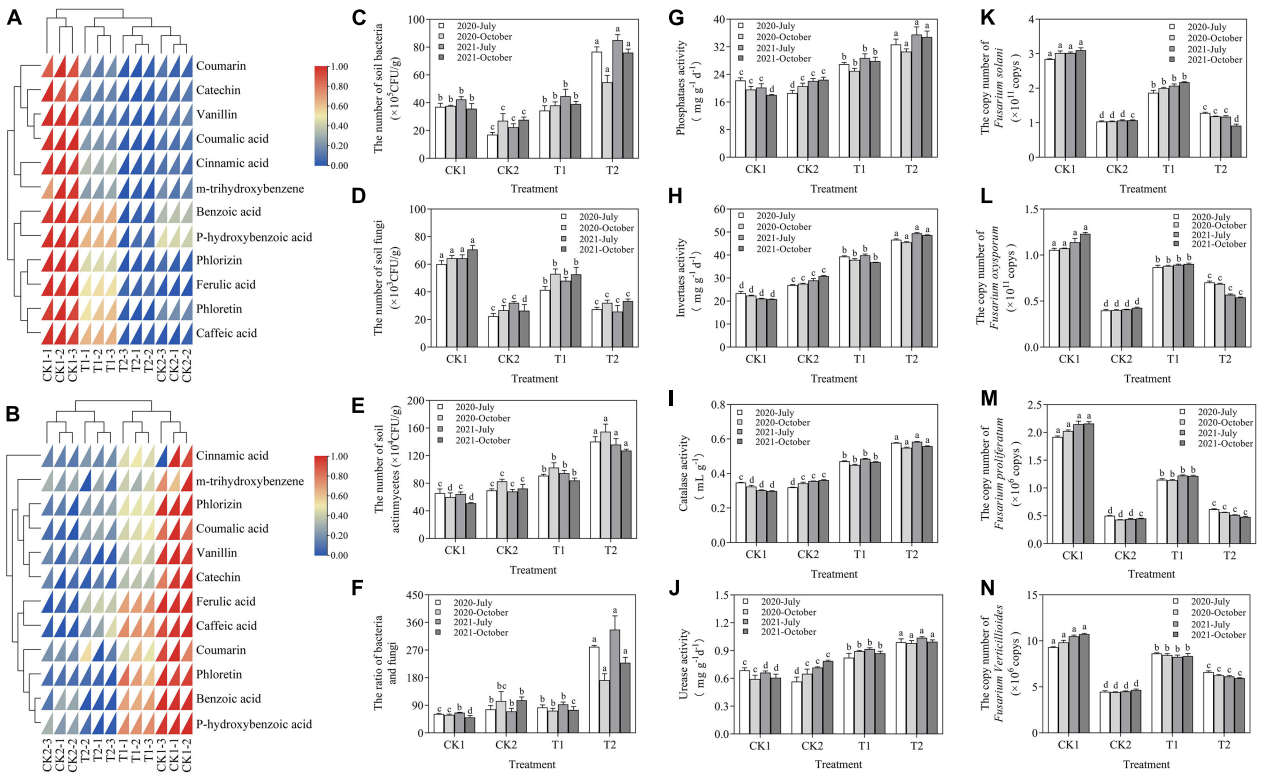

FIGURE 7 | (A) Effects of different treatments on soil phenolic acid in October 2020. **(B)** Effects of different treatments on soil phenolic acid in October 2021. **(C–F)** The effect of strain XNRB-3 on the density of microorganisms in the rhizosphere of young apple trees. **(G–J)** Effect of different treatments on soil enzyme activities. **(K–N)** Effect of different treatments on the gene copy number of four fungal pathogens by using real-time qPCR. CK1: 28-year-old orchard soil, CK2: Methyl bromide fumigation, T1: Fertilizer carrier, T2: XNRB-3 bacterial fertilizer. Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

by plants and promote root development (Richardson, 2001). Strain XNRB-3 can also produce enzymes that dissolve fungal cell walls (cellulose, pectinase, β1,3-glucanase, chitosanase, and protease), siderophores, antifungal compounds (2,4-di-tert-butylphenol and alpha-bisabolol), and low molecular weight metabolites (HCN) restrict the growth of pathogens. Among them, the production of siderophores can also induce the activity of plant root protection enzymes to defend against pathogenic fungi. The production of cellulase and pectinase can also help strain XNRB-3 better colonize the root system (Verma et al., 2001; Wahyudi et al., 2011; Yu et al., 2011). Therefore, adding the strain XNRB-3 under potted and field conditions can promote the growth of apple plants.

The production and transportation of BCAs are essential for successful biological control under field conditions (Thangavelu et al., 2004). An appropriate carrier can support the survival of BCAs while inhibiting the growth of target pathogens, thereby improving the performance of BCAs for plant disease control (Ling et al., 2010; Malusá et al., 2012; Wei et al., 2015). Smith (1992) found that dry inoculants can be produced using different types of soil materials (peat, coal, clays, and inorganic soil), organic materials (composts, soybean meal, wheat bran, and sawdust), or inert materials (e.g., vermiculite, perlite, kaolin, bentonite, and silicates). In this experiment, dry inoculants, as listed in **Supplementary Table 10**, were used to optimize the fermentation conditions of strain XNRB-3 using RSM, which significantly increased the survival rate and shelf life of strain XNRB-3 and complies with the Chinese bio-organic fertilizer production standard stipulating that the functional microorganism content should be greater than 2.0×10^7 CFU g^{-1} dry formulation after storage for 6 months at room temperature (Emmert and Handelsman, 1999). The raw materials (CDC and wheat straw) in the formula are cheap and easy to obtain, and the fermentation level is high, providing a good foundation for its large-scale industrial production.

The optimized strain XNRB-3 fermentation broth can significantly inhibit the mycelial growth and spore germination of *Fusarium*, and an abnormal structure of the mycelia (mycelia and conidia breakage, deformity, and dissolution) from the edge of the inhibition zone was observed using scanning electron microscopy (SEM) *in vitro* assays. This antagonism may be caused by the secreted antifungal compounds (Yndj protein, subtilisin, bacillomycin, iturin A, fengycin, and surfactin) (Cawoy et al., 2015; Harwood et al., 2018). Among these compounds, lipopeptides (surfactin, iturin, and fengycin families) and bacillomycin show potent antimicrobial activity against a wide variety of microorganisms *in vitro*, especially filamentous fungi

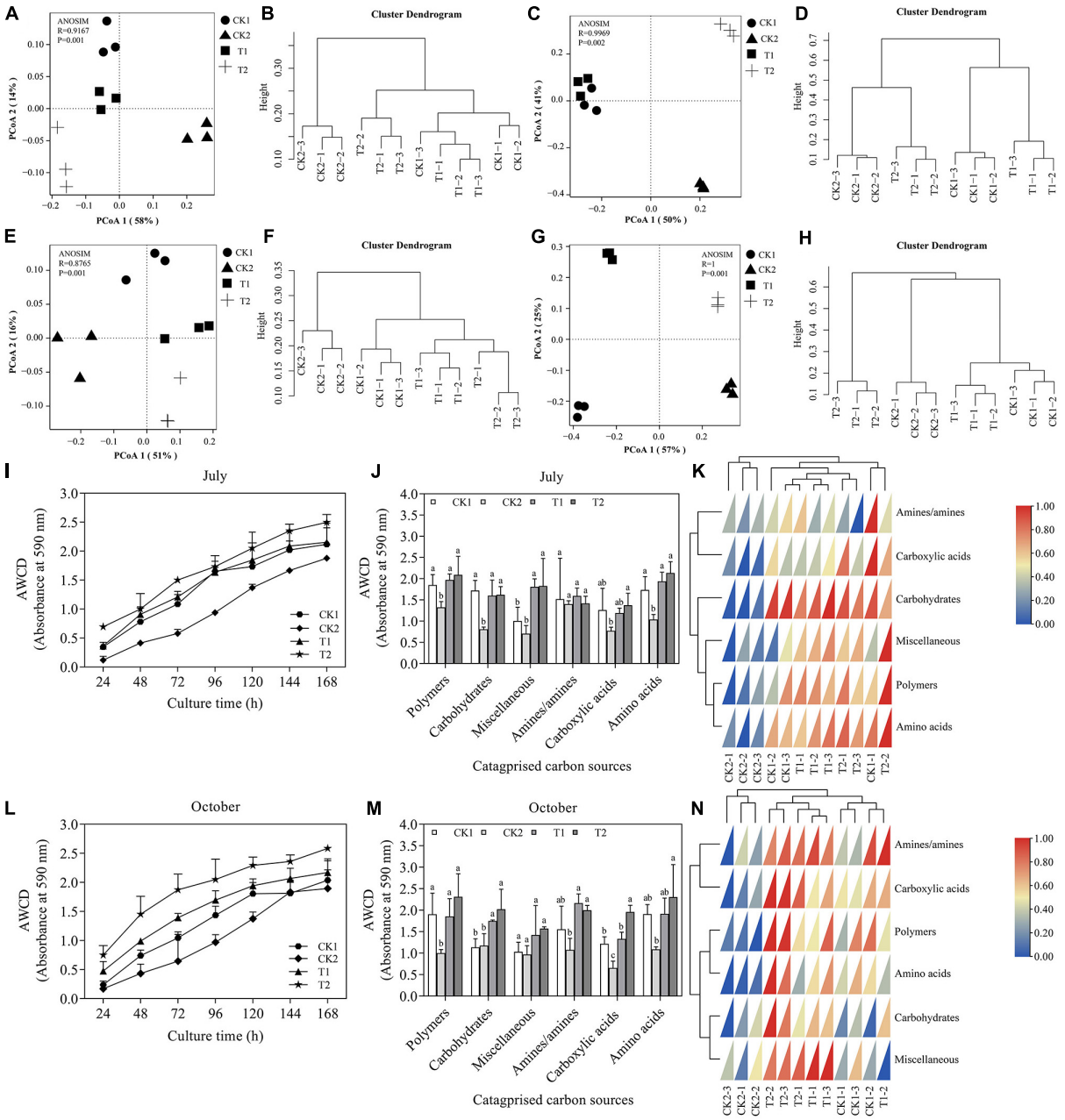

FIGURE 8 | (A,B,I–K) (October, 2020), **(E,F,L–N)** (October, 2021): Utilization of different carbon substrates by rhizosphere soil samples under different treatments using Biolog Eco plates. **(A,B,E,F)** The PCA of potential community functional diversity of soil microbes. **(I,L)** Variation in average well color development (AWCD) after 168 h of incubation. **(J,K,M,N)** Categorized substrates utilization pattern by microbial communities from rhizosphere soils after 96 h of incubation. **(G,H,** bacteria) and **(C,D,** fungi): Microbial community structures in the different treatments based on the T-RFLP data in October 2021. Principal coordinate analysis (PCoA) **(C,G)** and cluster analysis **(D,H)** plot based on the OTUs of Bray_Curtis distance. $R = 1 > 0$, $p < 0.05$ means that the difference between groups is greater than the difference within groups, and there are significant differences between different treatments. CK1: 28-year-old orchard soil, CK2: Methyl bromide fumigation, T1: Fertilizer carrier, T2: XNRB-3 bacterial fertilizer. Values in columns followed by the same letter are not significantly different according to Duncan test at $p < 0.05$. Values are mean ± SD ($n = 3$).

(*F. oxysporum*, *Verticillium dahliae*, *P. capsici*, and *P. nicotianae*) (Cao et al., 2012; Frikha-Gargouri et al., 2017). The production of lipopeptide substances might also be one of the important reasons why strain XNRB-3 can form a biofilm on the surface of the roots (Hofemeister et al., 2004; Chen et al., 2013). Strain XNRB-3 can also produce free amino acids (aspartic acid, glutamic acid, proline, and tyrosine) during the fermentation process. The production of amino acids is closely related to the biosynthesis of peptide antibiotics (Besson et al., 1990; Ren et al., 2012).

The formation of bacterial biofilms and their ability to colonize the rhizosphere and/or roots are closely related to successful field applications (Compant et al., 2010; Zhang et al., 2011; Mendis et al., 2018). This study found that strain XNRB-3 could colonize plant roots, and its fresh weight ranged from 10^5 to 10^7 CFU g^{-1} within 21 days, and it can also significantly reduce the abundance of *Fusarium* in the rhizosphere soil. These findings were consistent with the results of Cao et al. (2011), the pathogen density in the rhizosphere of cucumber seedlings inoculated with *B. subtilis* SQR9 was significantly reduced. Similar results were obtained in the PAS staining test. The roots treated with the fermentation broth of the strain XNRB-3 prevents the entry of the *Fusarium* into the vascular stele, and the mycelial growth was restricted to the epidermis and outer root cortex, indicating that strain XNRB-3 can colonize the roots of the plant and grow root epidermis, forming a biofilm that prevents *Fusarium* infection and improves the resistance of plants to infection (Benhamou et al., 1998; Duan et al., 2021). Infected roots can also produce a large amount of a sticky substance, which results in the deposition of formed callose and starch granules to form a mechanical barrier that inhibits the invasion of pathogens (Lagopodi et al., 2002; Grunewaldt-Stöcker et al., 2020).

Soil enzyme activity and soil attributes (e.g., SOC, TN, TP, AN, and AP concentrations) are often used to monitor changes in soil microbial activity and soil fertility (Kandeler et al., 2006; Liu et al., 2011; Song et al., 2012; Pan et al., 2013). This study found that the application of strain XNRB-3 significantly increased the activity of soil-related enzymes, and it increased after the second year of applying strain XNRB-3. This was consistent with the results of several previous studies. For example, the addition of PGPR can significantly improve the physical and chemical properties of soil and soil enzyme activity (Ren et al., 2019). PGPR strains can induce the production of lytic enzymes by utilizing carbon from the cell wall of microorganisms, thereby increasing soil urease activity (Karthik et al., 2017). The increase in urease activity indicates that the application of XNRB-3 may increase the gross N mineralization rate because urease can catalyze the hydrolysis of urea into CO_2 and NH_4^+, as well as promote the soil nitrogen cycle (Xu et al., 2015). The increase in invertase activity can promote the conversion of carbohydrates and increase the concentration of soil nutrients (e.g., N, P, and K) under the action of microorganisms and improve soil fertility (Han et al., 2017). Strain XNRB-3 can increase AP activity, which increases the availability of soluble P and promotes plant growth (Krämer, 2000). After adding the strain XNRB-3, the contents of N, P, K, and SOC in the soil and the activity of soil-related enzymes were consistent. The change in soil pH value was opposite, which was consistent with the results of previous studies (Xie et al., 2017; Fu et al., 2018; Ren et al., 2019). The increase in the nutrient concentration in the soil might also be related to the characteristics of strain XNRB-3, such as nitrogen fixation, phosphorus dissolution, and potassium dissolution.

The functional diversity and overall activity of microbial communities in soil can reflect soil quality (Islam et al., 2011). Biological methods are some of the main methods used to measure the functional diversity and overall activity of microbial communities for their simple operation, high sensitivity and resolution, and rich data (Dang et al., 2015; Guo et al., 2015). Therefore, biological methods were used in this study to investigate the functional diversity and carbon source utilization of the rhizosphere soil microbial community after treatment with strain XNRB-3, as well as evaluate the safety of its use in the soil environment. The AWCD value of rhizosphere soil was significantly higher after the addition of strain XNRB-3 compared with other treatments, and also significantly enhanced the use of carbon sources such as polymers, carboxylic acids, and amino acids, which might be related to the increase in the number of soil bacteria (e.g., *Pseudomonas* spp., *Enterobacter* spp.) and actinomycetes after the addition of strain XNRB-3 (Heuer et al., 1995; Grayston et al., 1998; Larkin, 2003). Biolog GEN III microplate identification revealed that strain XNRB-3 can use a wide range of carbon sources, which permits this strain to grow and reproduce in environments with different nutrient levels (Schutter and Dick, 2001).

Previous studies have shown that the occurrence of ARD is closely related to the structure and diversity of soil microbial communities. Increases in the number of rhizosphere soil pathogens and decreases in the number of beneficial microorganisms are also important factors leading to disease outbreaks (Kelderer et al., 2012; Mazzola and Manici, 2012). The T-RFLP data from this study showed that strain XNRB-3 can significantly alter the structure of the rhizosphere soil fungal and bacterial communities after treatment, which was similar to the community structure of fumigation treatment. Application of this strain also significantly increased the abundance and diversity of rhizosphere soil bacteria and fungi, reduced the relative abundance of *Fusarium*, provided a stable and beneficial rhizosphere ecosystem for plants, and promoted plant growth (Liu L. et al., 2018). The addition of strain XNRB-3 may promote the aggregation of some beneficial microorganisms or secrete some VOCs to inhibit the growth of soil fungi and increases soil bacterial biomass, thereby improving the soil microbial environment, promoting the replanted young apple trees growth, and reducing the damage of ARD (Ryu et al., 2003; Fernando et al., 2005; Yuan et al., 2017; Liu et al., 2021).

Imbalances in the physical and chemical properties of soil and the allelopathy of root exudates and residues are considered to be the main causes of soil sickness (Zhang Y. et al., 2010; Pant et al., 2013). Yin et al. (2017) found that the roots of apple plants under continuous cropping can secrete the same substances (such as phenolic acid autotoxic substances) for a long time, and these substances significantly affect the composition and distribution of the rhizosphere microflora, increasing the number of pathogenic fungi and inhibiting plant growth (Liu H. et al., 2019; Xu et al., 2020). Phenolic substances related to currently known ARD mainly include 2,4-di-tert-butylphenol, vanillic acid, benzoic acid, p-hydroxybenzoic acid, ferulic acid, cinnamic acid, and phlorizin (Ye et al., 2006; Qu and Wang, 2008; Yin et al., 2013, 2017; Chen et al., 2021). Phlorizin is a unique phenolic acid substance of apples that mainly exists in the roots, stems, bark, tender leaves, and fruits of apples. Hofmann et al. (2009) found that the diseased seedlings cultivated in ARD soil exuded significantly more phlorizin compared to

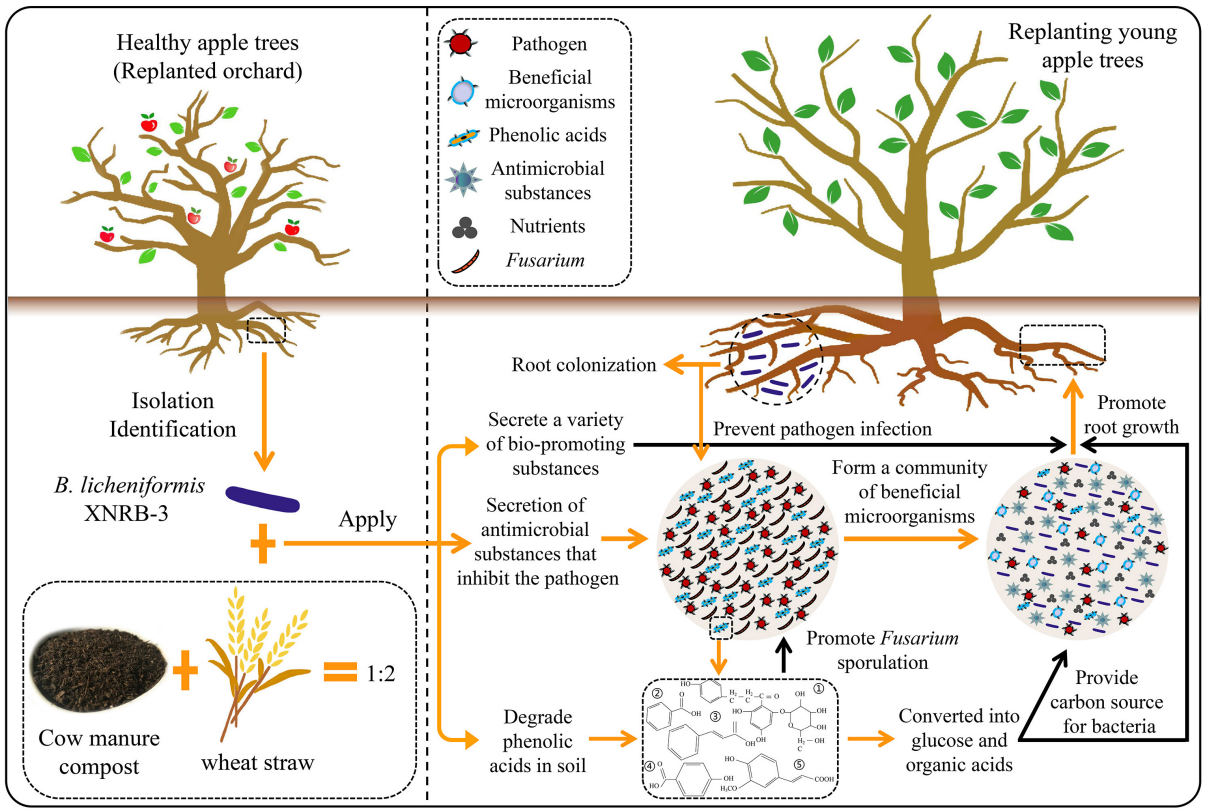

FIGURE 9 | Conceptual model displaying the potential role of strain XNRB-3 influencing the pathogen density (qPCR) in the soil and the occurrence of ARD.

healthy seedlings, indicating that the phlorizin in root exudates was closely related to the occurrence of ARD. In the study by Zhang et al. (2009), values for phlorizin contents secreted by the roots of apple seedlings are in a range of 0.442–1.583 μg per plant. Wang et al. (2015) found that the content of phlorizin in the soil was 6.0 mg kg^{-1}, which would destroy the antioxidant system of apple roots, thereby inhibiting growth. Ehrenkranz et al. (2005) found that the high concentration of phlorizin can reduce the rate of apple seedling photosynthesis and transpiration. It was also found that the growth and division of *F. moniliforme* were faster in the 1.0 mM phlorizin than in the 0.5 mM phlorizin (Yin et al., 2017). The above studies further illustrated that the long-term continuous cropping of apples can lead to the accumulation of excessive phlorizin in the rhizosphere, which directly damages the root system or indirectly affects the growth of plants by stimulating the growth of pathogenic fungi. Currently, the use of microbial degradation methods to degrade phenolic acids in the environment is becoming increasingly popular because of its various advantages, including low cost, high degradation efficiency, lack of secondary pollution, and environmental safety (Ma et al., 2018; Wang et al., 2021). Use of the medium containing phlorizin as the sole carbon source revealed that strain XNRB-3 can efficiently degrade phlorizin, and the phlorizin degradation rate could reach 68.83% after 60 h of culture under the condition of 2% inoculum. This was similar to the research results obtained by Liu et al. (2017), the phlorizin degradation rate of *Aspergillus terreus* can reach 88.96% when cultured for 96 h under the condition of 2% inoculum. Strain XNRB-3 can also effectively degrade phlorizin, cinnamic acid, ferulic acid, benzoic acid, and *p*-hydroxybenzoic acid in soil and culture fluid, thereby promoting the growth of apple seedlings. This finding was similar to the experimental results of Zhang Y. et al. (2010), which used a screening medium containing *p*-coumaric acid as the sole carbon source. Four microbes were isolated from plant soils, and these microbes could effectively degrade ferulic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde, as well as promote seedling growth. Phlorizin is degraded in the soil in two main ways. The first is through the hydrolysis of phloretin into phloroglucinol and *p*-hydroxyphenylpropionic acid by secreting a phloretin hydrolase, followed by the decomposition to phloretin and glucose by β-glucosidase, which is then used by bacteria (Chatterjee and Gibbins, 1969). Alternatively, it can be degraded to pyruvic acid by the protocatechuic acid pathway (Mohan and Phale, 2017), and pyruvic acid can be converted to acetyl CoA and enter the tricarboxylic acid cycle, which produces organic acids, such as citric acid, succinic acid, malic acid, and oxaloacetic acid (Priefert et al., 2001; Zhang Y. et al., 2010). These substances play an important role in promoting the absorption and transportation of certain nutrients and improving the photosynthetic efficiency of plants and the accumulation of nitrogen, phosphorus, and potassium (Liu et al., 2005). Therefore, the method of phlorizin degradation in the soil environment by

the strain XNRB-3 is thought to be an effective approach for overcoming the obstacles of continuous apple cropping.

CONCLUSION

The phlorizin-degrading bacterium *B. licheniformis* XNRB-3 was isolated from the roots of apple plants grown in a replanted orchard. Strain XNRB-3 features a variety of PGP characteristics and antagonistic traits, which confers it with high potential for practical use, including its ability to produce some antifungal substances and significantly inhibit the spore germination of *Fusarium*. Strain XNRB-3 could effectively colonize the root surface of plant seedlings and even enter roots after it was inoculated on the roots of plant seedlings. The addition of strain XNRB-3 under potted and field conditions can significantly promote the growth of apple plants; reduce the abundance of *Fusarium* and the content of phenolic acids in the rhizosphere soil; improve the structure of the soil microbial community; increase the available nitrogen, phosphate, and potassium in the soil; and improve soil health (**Figure 9**). This study provides new insight and a strain resource that could be used to aid in the prevention and control of ARD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MN726439.1; https://www.ncbi.nlm.nih.gov/genbank/, MT703801.1; https://www.ncbi.nlm.nih.gov/genbank/, MT713119.1; https://www.ncbi.nlm.nih.gov/genbank/, MT713122.1.

AUTHOR CONTRIBUTIONS

ZM and CY contributed to conception and design of the study. YD organized the database. YD, RC, RZ, and WJ performed the statistical analysis. YD wrote the first draft of the manuscript. LZ wrote sections of the manuscript. All the authors contributed to manuscript revision, read, 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.2022.839484/full#supplementary-material

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Safety Level of Microorganism-Bearing Products Applied in Soil-Plant Systems

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The indiscriminate use of chemical fertilizers adversely affects ecological health and soil microbiota provoking loss of soil fertility and greater pathogen and pest presence in soil-plant systems, which further reduce the quality of food and human health. Therefore, the sustainability, circular economy, environmental safety of agricultural production, and health concerns made possible the practical realization of eco-friendly biotechnological approaches like organic matter amendments, biofertilizers, biopesticides, and reuse of agro-industrial wastes by applying novel and traditional methods and processes. However, the advancement in the field of Biotechnology/Agriculture is related to the safety of these microorganism-bearing products. While the existing regulations in this field are well-known and are applied in the preparation and application of waste organic matter and microbial inoculants, more attention should be paid to gene transfer, antibiotic resistance, contamination of the workers and environment in farms and biotech-plants, and microbiome changes. These risks should be carefully assessed, and new analytical tools and regulations should be applied to ensure safe and high-quality food and a healthy environment for people working in the field of bio-based soil amendments.

Keywords: microbial inoculants, pathogens, risks of contamination, safety measures and regulations, organic matter

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INTRODUCTION

There is a generalized expert opinion that the major challenge facing agriculture is to increase crop productivity with a simultaneous reduction of environmentally damaging chemical fertilization. Intensive agricultural practices based on chemical fertilizers caused an adverse impact on autochthonous microbial communities (including plant beneficial microorganisms established in the rhizosphere) and microbial biodiversity with a simultaneous significant reduction of soil organic matter and mineral content (Mäder et al., 2002; Huang et al., 2019). With the aim of solving the problems arising from modern conventional agriculture and following the principles of sustainable agriculture and circular economy, the scientific efforts are focused on the development of less harmful strategies for stimulating plant growth and health by restoring soil fertility and

microbial diversity. These strategies include attempts to close the nutrient cycle at farm level by maximizing reutilization of by-products and wastes, and restore beneficial plant-biological interactions and processes by using compost, biofertilizers (BFs), and biocontrol agents (BAs). The use of plant beneficial microorganisms seems to be a very attractive strategy as they are known for their prebiotic, probiotic, and postbiotic functions and as an important part of plant development (Alegria Terrazas et al., 2016; Vassileva et al., 2020a). Recently, the microbial communities associated with the plant have been classified as plant microbiome, which also include viruses, archae, and nematodes (Orozco-Mosqueda et al., 2018). Microorganisms colonize plants creating specific interrelations, including pathogen protection and enhanced nutrient mobilization and acquisition (Lugtenberg and Kamilova, 2009; Gupta et al., 2022). Undoubtedly, these interrelations have been historically affected by the conventional agricultural activities aimed at increasing the yields of crops (Perez-Jaramillo et al., 2015). Introducing microorganisms and organic fertilizers into soil-plant systems is considered an important tool in overcoming problems associated with the excessive use of chemical fertilizers and pesticides (Bashan et al., 2014; Malusà et al., 2021; Shaji et al., 2021). By this reason, there is a strong tendency to stimulate application of microorganism-bearing products to re-establish and enhance soil fertility and crop production and quality particularly in a stressed environment (Shilev et al., 2019).

It should be distinguished between traditional microorganism-bearing fertilizers, such as compost or animal manure and formulated biofertilizers. While in the traditional microorganism-bearing fertilizers, there is a wide range of well-studied and categorized, including pathogenic, microorganisms, which in some cases are difficult to control, BF/biocontrol products normally containing one or more microbial cultures, with guaranteed quality and cell quantity and, in some products, controlled release after introduction into the soil-plant system (Venglovsky et al., 2006; Young et al., 2012; Malusà et al., 2021). It should be noted, however, that there is a discussion in the scientific literature on the potential risk for humans and animals when commercial plant beneficial microbial formulations are introduced into soil-plant systems (Deising et al., 2018). The main concern is that it is difficult to distinguish between plant beneficial and opportunistic pathogenic microorganisms as they have similar properties and characteristics (Berg et al., 2005, 2013; Lu et al., 2021).

Similarly, after application of organic fertilizers, such as compost or animal manure, particularly fresh fruit and vegetables have been repeatedly reported as vehicles of pathogenic microorganisms, such as *Listeria monocytogenes, Staphylococcus aureus* (Johannessen et al., 2002), *Enterococcus faecium, E. faecalis*, *L. monocytogenes* (Johnston et al., 2006), *Salmonella enterica* (Branquinho Bordini et al., 2007), *Escherihia coli* O157:H7 (Beretti and Stuart, 2008), and *E. coli* O104:H4 (Mellmann et al., 2011). In the latter case, the pathogen was found in seeds and caused the hospitalization of over 800 individuals and 53 deaths in Germany, followed by 4 other countries, which indicated the low level of attention to this problem of all players involved in the production chain (including scientific organizations). It is also important to note the negative results from microbiological tests of the suspected seeds (European Food Safety Authority, 2011) and, in general, the difficulty to determine pathogenicity through screening for virulence genes.

The aim of this mini-review was to reveal how safe are the above products, before and after their application following the 3-P approach (prebiotics, probiotics, and postbiotics). During the past years, the analysis of risk factors for both types of microorganism-bearing products is limited to single strains or single product (e.g., commercial organic waste or formulated product) without presenting a global view of all products containing pathogenic or beneficial microorganisms. Special, but limited, attention will be given to some regulatory issues concerning Spain but also those aimed at European harmonization of safety parameters of bio-based products, which are now not homogenous at national and regional levels. In fact, safety issues and measures in the field of microbially bearing fertilizers should be oriented in several main directions: a) before field application while treating organic fertilizers or producing BFs; b) during application of the microbial products; and c) during harvesting operations and postharvesting services. Here, the first two key points are discussed. We consciously do not discuss here the effect of microorganism-bearing products on plant microbiome as interactions between various biotic and abiotic factors in soil-plant systems, host preferences, selection of highly competitive smart microbial consortia, and their suitable formulations for preserving cell viability after storage and delivery need more studies (Orozco-Mosqueda et al., 2018).

RISKS OF TRADITIONAL MICROORGANISM-BEARING FERTILIZERS

Prebiotic materials, such as biosolids, animal manure, and compost, alone or combined, are the most applied organic-waste-based fertilizers, as they increase the input of carbon and nutrients to the soil (EIP-AGRI, 2015). However, due to the presence of microorganisms in their composition and despite the official statements in many countries that these products are microbiologically safe before application to soil, the scientific community continuously publishes data or opinions of concerns (refer to **Figure 1**). The main reason is the difference between the recommended treatment procedures by the authorities and their practical use. For example, in a recent analysis, Ramos et al. (2021) reported that farmers frequently consider the manure aging (storage time needed to reduce the manure pathogenicity) as composting thus introducing live microorganisms existing in the "treated" material into the soil. Moreover, particularly in Spain, illegal or sub-standard landfilling is still widespread practice with all risks of water, air, and soil contamination and potential health problems for animals and humans (EC Country Report Spain, 2019). Since food safety problems provoked by pathogen-contaminated roots or leaves increase (particularly in fresh produce and minimally processed crops), the European Commission investigates such cases strictly and takes measures through well-established procedures.

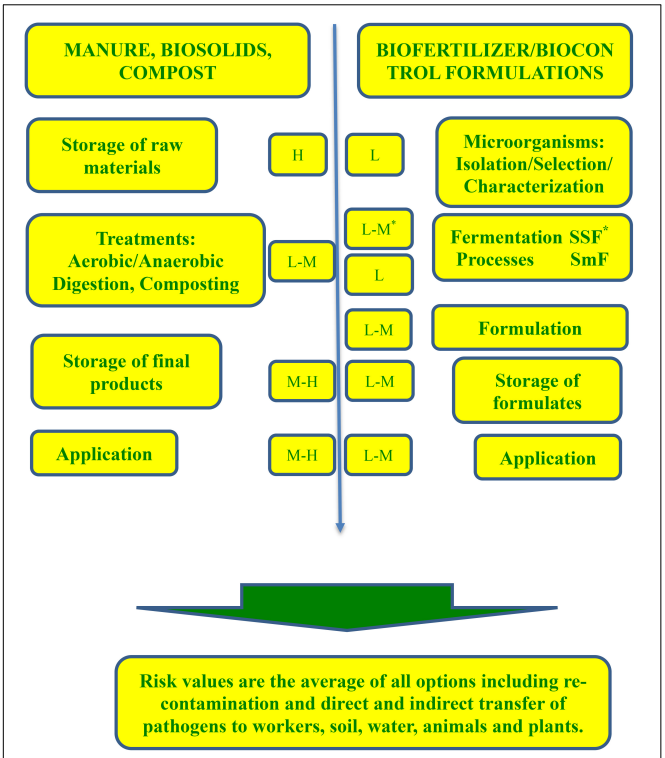

FIGURE 1 | Risks of contamination of microorganism-bearing fertilizer preparations during different stages of their production (L-low risk; M-medium risk; H-high risk).

Biosolids

Biosolids are "nutrient-rich organic materials resulting from the treatment of domestic sewage in a treatment facility that can be recycled and applied as fertilizer to improve and maintain productive soils and stimulate plant growth" (EPA, 1994). The introduction of sewage sludge (i.e., biosolids) in agricultural soil-plant systems is a subject of criticism, particularly as a source of heavy metals and human pathogens. However, it is also well established that after the treatment of sewage in waste-water treatment plants, the number of pathogenic microorganisms in the effluent is greatly reduced. After analyzing the microbial community structure of several sewage sludges, two clusters of dominant genera were found: one included *Propionibacterium*, *Comamonas*, *Brevundimonas*, *Methylobacterium*, *Stenotrophomonas*, and *Cloacibacterium*, while the other cluster included *Clostridium*, *Treponema*, *Desulfobulbus*, and *Syntrophus* (Nascimento et al., 2018). Sludges revealed high bacterial diversity, but their sources and redox operating conditions, as well as liming, did not consistently affect bacterial community structures. Particularly efficient for the reduction of bacteria, fungi, helminth eggs, and viruses are composting, settling ponds, dewatering drying, and pH elevation (>9) (Adegoke et al., 2016). Nevertheless, biosolid-derived pathogens can easily enter the soil-plant-food chain, which might provoke safety decrease (Makádi et al., 2007; Bastida et al., 2008). In addition, a substantial amount of antibiotic-resistant bacteria (ARB), antibiotics, heavy metals, and antibiotic-resistant genes (ARG) remain after treatment and further invade soil-plant systems and microbial community (Karkman et al., 2018; Nunes et al., 2021). Heavy metals in biosolids and other wastes exist, including after treatment in the form of a molecule or ion, thus ensuring horizontal gene transfer between different bacteria (Ezugworie et al., 2021). Recently, thermally dried anaerobically digested sewage sludge has been proved as a source of ARGs and mobile genetic elements (MGEs), thus increasing the risk of antibiotic resistance dissemination in agricultural soils (Jauregi et al., 2021).

Manure

Manure is one of the most important organic sources of nutrients, containing microorganisms, and deserves special attention. Livestock manure, according to the last EC Directive, means "waste products excreted by livestock, or a mixture of litter and waste products excreted by livestock, even in processed form" (EU Commission, 1991). Manures are different and, depending on their origin, can be solid, semisolid, and liquid, containing mixtures of feces, urine, bedding materials, including various chemical or physical wastes (Shober and Maguire, 2018). The total production of manure in Europe is 140 million tons on a dry matter basis (Scope Newsletter, 2014), which European farmers, following the European Environmental and Fertilizer policy measures, should process before application in soil (EU Commission, 2013). Manure contains a wide variety of microorganisms (about 10^8–10^{10} CFU/g), including pathogens, which present health risks for animals or humans. Among viruses, fungi, and bacteria in manures, typical pathogens, including *Salmonella* sp., *E. coli* O157 H7, *Campylobacter jejuni, Yersinia enterocolitica,* and *C. perfringens. Salmonella,* are *Enterobacteriaceae,* which are widely distributed and include more than 2,000 serotypes (Venglovsky et al., 2006) (for detailed microbial characteristics of manure see Black et al., 2021). The initial number and profile of pathogens in manures and their characteristics change as a function of the manure type, storage conditions, and after treatment (Hutchison et al., 2005). The EC legislation permits the use of all types of manure except "factory farming" manures (EC Regulation 889, 2008), and in many countries, the application of liquid manure is not allowed. Therefore, it is important to develop and select strategies to manage not only the nutrient content in manure without affecting soil, water, and air but also reduce pathogenic microorganisms by microbiological, chemical, or physical methods (Heinonen-Tanski et al., 2006). In general, most enteric microorganisms do not multiply and survive out of the host due to the enormous stress conditions and after a long period of storage, but early studies reported that low temperatures and high solid concentrations increase the probability of survival of many pathogens (Feachem et al., 1983; Kudva et al., 1998). For these reasons, and particularly in case of factory farming, it is recommended to clean manure, normally by biological or thermal treatment (Venglovsky et al., 2006). In any case, management of manure during storage and further treatment (e.g., by composting) and its proximity to plants from one side, and the always existing possibility of runoff, formation of contaminated dust, and animal movements from the manure

storage to crop fields, on the other side, should be an important consideration when analyzing all safety risks on farm sites (Ramos et al., 2021).

Composting

Composting is the biological process of choice for manure and sludge treatment as it is cost-effective and decreases pathogenic bacteria, fungi, and helminthic eggs, thus producing high-quality products enriched with humic acids. Slurry and sludge treatment may include aerobic stabilization when the temperature rises to 70°C thus becoming free of pathogens (Soares et al., 1995). It was reported that in composting reactors or aerated static-piles at a temperature of up to 60°C/3 days and following the existing regulations, pathogen destruction reached the most probable number of 1,000 coliforms/g dried solids and $\leq$ 3 salmonellae/4 g dried solids, but these processes should be well controlled as materials that are treated many times do not meet these standards, thus allowing pathogen regrowth.

It seems impossible to achieve organic fertilizers and ambient surrounding the storage/treatment facilities totally free of pathogenic or potentially pathogenic microorganisms. Enteric viruses and *Salmonella* spp. were found in liquid manure after the anaerobic bio-digestion process (Fongaro et al., 2014). It has been also reported that viable bacteria and viruses immobilized on air-dust particles have a greater ability to survive and affect human and animal health (Takai et al., 1998). Bio-aerosol, containing mainly Firmicutes and Actinobacteria, is continuously released starting with the pretreatment of raw materials and during the open-air process of composting without any form of control (Wery, 2014). Around the composting facilities, the bacterial diversity reaches 1.5–15.3% bacterial cells, but other microorganisms, including *Aspergillus* and *Penicillium*, can also be found in the air (Walser et al., 2015). The selection of specific raw materials and effective process management produced different levels of bacterial and fungal diversity (Hernández-Lara et al., 2022). Therefore, the risk of biological contamination of farmworkers is high, and safety measures should be continuously improved (OECD, 2012). Approaches related to determining the number of cells/spores in environments surrounding production facilities thus monitoring, for example, airborne particles could be included in disease management systems when working with organic residues (Mahafee, 2014).

Another point of attention, which was mentioned in the manure part of the mini review, concerns antibiotic resistance in soil (Wang and Tiedje, 2020). Particularly intensive are studies on manure as a vehicle of ARGs, which, once transmitted, potentially are a great risk to public health. Moreover, MGEs boost the horizontal gene transfer of ARGs in the environmental microorganism. It was found that the wide application of animal manures in organic agriculture inevitably enriches the already existing ARGs pool in soils but also additionally introduces exogenous ARGs, which can be found in soil for a period ranging from few weeks to several months depending on the manure and soil characteristics. There are a wide number of studies on the mechanisms and interactions in manure- or compost-enriched soil, which try to explain the regulation or control of the persistence of ARGs in soil for different periods of time, but the fate of ARGs where manure from different sources has been repeatedly implemented is not fully understood although some ARGs could be found in deep soil carried by their host bacteria (Li et al., 2022).

BIOFERTILIZER AND BIOCONTROL PRODUCTS, PATHOGEN-FREE OR PATHOGEN STIMULATING?

A biofertilizer can be defined as the formulated product containing one or more microorganisms that enhance the nutrient status (and the growth and yield) of the plants by either replacing soil nutrients and/or by making nutrients more available to plants and/or by increasing plant access to nutrients (Malusa and Vassilev, 2014). BAs can be defined as living organisms or natural products derived from living organisms, including microorganisms, that are used to suppress plant pathogen pest populations (Panpatte et al., 2016). All these products are based on the activity of one or more microorganisms and can be commercialized in liquid or granular form (Bashan et al., 2014). Contamination of the BF/BA could be observed in the production/formulation stage as well as during the storage. In fact, contamination is one of the main reasons for unsuccessful field application of plant beneficial microorganisms; an early study by Herrmann et al. (2013) demonstrated that 37% of the tested formulated products could be considered as "pure"; 63% were contaminated with bacteria and 40% contained only contaminants.

The schemes of selection, production, and formulation of microbial plant beneficial products are well developed (Vassilev et al., 2001a, 2015, 2017a; Malusá et al., 2012; Bashan et al., 2014; Vassilev and Mendes, 2018; Vassileva et al., 2020a). Usually, at least one, two, or three different microbiome members can be included in the final product (Vassilev et al., 2001b,c,d, 2020; Sahu and Brahmaprakash, 2016). All operations starting from the inoculum preparation, fermentation process, and downstream stage, including the product formulation as well as packaging, are carried out in sterile conditions, and, therefore, these biotechnological products should be free of contaminants. The production of spores, biomass, or metabolites is normally carried out in closed liquid submerged (e.g., batch and fed-batch) or solid-state fermentation systems with well-controlled parameters and improved quality of the final product (Vassileva et al., 2021). However, there is a risk of biological contamination in each one of the production process stages deriving from water, air, equipment, nutrient media, and laboratory/plant technical staff. The starter inoculum should be carefully managed to avoid contamination, mutation, and phenotypic changes during the fermentation process that may result in the production of ineffective BF or postbiotic (i.e., metabolic) biostimulants with different characteristics (Nims and Price, 2017). Contamination is also possible during the formulation stage or after introduction into the soil. For example, two endophytic fungi (i.e., *Muscodor albus* and *M. roseus*) producing volatile myco-fumigants were formulated in a mixture of water-absorbent starch, corn oil, sucrose, and fumed silica

(Stinson et al., 2003). The produced formulations reduced the disease incidence of soilborne pathogens, but plant growth reduction was observed due to the growth of deleterious rhizobacteria on some components of the complex carrier.

Is There Any Risk of Pathogen Contamination in the Chain "Plant Beneficial Microbial Products-Soil/Plant/Food/Humans/Animals"?

During the past years, serious doubts appeared in the security and safety of plant beneficial microorganisms. As mentioned above, Deising et al. (2018) suggested possible changes in microbial community profile and appearance of secondary metabolites, such as aflatoxin, ochratoxin, patulin, and mycotoxins, after the introduction of plant beneficial microorganisms in soil-plant systems. It is interesting to note, in this sense, that risk assessment of plant beneficial microorganisms is not included in the corresponding legislations although many plant growth- and health-stimulating microorganisms are suggested as opportunistic human pathogens (Berg et al., 2005). The dual behavior of soil microorganisms was frequently described, thus increasing the need for serious preliminary testing. For example, *Aspergillus terreus*, known as both the plant growth stimulator and BA, produced terrain, one of the numerous genome-analyzed secondary metabolites released by *A. terreus* (Gressler et al., 2015). Terrain inhibits seed germination and plant growth, provokes plant surfaces' damage, and inhibits the growth of competitors, thus facilitating the fungal invasion in the respective environmental niche (Vassileva et al., 2020b). When introduced into the human body, like other members of the genus *Aspergillus*, *A. terreus* can cause aspergillosis infection with a high level of mortality particularly in immunocompromised persons (Bartash et al., 2017). Similarly, *Stenotrophomonas* are present in manure samples and particularly *S. rhizophila,* after physiological and molecular studies, are found safe and have a high plant beneficial potential without human pathogenic traits. Recent studies propose *S. rhizophila* as a promising PGP and biocontrol product. However, some *Stenotrophomonas* species demonstrated dual characteristics, promoting plant growth and health with a simultaneous multidrug resistance affecting immunosuppressed patients, which was further confirmed by a genome analysis (Denton et al., 1998). It should be noted the bioaerosol concentration during the biostabilization of sewage sludge ranged from 160 to 1,440 cell/m^3, and species, such as *S. rhizophila* and *Fusarium graminerum,* with high bioaerosolization indexes that could be threats to human health were recently identified (Lu et al., 2021). Other microbial strains belonging to the most studied and commercially available genera *Pseudomonas, Enterobacter, Serratia,* and *Burkholderia*, among others, are also known to colonize both plants and humans and should be tested at least before starting serious biotechnological experimental work on their mass production, formulation, and application (Zachow et al., 2009). A well-studied case is that of the *Burkholderia cepacia* complex, a group of phenotypically associated bacterial species that have known PGP traits, including N_2 fixation, but can also be opportunistic human pathogens (Eberl and Vandamme, 2016). Another intensively debated bacterial genus is *Pseudomonas*, which encompasses several PGP species (e.g., *P. fluorescens, P. putida, P. putrefaciens*, *P. stutzeri*, and *P. pseudoalcaligenes*) but also the pathogenic species *P. aeruginosa*, an opportunistic pathogen causing respiratory tract infections in humans (Mendes et al., 2013).

Therefore, there are two well-defined tendencies related with the potential pathogenicity of plant beneficial microorganisms. The first one proposes more control and a risk assessment test of all microorganisms before their commercialization, while the second does not see any reason for concerns about their safety and changes in the actual registration rules (Koch et al., 2018). Which point should the authorities rely on when defining the safety measures for workers dealing with the production and application of manure, compost, and BFs? In the latter case, the new EC Regulation, which is foreseen to start in 2022, states that a microbial plant biostimulant should be reduced to mycorrhizal fungi, *Azotobacter* spp., *Azospirillum* spp., and *Rhizobium* spp., thus limiting the possibility of introducing opportunistic pathogens into the food chain. In contrast, from farm-workers-safety point of view, the formulation form (e.g., liquid or solid) of all products containing plant beneficial microorganisms is important. In case of gel-based formulations, immobilized cells are embedded in a polysaccharide matrix (Vassilev and Vassileva, 2005), thus reducing the risk of direct contact between cells and workers handling the product although in this scheme, a potential plant-soil contamination is possible. For example, additives included into the carrier matrix (Vassilev et al., 2020) could attract other microorganisms, including pathogenic strains, during storage or transportation. However, to avoid contamination, carriers, such as k-carrageenan, can be used, which after drying reduce their volume and water content. For these reasons, it is recommendable to do a thorough risk assessment of all these amendments for environment-animals-humans before the process of registration regardless of their risk group and plant beneficial effects.

MICROBIALLY BASED FERTILIZERS' ASSESSMENT AND SAFETY MEASURES

When analyzing the above information, two modes of contamination by plant beneficial microorganism-bearing products can be distinguished, namely, (1) direct contamination of people working on their production and application and (2) contamination of mainly fresh production grown in environment contaminated by these products with further effects on consumers.

As recently summarized by the COST Action SACURIMA (Leppälä et al., 2021), the European Community described high rates of injury, occupational disease, and exposures in Agriculture. Each year, there are about 6 reported accidents per 100 workers and 12 reported fatal accidents per 100,000 workers in Agriculture (Eurostat, 2019). Over 40% of agricultural workers feel unsafe at work. Over 15% report exposure to skin and respiratory diseases. About 4% suffer from work-induced respiratory illnesses. In addition, foreign workers (mainly

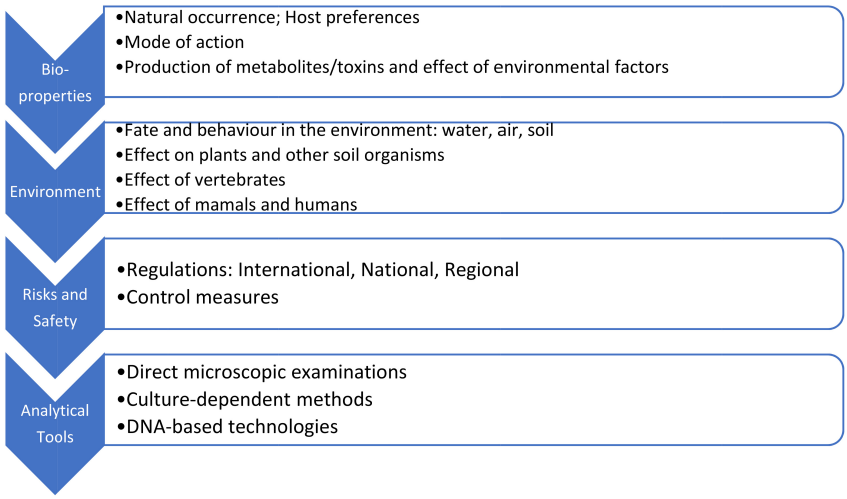

FIGURE 2 | Analytical tools and measures for characterization and monitoring of microorganisms before and after their release in the environment.

migrants) have a higher risk for occupational injuries than native workers (Casey et al., 2015). Bearing the above in mind, we should first assess how the fertilizers containing microorganisms affects the manpower.

Management of safety measures in both preparation and application of microorganism-bearing organic fertilizers and biotechnologically produced BFs/biocontrol formulates is based on the understanding of how microorganisms from microbially-derived products or their derivatives could reach and enter the human body (**Figure 2**). As a rule, it is not possible to know if a given microorganism is completely "safe." Therefore, the general measure is to avoid contact with microorganisms and their metabolites. One of the most important actions to follow includes assessing and avoiding all possibilities of inhalation of contaminant's aerosols and airborne particles (as mentioned earlier) and contamination of hands, eye-hand contact, or absorption through intact skin (Liberman, 1984; Peng et al., 2018). In contrast, some producers of BFs apply techniques, which are far from the biotechnological normal and safe procedures, using open-air fermenter-like vessels, or inoculate microorganisms directly to open-air storage reservoirs of waste substrates (corresponding author information), thus allowing growth and regrowth of both beneficial and pathogenic microorganisms (including spore-forming). Similar regrowth can be observed in composting processes when *Salmonella* spp., *E. coli*, and *Listeria* sp. are present in not-matured composts. All these and similar practices should be controlled and forbidden.

In general, the concept of biosafety is applied first to ensure the safe work with pathogenic microorganisms in the laboratory. Starting from the production of BFs and BAs, it should be mentioned that in biotechnological laboratories and plants, there are many operations, such as centrifugation, homogenization, mixing, blending, aeration of liquids, release of liquids under pressure, and handling of solids, which can form highly contaminated aerosols (Lelieveld et al., 1995). Similar is the contact with biological substances, which occurs during the handling of manure and other organic materials before and after composting due to exposure to liquid/solid particles containing microorganisms or microbial metabolic products. The most frequent diseases in the agricultural sector by biological agents are provoked by bacteria, fungi, and viruses that enter the body through the respiratory, dermal, or digestive routes: Allergies or sensitization processes, such as the farmer workers' lung; Aspergillosis; toxic organic dust syndrome due to worker exposure mainly by inhalation of microbially derived proteins and toxins; and carcinogenic, mutagenic, immuno-toxic, neuro-toxic, hemato-toxic, and hormonal disorders caused by filamentous fungi, such as *Aspergillus*, *Fusarium,* and *Penicillium* producing mycotoxins.

One of the main reasons is the difficulty of controlling the spread of various pathogenic microorganisms, which are resistant to high temperatures, such as fungi and/or sporulating bacteria, before and after treatment in plants and during storage (EU-OSHA, 2009). As a special case/option, it could be mentioned that a microbially treated organic matter enriched with plant beneficial *A. niger* and solubilized P could be partially incinerated at 350–500°C to reduce its volume and, consequently, increase P concentration but also to avoid the presence of microbial biomass/spores (Mendes et al., 2015).

In the production, storage, and application of formulated BF and biocontrol products, workers and biotechnologists should follow the Good Microbiological Practice (GMP) rules, which are normally in use when working with low-risk microorganisms belonging to class I (i.e., non-pathogenic microorganisms). This set of rules is developed to prevent laboratory workers from exposure to the microorganisms and, simultaneously, prevent microorganisms from environmental factors, including contamination by other biological material; it is known that 80% of lab operators carry mycoplasma, which is highly infectious and changes cell metabolism and growth (Falkow, 2008). One of the most important points of the safeguarding strategies should be the information of lab/bio-plant workers and farmers: information, in sense of education and communication, as suggested by the Final

Report of the EIP-AGRI (European Innovation Partnership Agricultural Productivity and Sustainability) focus group on soil organic matter in Mediterranean regions (EIP-AGRI, 2015). They should be well informed about the production details, product quality, product composition, microbial content, potential microbial pathogens and their effects on human health, protection measures, mode of product action in soil-plant systems, and advantages of product application. Authorities and producers should inform farmers on the maximum permitted and real number of pathogens. For example, the Spanish Directive on Fertilizers (Real Decreto 506/2013) fixes the maximum amount (CFU) of the most abundant pathogens in traditional microorganism-bearing organic fertilizers, *Salmonella* and *Escherichia coli*, what is in accordance with the Proposal for a European Commission, 2009. However, information on other potentially pathogenic microorganisms in organic fertilizers is missing. In the recent EC Regulation (Regulation EU 2019/1009 of the European Parliament and of the Council, 2019), organic and organo-mineral fertilizers should be free of *Salmonella* and *Escherichia coli*. Additionally, plant biostimulants should be free of *L. monocytogenes, Vibrio* spp., *Shigella* spp., and *S. aureus*, while for *Enterococcaceae*, anaerobic microorganisms, yeasts, and molds, the CFU can be 10, 105, and 1,000 per g or ml. To fulfill these requirements, and particularly in case of organic fertilizers, all crude materials should be treated in special installations (e.g., plants). Therefore, farmers and workers should be informed about the whole production process starting with the initial operations of recollection and ending with the application of the commercial products and in contrast, correspondingly equipped.

A recently published review questioned the whole process of production and application of microbially based fertilizers and the basic understanding on what pathogenicity is (Hardoim et al., 2015). In contrast, the wide ecological and metabolic diversity in microbes, their relatively short generation time, and their ability to rapidly adapt to and colonize highly specific niches, including specific compartments of animals, humans, and plants, allow some microorganisms to cause disease. For this reason, in the new EU Regulation, the label of potentially dangerous microbial fertilizers (i.e., biostimulants) shall contain the following phrase: "Microorganisms may have the potential to provoke sensitizing reactions" (Regulation EU 2019/1009 of the European Parliament and of the Council, 2019). Special attention and measures need the presence of antibiotics in the microorganism-bearing natural organic amendments and their antibacterial activity, which could affect the structure and function of natural microbial communities thus promoting the accumulation in soil of antibiotic-resistant bacteria ARB and ARGs. The recently adopted EU Commission, 2019/6 on veterinary medicinal products requires that any risk associated with the development of antimicrobial resistance (AMR) must be considered (EU Commission, 2019). However, there is no generally accepted approach for assessing the risk of development or dissemination of AMR in the environment. This problem could be easily solved if antibiotics use is controlled every day individually based on the administration history and the farm environment is strictly managed (Suzuki et al., 2022).

Field workers dealing with potentially dangerous plant beneficial products containing microorganisms could be divided into groups according to the tasks: general tasks, including work with soil, seeds, planting, watering, treating plants with chemical and organic fertilizers, and other groups working directly with animals and derived wastes or working with microorganisms (in case of Spain: Instituto Nacional de Seguridad e Higiene en el Trabajo, 2014). Each group has its specific norms, National but also local Directives prepared by each Spanish province with instructions on how to work with materials contaminated by or bearing microorganisms and how to react in case of emergencies. Therefore, this part of the safety measures is administratively/normatively well-organized for the local workers. However, with the recent migrants' entrance in many EC countries, including Spain, some of them are introduced into diverse agricultural activities thus changing the traditional workforce profile. These newcomers will need special attention as inadequate and unskilled, inexperienced human resources may be easily subjected to microbial contamination when working with microbially bearing products (Itelima et al., 2018). In any case, authorities, biotech producers, and farm workers should be prepared to rapidly analyze scientific elaborated risk assessments of microbially bearing fertilizers concerning human, animal or plant health, safety, or the environment (for more detailed information regarding regulations in the field of BFs, refer to Malusa and Vassilev, 2014).

There is another very important issue when assessing all potential risks of applying microorganism-bearing materials as soil amendments thus potentially entering determined strains in the plant/human/animal microbiome. A recent study analyzed the intime role of microorganisms in mechanisms of development and progression of cancer (Parhi et al., 2020). Microbial species, including *Streptococcus gallolyticus*, *Enterococcus faecalis*, enterotoxigenic *B. fragilis*, enteropathogenic *Escherichia coli*, and *Fusobacterium* spp., were registered in these studies. A typical example is the bacterium *Helicobacter pylori*, which increases the risk of cancer in the stomach. Enterococci are part of the intestinal microbiota in a great variety of hosts. They are particularly abundant in feces of warm-blooded animals and demonstrated a long-term survival in the environment (Wasteson et al., 2020). The question that appears is: Is there any risk of contamination with microorganisms resistant to treatments found in human and animal cancers through sewage sludge/manure/composts applied in the soil-plant-food chain?

CONCLUSION

The main conclusion that could be formulated at the end of this short mini-review is the need of well-regulated and controlled circulation of microorganisms in agricultural ecosystems with further health-beneficial effects on consumers. This approach could ensure highly efficient and safe microbially based and chemicals-free sustainable agriculture. Better structured safety assessment and risk management measures should be developed based on existing knowledge of the microorganisms. From

safety point of view, the first reason of concern is the nature, characteristics, and mode of treatment or production of fertilizer with microorganisms in its composition. Manure and sewage sludge are the main natural sources of potential risks, but serious safety measures are foreseen at the European and National levels. Incentive actions are offered to reduce their potential field application while enhancing alternative uses in composting plants or energy generation and further use of the resulting products (digestates, biochar, etc.). Plant microbiome consists not only of beneficial but also pathogenic microorganisms. Therefore, another important point is the fate of the introduced microorganism-bearing fertilizers, particularly biostimulants, in soil and their effect on the microbial community structure, including autochthonous soil pathogens. In general, we should know the ecological behavior of introduced microorganisms, and the possibility of interactions must be considered in risk assessment actions (Malusà et al., 2021). As suggested by van Elsas et al. (2012), soil microbial diversity and the level of its metabolic activity is the key regulating the fate of a given microorganism introduced in soil. Soil biodiversity dynamics is a multidirectional process where soil management, applied microbial biostimulants, and organic matter interact with the autochthonous microflora within a functioning ecosystem (Vassilev et al., 2021). In short term, the introduction of nutrients derived from the organic fertilizer (compost, treated manure, etc.) or metabolites released from introduced microbial biostimulants might stimulate the growth of whichever microorganism from the plant microbiome, including plant-associated pathogens (Berg, 2009). Therefore, microbial toxicological data, metabolite profile in field conditions, and long-term experiments on assessing the risk for the environment should be performed. Particularly important are risk studies and determination of potentially invasive fungi and bacteria able to survive in stress conditions (Vassilev et al., 2012; Alavi et al., 2014).

Another important conclusion of this short analysis of the development of measures for safe production and use of microbial-based fertilizers is that this field of research and biotechnological/agronomical activity needs a strict but flexible legal framework based on the available database to further support the transition toward more sustainable agriculture. In this sense, a better methodological approach is needed to determine the potential pathogenic power of the plant beneficial microorganisms before their direct industrial production and formulation. In a recent article, Vílchez et al. (2016) proposed a simple, cheap, and efficient strategy to evaluate the potential risk of plant growth-promoting microorganisms for human, animal, and plant health, avoiding the use of vertebrate animals. Another important approach could be the limited use of potentially pathogenic organic materials and their substitution by carefully risk-tested beneficial microorganisms combined with organic matter used in their production process. Manure could be applied after more strict treatments, while compost could be enriched with safe beneficial microorganisms. Although many basics of microbiome biology remain unresolved, could we manipulate the animal microbiome through animal breeding and dietary control thus preventing the presence of pathogens in the manure (Huws et al., 2018)? In contrast, following the principle of One Health approach, microorganisms derived from human gut microbiota can be considered in the near future as a PGP-biocontrol option. By applying this scheme, we could produce safe fresh products rich in probiotics. Thus, the natural circle of soil-plants-humans-soil could be reactivated. In some cases, plant beneficial metabolites (postbiotics) could be applied instead of their producers (Mendes et al., 2017; Vassilev et al., 2017b), thus avoiding the direct application of microbial cells but assessing the effect of all microbial metabolites.

AUTHOR CONTRIBUTIONS

MV and NV designed and drafted the work. SM, LC, EM, LG, VM, and EF-P contributed to the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Antimicrobial Terpenes Suppressed the Infection Process of *Phytophthora* in Fennel-Pepper Intercropping System

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The interactions between non-host roots and pathogens may be key to the inhibition of soilborne pathogens in intercropping systems. Fennel (*Foeniculum vulgare*) can be intercropped with a wide range of other plants to inhibit soilborne pathogens in biodiversity cultivation. However, the key compounds of fennel root exudates involved in the interactions between fennel roots and pathogens are still unknown. Here, a greenhouse experiment confirmed that intercropping with fennel suppressed pepper (*Capsicum annuum*) blight disease caused by *Phytophthora capsici*. Experimentally, the roots and root exudates of fennel can effectively interfere with the infection process of *P. capsici* at rhizosphere soil concentrations by attracting zoospores and inhibiting the motility of the zoospores and germination of the cystospores. Five terpene compounds (D-limonene, estragole, anethole, gamma-terpenes, and beta-myrcene) that were identified in the fennel rhizosphere soil and root exudates were found to interfere with *P. capsica* infection. D-limonene was associated with positive chemotaxis with zoospores, and a mixture of the five terpene compounds showed a strong synergistic effect on the infection process of *P. capsici*, especially for zoospore rupture. Furthermore, the five terpene compounds can induce the accumulation of reactive oxygen species (ROS), especially anethole, in hyphae. ROS accumulation may be one of the antimicrobial mechanisms of terpene compounds. Above all, we proposed that terpene compounds secreted from fennel root play a key role in *Phytophthora* disease suppression in this intercropping system.

Keywords: synergistic antimicrobial ability, intercropping, *Phytophthora capsici*, terpene compounds, reactive oxygen species

INTRODUCTION

Crop diversity is a global trend (Keesing and Ostfeld, 2015), and it has been confirmed that increasing plant diversity in ecosystems not only enhances stability but also prevents biological stresses (Flombaum and Sala, 2008; Newton et al., 2009). Compared with monoculture, biodiversity intercropping can improve utilization of land (Homulle et al., 2021) and enhance nitrogen

absorption to increase yield (Gómez-Rodrìguez et al., 2003), especially for disease management in the field (Zhu et al., 2000). For example, cereal/fava bean (*Vicia faba* L) mixtures decrease the disease incidence of yellow rust and mildew in wheat (Zhang et al., 2019), and cotton (*Gossypium spp*)/cucumber (*Cucumis sativus* L.) intercropping can suppress cotton root rot and wilt (Armanious, 2000). Maize (*Zea mays* L) grown between pepper (*Capsicum annuum*) rows reduced the levels of *Phytophthora* blight of pepper (Yang et al., 2014), and rice-water spinach (*Spinacia oleracea* L.) intercropping effectively controlled rice sheath blight and leaf folders (Ning et al., 2017).

Root exudates of intercropped species can protect neighboring crop plants by directly inhibiting spore germination and mycelial growth, thus reducing pathogen populations in the soil (Zhu and Morel, 2019). *Phytophthora* (pepper blight) is one of the most wide spread and devastating soil-borne pathogens of pepper (Hwang and Kim, 1995). Pepper intercropping with other crops is an effective and economic measure to suppress the spread of this pathogen in the soil (Gao et al., 2014; Yang et al., 2014). Non-host maize plant roots can form a "root wall" below the ground that attracts zoospores of *P. capsici* and simultaneously secretes antimicrobial compounds to kill zoospores, similar examples include garlic (*Allium sativum* L.) root against *P. capsica* (Liao et al., 2015), maize root against *Phytophthora sojae* and rape (*Brassica napus* L.) root against *Phytophthora nicotiana* (Gao et al., 2014; Fang et al., 2016). In addition, the rotation of fennel (*Foeniculum vulgare*) with tobacco (*Nicotiana tabacum* L.) suppressed tobacco black shank caused by *Phytophthora parasitica* (Zhang, 2012). However, there are limited data on the underlying mechanisms by which fennel roots interfere with *Phytophthora* infestation in diversity systems.

Root exudates, including flavone phenolic alcohols, terpenes, isothiocyanates, and glucosinolates (Ren et al., 2003; Kliebenstein, 2009; Wu et al., 2009; Sanchez-Vallet et al., 2010; Cruz et al., 2012; Liu H. et al., 2021), improved plant growth and weakened soil-borne pathogens. Among those compounds, terpenes are hydrocarbons that contain one or more carbon–carbon double bonds and share the same elementary unit of isoprene (2-methyl-1,4-butadiene), with additional pollinator-attractive properties and strong antimicrobial activity against microorganisms (Dalleau et al., 2008; Pandey et al., 2014). Thus, we hypothesized that terpenes may be the key component of the interaction between non-host plant roots and pathogens, and their underlying mechanisms remain to be further studied.

Reactive oxygen species (ROS) are considered to be important in signal transduction pathways in the interaction between plants and pathogens (Voges et al., 2019). Under normal conditions, ROS production and ROS elimination are balanced; however, oxidative stress represents an imbalance due to an increase in ROS (Sies, 2018). Previous studies have found that oxidative stress may play an important role in the antimicrobial mechanisms of natural active products of plants, such as cinnamaldehyde and vanillin, which can inhibit hyphal growth and induce ROS accumulation in *P. nicotianae* (Scott and Eaton, 2008; Torres, 2010; Finkel, 2011; Mittler et al., 2011; Yang et al., 2021).

The aims of this study were to use fennel/pepper-*P. capsici* as a model (1) to study fennel and pepper intercropping for disease suppression in a greenhouse; (2) to observe the interaction between fennel roots and *P. capsica* and identify the terpene compounds in root exudates; and (3) to determine the accumulation of ROS in *P. capsici* hyphae and illustrate the antimicrobial mechanism to reveal the potential mechanism of infection behavior suppression in *P. capsici* blight by non-host plants.

MATERIALS AND METHODS

Plants and Pathogen

The pepper (cultivar "Tianjiao 6") and fennel (cultivar "Siji") seeds, and *Phytophthora capsici* (strain 501) were provided by the State Key Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Agricultural University. *P. capsici* was cultured on V8 medium (30 mL of V8 juice, 6.0 g of agar, 0.5 g of $CaCO_3$, 300 mL of ddH_2O, 121°C for 20 min) at 25°C under a 12-h light-dark cycle for 8 days to produce sporangia. Then, 15 mL of sterilized water was added to the plate and placed in a 4°C refrigerator for 30 min before returning to room temperature for 30 min to release zoospores. The zoospore suspension was collected after filtration with gauze (8 m × 0.84 m) and diluted to a concentration of 10^5 zoospores/mL for further use.

Greenhouse Assay

To determine the effect of the pepper and fennel intercropping system on *Phytophthora* pepper disease suppression, greenhouse experiments were designed. Pepper and fennel seeds were surface sterilized with 3% sodium hypochlorite and then pregerminated in a humid chamber for 3 days at 25°C in the dark. Pepper seeds were sown after the fennel seeds were planted for 7 days in a plastic basin (50 by 30 cm). The greenhouse temperature range was approximately 25–30°C. To confirm the role of fennel plants in the intercropping system, pepper/fennel intercropping was performed (P2/F2), as shown in **Figure 1A**. In the second experiment, all pepper plants were planted with the same row spacing (P2/F2-) to eliminate the influence of physical distance (**Figure 1B**). In the third experiment, a pepper monoculture (P2) was designed (**Figure 1C**). Each treatment was replicated three times. One milliliter of a zoospore suspension (10^5 mL^{-1}) of *P. capsici* was injected into the central pepper seedling in the central pepper row after 30 days (seeding stage). Intercropping borderlines (IB) and monoculture borderlines (MB) indicate the borderlines (indicator line) in the intercropping and monoculture fields, respectively. Intercropping centerlines (IC) and monoculture centerlines (MC) indicate the centerlines (inoculate online) in intercropping and monoculture fields, respectively. Disease incidence in inoculation and indicator rows was investigated from 3 dpi (days postinoculation) until all pepper plants in the inoculation rows were dead.

Disease incidence rate of pepper border row = 100 × [Number of infected plants in border row/Total number of investigated plants in border row]

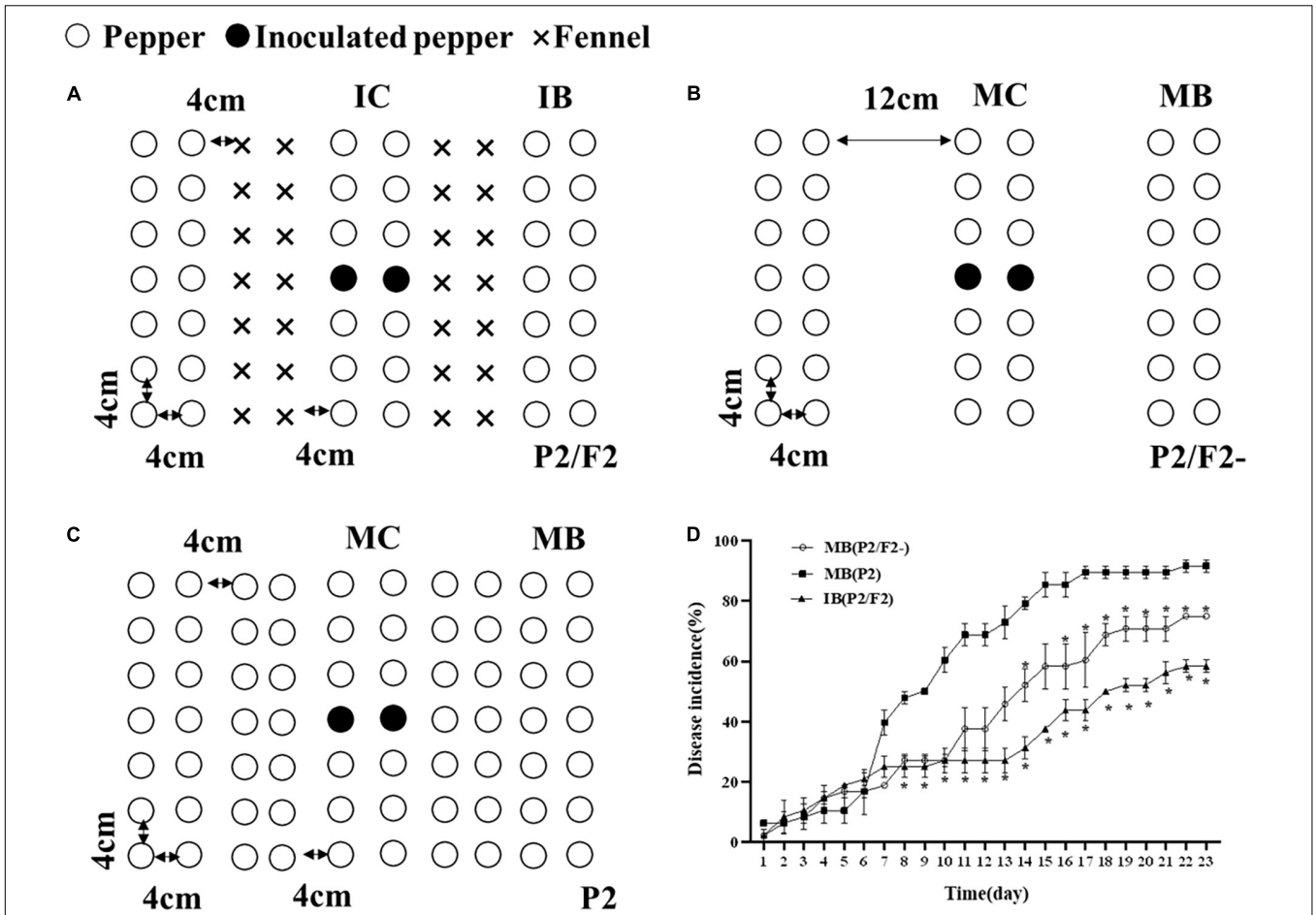

FIGURE 1 | Pepper and fennel intercropping patterns and their effects on pepper *Phytophthora* blight control in the field. **(A)** Pepper and fennel intercropping; **(B)** pepper/blank intercropping; **(C)** pepper monoculture; **(D)** disease incidence of *Phytophthora* pepper blight in monoculture systems, intercropping systems and the intercropping spacing without fennel rows. IB and MB indicate the borderlines (indicator line) in the intercropping and monoculture fields, respectively. IC and MC indicate the centerlines (inoculate online) in the intercropping and monoculture fields, respectively. Significant differences are based on Chi square test ($p < 0.05$). The error bars indicate the standard errors of the means ($n = 3$). The significant differences were compared between monoculture (P2), intercropping with fennel (P2/F2), and peppers spaced as in intercropping but without fennel (P2/F2-). *Indicate $p < 0.05$, respectively.

Field Experiment

A field trial was conducted in 2021 at the Xundian County, Kunming City (N25°20′, E102°41′), Yunnan Province, China. To determine the effect of the pepper and fennel intercropping system on pepper *Phytophthora* blight suppression, we conducted field experiments using a single factor randomized block design with three replicates. And they include pepper monoculture and removing fennel rows from the intercropping system and pepper/fennel intercropping. Each treatment was replicated three times. All the plots were located in the same field and arranged using a randomized block design. When the pepper plants had grown to the fruit period, Phytophthora capsicum cake was used to inoculate the pepper. In brief, the pepper plants were cut with a blade at 0.5 cm below the stem base, and then the cake was put in the wound. The incidence rates of pepper *Phytophthora* disease in the center and border rows were surveyed to show the ability to spread in and across rows. The incidence was calculated by using the following formula.

Disease incidence rate of pepper in the center row = Number of infected plants in center row/Total number of investigated plants in center row × 100%.

Disease incidence rate of pepper border row = Number of infected plants in border row/Total number of investigated plants in border row × 100%.

The Interaction Between Fennel Root and Zoospores of *P. capsici*

A special apparatus was used to monitor the interaction between the spores of *P. capsici* and fennel roots (Yang et al., 2014). Briefly, a U-shaped chamber was formed by placing a bent capillary tube on a glass slide and covering this with a coverslip. The root cap side of fennel roots was inserted into a zoospore suspension (10^5 spores mL^{-1}) in the chamber. The behavior of the zoospores in the root cap was recorded every 5 min for a period of 30 min by taking five photographs of each zone (Olympus BX43F, Japan). A capillary tube replaced the

root as the control. The numbers of zoospores, cystospores and ruptured cystospores in the different root zones were counted. The chemotactic ratio (CR) was calculated as "scores of the zoospores and cystospores on the test root" divided by "scores of the zoospores and cystospores in the control" (Halsall, 1976). CR > 1 indicated positive chemotactic activity. Each interaction assay was replicated three times. Then, the inhibition ratio of swimming zoospores and cystospore germination were calculated (Zhang et al., 2020).

Ratio of zoospore rupture (%) = (Number of ruptured zoospores)/(Number of total zoospores) × 100.

Collection of Fennel Root Exudates

Fennel plants were cultured using a previously described method (Yang et al., 2014), and root exudates were collected by the water culture method (Yuan et al., 2015). The fennel seeds were sown in black plastic pots with 40% humus soil and 60% field soil (poor soil outside the planting area) and grown for 45 days. The collected liquids were filtered, extracted twice with ethyl acetate, and concentrated under reduced pressure (Rotavapor R-200, Buchi). Finally, the concentrate was weighed, redissolved in 1 mL of methanol and filtered through a 0.22 μm filter. The concentration of the fennel root exudate stock was 10,000 μg/mL and that of the collected fluids before concentration was 25 μg/mL. The root exudates were prepared for antimicrobial assays.

Identification of Terpene Compounds in Fennel Root

Headspace solid-phase microextraction (HS-SPME) of fennel root volatiles was conducted. The volatiles were collected from fennel roots that had been growing for 45 days. First, 20 fennel roots were put into the collection flask, and the SPME head was inserted at the top of the collection flask and collected at 25°C for 1 h (Li et al., 2018). Then, the head of extraction for collecting volatiles was directly inserted into the thermal analytical injection port for gas chromatography–mass spectrometry (GC–MS) analysis. The GC–MS fingerprints of the root volatiles were obtained on a GC–MS instrument (Shimadzu QP2010, Japan) (Block, 2010). Then, fennel root volatiles were separated on an SH-Rxi-5Sil MS capillary column (221-75954-30, 30 m × 0.25 mm × 0.25 μm, Shimadzu, Japan). The pressure was maintained at 49.5 kPa, giving a column flow of 1 mL/min. The injection volume was 1 μL in split-less mode, and the injector temperature was 250°C. The initial column temperature was 40°C (hold 2 min) and programmed to increase at a rate of 3°C/min to 80°C and then continue increasing to 270°C at a rate of 5°C/min, where it was then held for 10 min. The ion source temperature was 230°C with an interface temperature of 250°C. Helium (99.999% purity) was used as the carrier gas at a flow rate of 1 mL/min. Mass spectra were obtained in electron impact (EI) ionization mode at 70 eV by monitoring the full-scan range (m/z 35-500). The compounds were identified by matching the obtained mass spectra with those of reference compounds stored in the NIST14 library, except for the compounds that appeared in the control. Then, the standards of terpene compounds were analyzed by GC–MS under similar conditions (Zhang et al., 2020).

Identification of Terpene Compounds in Fennel Rhizosphere Soil

The collection of rhizosphere soil was based on the methods of Bai et al. (2015) with slight modifications. The collected soil (with cultured fennel) and control soil (without any plants) were dried in an oven at 60°C for 12 h. At this time, the water content of the collected soil was 43.02%, and the water content of the control soil was 33.4%. Based on this result, the content of terpenes in the soil water could be calculated.

Experiments were designed to obtain the final concentration range of terpene compounds in fennel rhizosphere terpene standards with different concentrations (0, 1, 10, 20, 30, 100 μg/mL) were added to the blank dry soil (B-soil) to reach the same soil water content as the rhizosphere soil of fennel, and the method of collecting B-soil volatiles and determining the GC–MS fingerprint were the same as those described for fennel root exudates. The regression equation between the terpene standards with different concentrations and the GC–MS fingerprints of the fennel rhizosphere soil were obtained. The concentration-peak area standard curve was used to obtain the contents of terpenes in the soil sample to be measured.

Antimicrobial Activity of Fennel Root Exudates Against Zoospore Motility and Cystospore Germination

The fennel root exudate stock (10,000 μg/mL) was diluted 2, 5, 10, 20, 50, and 100 times, and distilled water containing the same concentration of methanol was used as a control treatment. The chemotaxis of fennel root exudates on zoospores of *P. capsica* was observed using a previously reported method (Fang et al., 2016). A square groove was made with a capillary of 1 mm in diameter, and then the square groove was placed on a glass slide (length 25 mm × width 25 mm × height 1 mm). Zoospore suspension at a concentration of 10^5 spores/mL was added to the tank. One end was treated with a capillary tube to which a diluted root exudate was added, and a capillary containing the same concentration of methanol at one end was used as a control. The behavior of zoospores was observed under a microscope with 40-fold magnification. The experiment was performed three times, each time in triplicate.

The zoospore motility and cystospore germination effects of the root exudates on *P. capsici* were tested as described by Yang et al. (2014). Briefly, 5 μL of root exudates and 20 μL of zoospore suspension (10^5 zoospores/mL) or cystospore suspension (10^5 cystospores/mL) were immediately mixed in glass slides. Then, the final concentration of the root exudates was diluted 5, 10, 25, 100, 150, and 250 times, and the concentrations reached 100, 50, 20, 10, 5, and 2 μg/mL in the slide. The slides were placed in Petri dishes containing moist filter paper and incubated at 25°C in the dark.

Photographs of immobilized zoospores and germinated cystospores were taken under a light microscope. Then, the percentage of zoospores encysted into cystospores was recorded

every 1 min for a period of 5 min, and the percentage of germinated-cystospores was calculated after incubation for 1.5 h. Three fields were observed in each treatment, with 100–150 spores per field and three replicates per treatment.

Antimicrobial Activity of Terpene Compounds Against *P. capsici*

Based on actual concentrations of the rhizosphere measured by GC–MS, the antimicrobial effect of the terpene compounds from the fennel exudates on the infection behavior of *P. capsici* (chemotaxis, zoospore motility, cystospore germination, and hyphal growth) was determined. The terpene compounds were prepared as 1 × and 2 × mixed terpene compounds (MTCs) according to their concentration proportions in the rhizosphere soil of fennel, and each single terpene compound at different concentrations was tested separately using the method with a few modifications described below (Miller et al., 1983).

Six mycelial plugs (7 mm in diameter) of *P. capsici* were placed in 100 mL flasks containing 60 mL of carrot liquid medium and cultured for 36 h at 28°C and 140 rpm. Then, 600 μL of terpene compound solutions of different concentrations were added to each flask, and 600 μL of methanol solution without terpene compound was added as the control; each treatment was repeated four times. After continuous culturing for 12 h, the liquid medium was removed by filtration; the hyphae were then wrapped in filter paper, dried and weighed to calculate the inhibition rate of the hyphae. The calculation method was as follows: mycelium inhibition rate (%) = (mycelia weight of control 1- treated mycelia weight)/(mycelia weight of control 1- mycelia weight of control 2) × 100. Control 1 was the mycelial weight after treatment with 600 μL of methanol solution without terpene compounds, and control 2 was the mycelial weight after culturing for 36 h at 28°C and 140 rpm.

Measurement of Intracellular Reactive Oxygen Species

Intracellular ROS generation was monitored by fluorescence microscopy using a 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent probe. The non-fluorescent compound can be oxidized by cellular oxidants into the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF), which is trapped inside the cells (Murphy et al., 2011). Thus, the fluorescence intensity is proportional to the level of peroxide produced by the cells. Briefly, the mycelium was washed with PBS and then stained with 10 μM H_2DCFDA at 25°C for 30 min after treatment with fennel root exudates and terpene standard compounds for 24 h (Xiao et al., 2013). The fluorescence of DCF was detected by an inverted fluorescence microscope (Leica DM 2000, Germany).

Data Analysis

Statistical analysis was performed with SPSS statistics 24.0 (Stanford University, Stanford, CA, United States) using Chi square test, one-way ANOVA and Duncan's multiple comparisons test, and figures were generated by GraphPad Prism 8 for Windows 10 (Microsoft Corporation, Redmond, WA, United States).

RESULTS

Effect of Fennel and Pepper Intercropping on *P. capsici* Disease Control

As **Figure 1** shows, the intercropping of fennel and pepper (P2/F2) significantly decreased pepper *Phytophthora* blight in the indicator rows ($p < 0.05$) (**Figures 1A–C**) compared with pepper monoculture (P2). Moreover, even though the pepper plants in the monoculture (P2/F2-) were planted with the same row spacing used in intercropping (**Figure 1B**), the disease incidence in the indicator row of the intercropping group was significantly lower than that in the monoculture, and the incidence of IB (P2/F2), MB (P2/F2-) and MB (P2) was 58%, 75%, 91.67%, respectively (**Figure 1D**), during the 23-day observation period. Therefore, the intercropping of fennel and pepper could significantly inhibit the occurrence and spread of *Phytophthora* pepper blight.

Fennel Roots Interfere With the Infection Behavior of Zoospores

The interaction between the fennel roots and *P. capsici* spores was observed under a microscope. As shown in **Figure 2**, zoospores of *P. capsici* showed strong chemotaxis to fennel root (**Figure 2A**). The dynamic chemotactic process showed that zoospores were significantly attracted by fennel root in 5 min, with chemotaxis ratios (CR) of 4.8 (**Figure 2D**). After approaching the fennel roots, the zoospores rapidly lost their swimming ability and transformed into cystospores, and the ratio of zoospore motility was only 4.14% (**Figures 2B,E**). After 30 min of incubation, all zoospores stopped swimming compared with those in the control treatment. After 120 min, fennel roots effectively promoted the germination of cystospores, which was 62.23% higher than that of the control (**Figures 2C,E**).

Fennel Root Exudates Interfere With the Infection Behavior of Spores

The fennel root exudates showed a strong ability to attract zoospores of *P. capsici* in a 20 μg/mL methanol solution, and the chemotaxis index of *P. capsici* was positively correlated with the concentration of root exudates (**Figure 3A**). Fennel root exudates inhibited the motility of zoospores and germination of cystospores in a dose-dependent manner. When the concentration of root exudates reached 20 μg/mL (close to the concentration of the root exudates before concentration), the inhibition rates for zoospore motility and cystospore germination were 40.5% and 13.2%, respectively (**Figures 3B,C**).

Compounds Identified in Fennel Rhizosphere Soil

As **Figure 4** shows, the GC–MS profiles of fennel root volatiles and rhizosphere soil showed five peaks at the following retention times (RT): 12.25 min (beta-myrcene), 13.80 min (D-limonene), 16.30 min (gamma-terpinene), 20.25 min (estragole), and 23.10 min (anethole) (**Figures 4A,B**). The characteristic

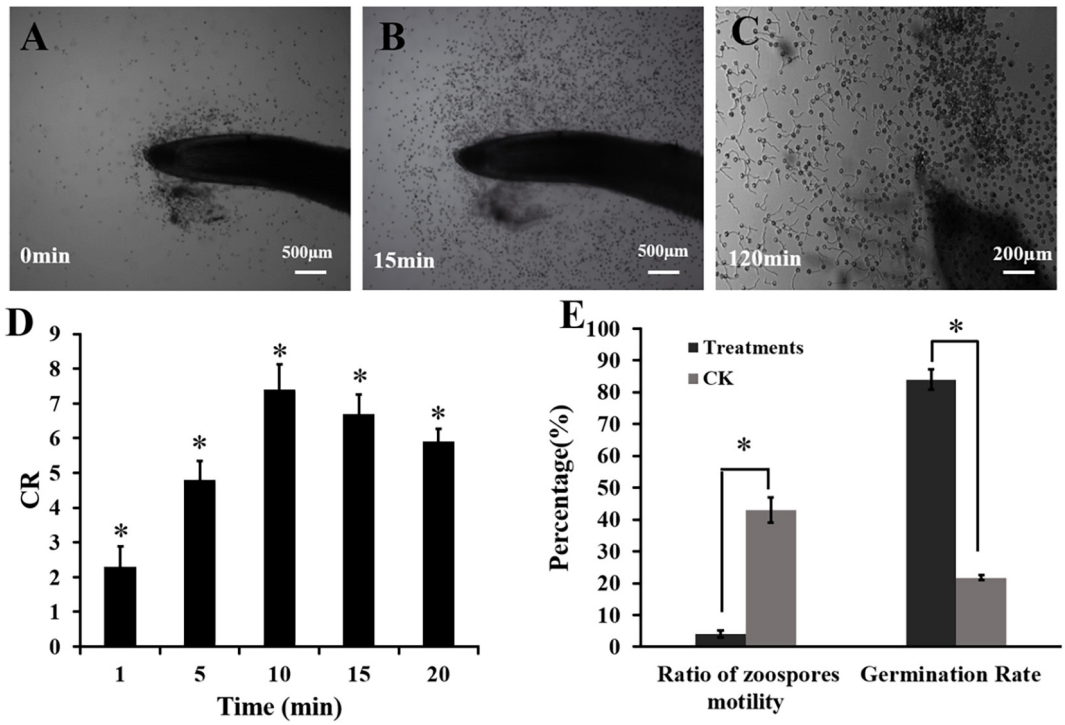

FIGURE 2 | Effects of fennel roots on zoospore behavior of *P. capsici.* **(A,B)** Dynamic process of *P. capsica* zoospore attraction by fennel roots. **(C)** Cystospore germination. **(D)** Chemotaxis ratios (CR): fennel root showed significant attraction to zoospores. **(E)** Germination rate and motility rate in fennel root and zoospore interactions. CK was a capillary tube replacing the root as the control. Significant differences were based on Chi square test ($p < 0.05$). The error bars indicate the standard error of the means ($n = 3$). *Indicates significant differences between the control and plant root treatment at the 0.05 level.

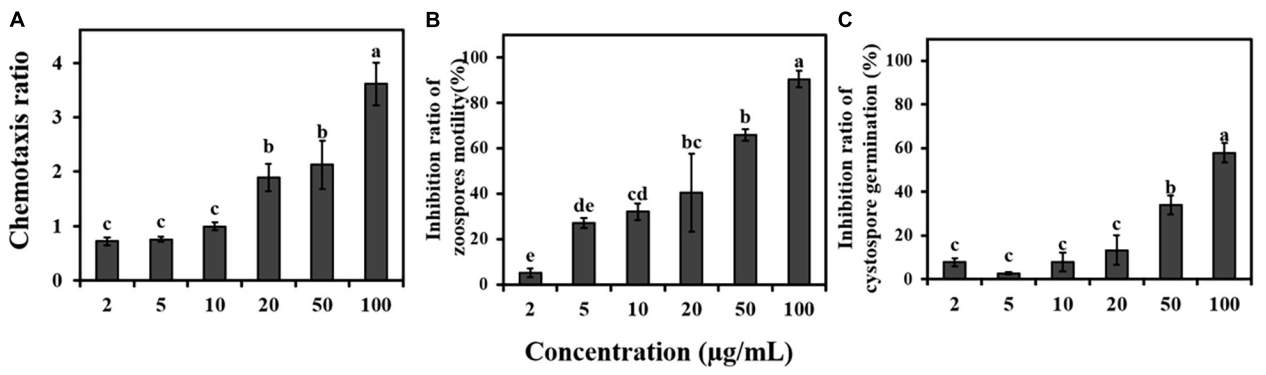

FIGURE 3 | Effects of fennel root exudates on zoospores at different stages. **(A)** Chemotaxis. **(B)** Motility. **(C)** Cystospore germination. Significant differences are based on one-way ANOVA. The error bars indicate the standard error of the means ($n = 3$). Lower case letters show significant differences in the inhibitory effect at different stages at the 0.05 level.

fragment of terpene compounds showed more than 90% similarity in root exudates or rhizosphere soil compared with the standards of terpene compounds. However, none of the five terpenes were detected in the control soil (**Figure 4C**). The contents of D-limonene, estragole, anethole, gamma-terpinene and beta-myrcene were 67.48, 13.54, 18.30, 66.30 and 6.13 μg/g, respectively, in fennel rhizosphere soil. Quantitative analysis of terpenes in rhizosphere soil showed that the content of D-limonene in the rhizosphere soil of fennel was the highest (67.48 ± 12.99 μg/g), and the content of beta-myrcene was the lowest (6.13 ± 0.70 μg/g) (**Table 1**).

Terpene Compounds Interfere With the Behavior of *P. capsici*

As shown in **Figures 5A–E**, the CR of each compound exhibited the ability to attract zoospores of *P. capsici* to a certain extent. Among the five terpenes, D-limonene (100 μg/mL), estragole (10 μg/mL), and anethole (50 and 100 μg/mL) were

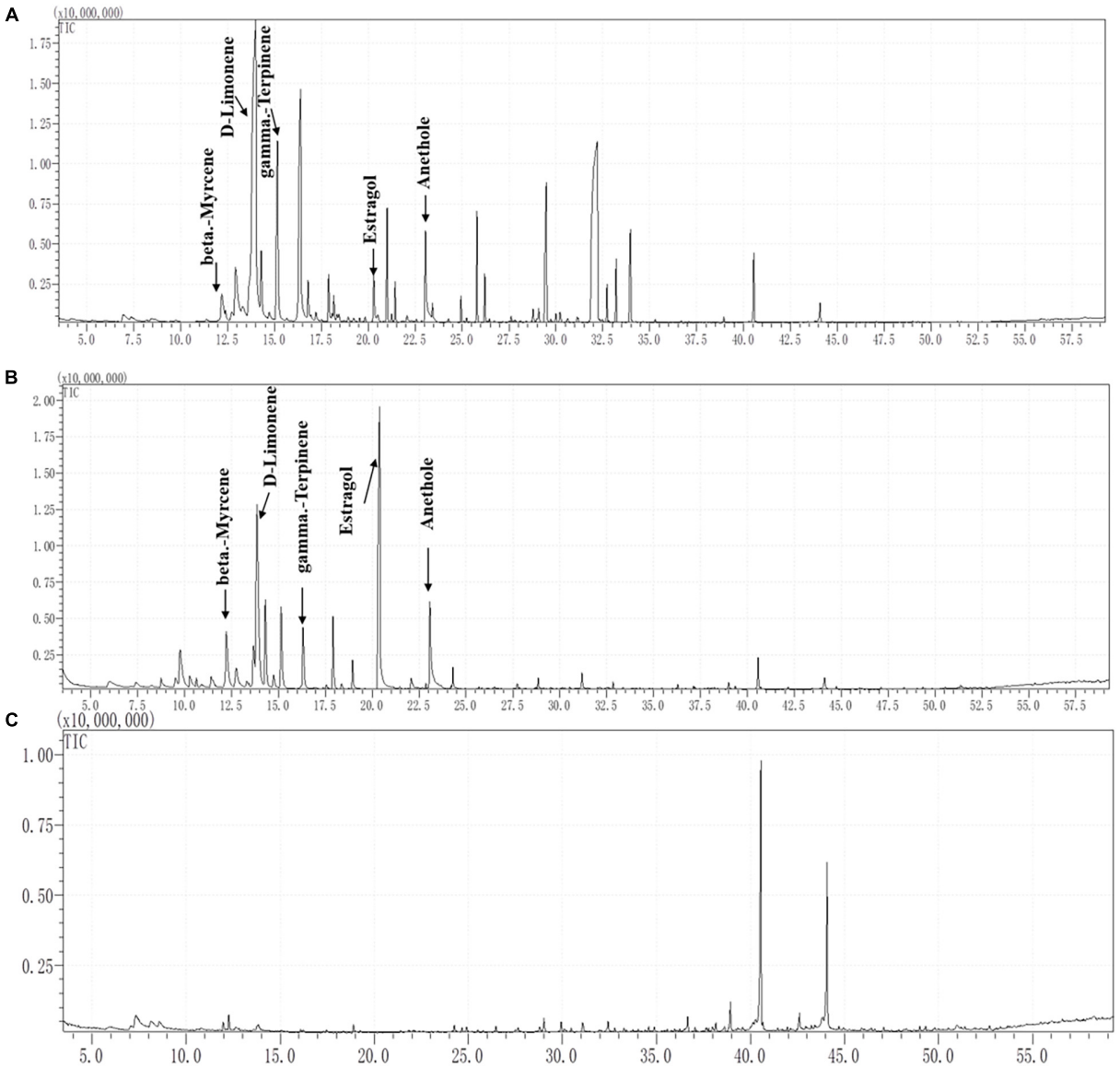

FIGURE 4 | Separation and characterization of terpene compounds from fennel root volatiles and fennel rhizosphere soil by gas chromatography (GC) mass spectrometry (MS) analysis. **(A)** GC–MS profiles of root volatiles, **(B)** GC–MS profiles of rhizosphere soil, and **(C)** GC–MS profiles of the control soil.

TABLE 1 | Quantitative analysis of terpenes in rhizosphere soil.

Analyte	Retention time (RT) (min)	Molecular weight	Calibration curve	R^2	Actual concentration (μg/g)
Beta-Myrcene	12.33	136.23	y = 313474x − 1E + 06	0.9958	6.13 ± 0.70
D-limonene	14.02	136.23	y = 82102x + 648887	0.9973	67.48 ± 12.99
gamma-Terpinene	15.26	136.23	y = 27272x + 26867	0.9992	66.30 ± 0.96
Estragole	20.43	148.2	y = 425864x − 4E + 06	0.9989	13.54 ± 0.71
Anethole	23.22	148.2	y = 217035x − 86524	0.9941	18.30 ± 3.38

Control, soil without plant culture. The error bar indicates the standard errors of the means (n = 3).

associated with positive chemotaxis, and gamma-terpinene and beta-myrcene showed chemotaxis ratios close to 1 at 1, 10, 50, and 100 μg/mL. However, the five terpenes had strong inhibitory effects on the motility of *P. capsici* zoospores at 100 μg/mL. The inhibition ratios of swimming ability for each of the five terpenes were 50.88, 66.83, 32.16, 55.27, and 79.05% at 100 μg/mL, respectively (**Figures 5F–J**). In addition, D-limonene and anethole inhibited cystospore germination in *P.*

capsici; the inhibition ratio was the highest at a concentration of 100 μg/mL; and estragole showed strong activity against cystospore germination at concentrations of 1, 10, 50, and 100 μg/mL (**Figures 5K–O**). These five terpenes significantly inhibited the mycelial growth of *P. capsici* within the effective concentration range, and the inhibition ratio reached 96.5% at 10 μg/mL anethole. Except for anethole and estragole, the higher the concentration of the other three terpene compounds was, the higher the mycelial inhibition (**Figures 5P–T**).

To further explore the effect of terpenes on the behavior of *P. capsici*. The five terpene compounds were mixed according to their concentration proportions in the rhizosphere soil of fennel. As shown in **Table 2**, the five terpene compounds were not attractive to zoospores, and the terpene mixture was slightly more attractive than its corresponding single compound. In contrast to the ability of the terpene mixtures and D-limonene to attract *P. capsica* zoospores, the other four terpene compounds could not attract zoospores at twice the rhizosphere concentration. The inhibitory effect of the mixture on zoospore motility was significantly higher than that of any terpene compound alone, and both 1 × and 2 × MTC reached 100% (**Table 2**). Analysis of the inhibitory effect of the terpene mixtures on cystospore germination also showed that 2 × MTC had a 19.08% stronger effect than 1 × MTC, which was significantly higher than that of any terpene compound alone (**Table 2**). Regarding the inhibition of hyphal growth, estragole and anethole showed similar activity against hyphal growth and exhibited no significant difference from the terpene mixtures. However, the terpene mixtures of 1 × and 2 × MTC showed the highest ratios of zoospore rupture, 89.43% and 98.85%, respectively, compared to a corresponding single compound.

Fluorescence Detection of Reactive Oxygen Species Accumulation

As determined by the DCHF-DA signal (**Figure 6**), fennel root exudates and the five terpene compounds (D-limonene, beta-myrcene, anethole, estragole, and gamma-terpene) significantly induced intracellular ROS accumulation, whereas almost no fluorescence was detected in the control group, suggesting that ROS accumulation may play a key role in hyphal growth inhibition or cell death.

DISCUSSION

In this study, intercropping fennel and pepper successfully prevented the spread of *P. capsici* and reduced the incidence of *Phytophthora* blight disease in the field (**Figure 1**). This intercropping model, using short rod-shaped fennel to control pepper blight, provides a new reference for spatial layout based on biodiversity theory compared with the intercropping pattern of "pepper blight control by high rod-shaped maize" (Yang et al., 2014). Furthermore, we found that the roots and root exudates of fennel could interfere with the infection process of *P. capsici* by attracting zoospores and then inhibiting motility (**Figures 2**, **3**), which may play an important role in the inhibitory effect of fennel and pepper intercropping on pepper *phytophthora* blight. This hypothesis can be proven by previous research showing that some other non-host plants, such as maize and rapeseed, have also been reported to inhibit the swimming or germination of *Phytophthora* zoospores and suppress blight disease in the field (Fang et al., 2016; Zhang et al., 2020). However, the germination of cystospores was suppressed by root exudates in a dose-dependent manner but not by fennel roots (**Figure 3**), which may be related to the higher concentration of antimicrobial compounds in fennel root exudates.

Terpenes are the largest class of natural products and perform a variety of roles in mediating antagonistic and beneficial interactions among organisms (Gershenzon and Dudareva, 2007). In this research, five terpene compounds with the highest abundance were identified both in the fennel root volatiles and the fennel rhizosphere soil (**Figure 4** and **Table 1**). Interestingly, D-limonene, gamma-terpinene, and beta-myrcene with the chemical formula $C_{10}H_{16}$ and estragole and anethole with the chemical formula $C_{10}H_{12}O$ were isomers. Moreover, at the concentrations of these compounds in rhizosphere soil, only D-limonene showed significant ability to attract zoospores of *P. capsici*, similar to that of the mixture with five terpene compounds (**Figure 5** and **Tables 1**, **2**). These results indicated that D-limonene might be the main chemotactic substance in the terpene group that attracts zoospores of *P. capsici*. In addition, all five terpenes with concentrations in fennel rhizosphere soil could significantly inhibit the zoospore motility, cystospore germination and hyphal growth of *P. capsici.* However, estragole and anethole, which are aromatic oxygenated monoterpenes, showed greater suppression of cystospore germination and hyphal growth than monoterpenes, including D-limonene and beta-myrcene. These results indicated that benzene rings or oxygenated monoterpenes might improve the antimicrobial ability, which was consistent with previous reports that oxygenated monoterpenes exhibit better antibacterial activity than non-oxygenated monoterpenes (Campos-Requena et al., 2015; Guimaraes et al., 2019).

Chemotaxis is defined as oriented movement toward or away from a chemical stimulus (Bi and Sourjik, 2018), and we speculate that the taxis of *P. capsici* zoospores may be a complex process that consists of chemotaxis and electrotaxis. For example, high limonene doses exert an important signaling effect to attract the bacterium Xanthomonas citri subsp. citri and the fungus Penicillium digitatum (Rodríguez et al., 2011); the K^+ concentration, bioconduction and electric fields (electrotaxis) share responsibility for this process (Ochiai et al., 2011; Galiana et al., 2019). Therefore, the mechanism of chemotaxis of terpene components still needs to be further investigated.

Synergistic antimicrobial reactions are common in the interaction between antimicrobial compounds and pathogens (Campos-Requena et al., 2015). This point supports our approach of performing an antimicrobial assay on mixtures of the five terpenes, which showed higher suppression of zoospore motility than the same concentrations of individual compounds (**Table 2**). More importantly, the terpene mixture could also cause rupture of almost all zoospores when individual compounds showed little effect (**Table 2**), indicating that the infection process of *P. capsici*

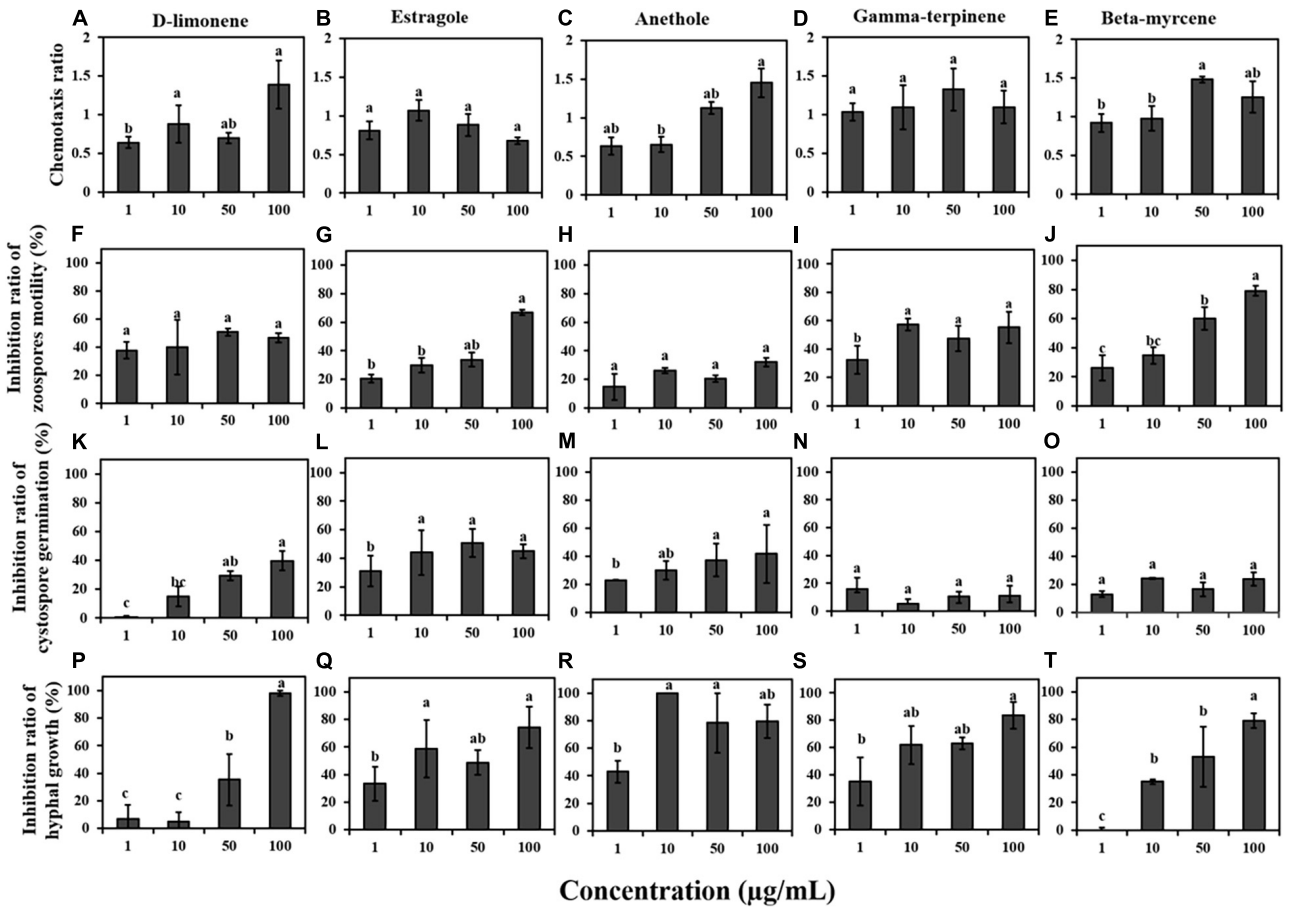

FIGURE 5 | (A–E) Chemotaxis of terpene compounds on zoospores. **(F–J)** Inhibition of terpene compounds on zoospore motility of *P. capsici*. **(K–O)** Inhibition of terpene compounds on cystospore germination. **(P–T)** Inhibition of terpene compounds on hyphal growth. Significant differences are based on one-way ANOVA ($p < 0.05$). The error bars indicate the standard errors of the means ($n = 3$).

TABLE 2 | The antimicrobial activity of a mixture of five terpene compounds against *Phytophthora capsici* infection according to their concentrations in the rhizosphere soil of fennel.

			Inhibition ratio (%)			
Concentration	**Compound**	**Chemotaxis**	**Zoospore motility**	**Cystospore germination**	**Hyphal growth**	**Ratio of zoospore rupture (%)**
× 1(1 time)	D-limonene	0.71 ± 0.12a	45.86 ± 1.23b	21.85 ± 9.22c	27.22 ± 4.12a	4.67 ± 1.62b
	Estragole	0.85 ± 0.08a	36.79 ± 3.50c	28.51 ± 1.72b	30.32 ± 5.90a	4.89 ± 2.00b
	Anethole	0.57 ± 0.14a	41.69 ± 1.63b	41.14 ± 8.49ab	49.18 ± 9.73a	7.92 ± 1.28b
	gamma-Terpinene	0.95 ± 0.47a	44.42 ± 1.80b	28.01 ± 14.36ab	56.91 ± 5.14a	0.58 ± 0.66b
	beta-Myrcene	1.01 ± 0.40a	34.1 ± 2.18b	18.85 ± 2.06c	34.97 ± 12.05a	3.97 ± 1.16b
	1 × MTC	1.00 ± 0.35a	100 ± 0a	64.88 ± 9.41a	51.73 ± 1.11a	89.43 ± 1.32a
× 2 (2 times)	D-limonene	1.41 ± 0.23a	44.175 ± 1.18b	30.31 ± 5.38c	51.18 ± 7.11a	6.79 ± 2.46b
	Estragole	1.04 ± 0.36b	39.0 ± 1.91bcd	65.02 ± 8.00ab	64.59 ± 2.00a	1.84 ± 1.24b
	Anethole	0.36 ± 0.06ab	34.82 ± 1.03cd	46.26 ± 3.61bc	72.95 ± 8.84a	7.23 ± 3.27b
	gamma-Terpinene	0.86 ± 0.35ab	34.1 ± 2.38d	36.40 ± 2.99c	50.38 ± 6.82a	6.62 ± 2.65b
	beta-Myrcene	0.81 ± 0.29ab	40.8 ± 2.81bc	25.55 ± 3.57d	38.71 ± 8.30a	7.84 ± 2.84b
	2 × MTC	1.38 ± 0.17a	100 ± 0a	83.80 ± 11.46a	69.67 ± 14.11a	98.85 ± 0.94a

1 × MTC and 2 × MTC indicate that the concentrations of the mixture were 1 and 2 times the rhizosphere soil concentrations, respectively (mixture = 6.13 μg/mL beta-myrcene + 67.48 μg/mL D-limonene + 66.30 μg/mL gamma-terpinene + 13.54 μg/mL estragole + 18.30 μg/mL anethole).
Different letters in the same group indicate significant differences as determined by one-way ANOVA ($p < 0.05$).
The error bars indicate the standard errors of the means (n = 3).

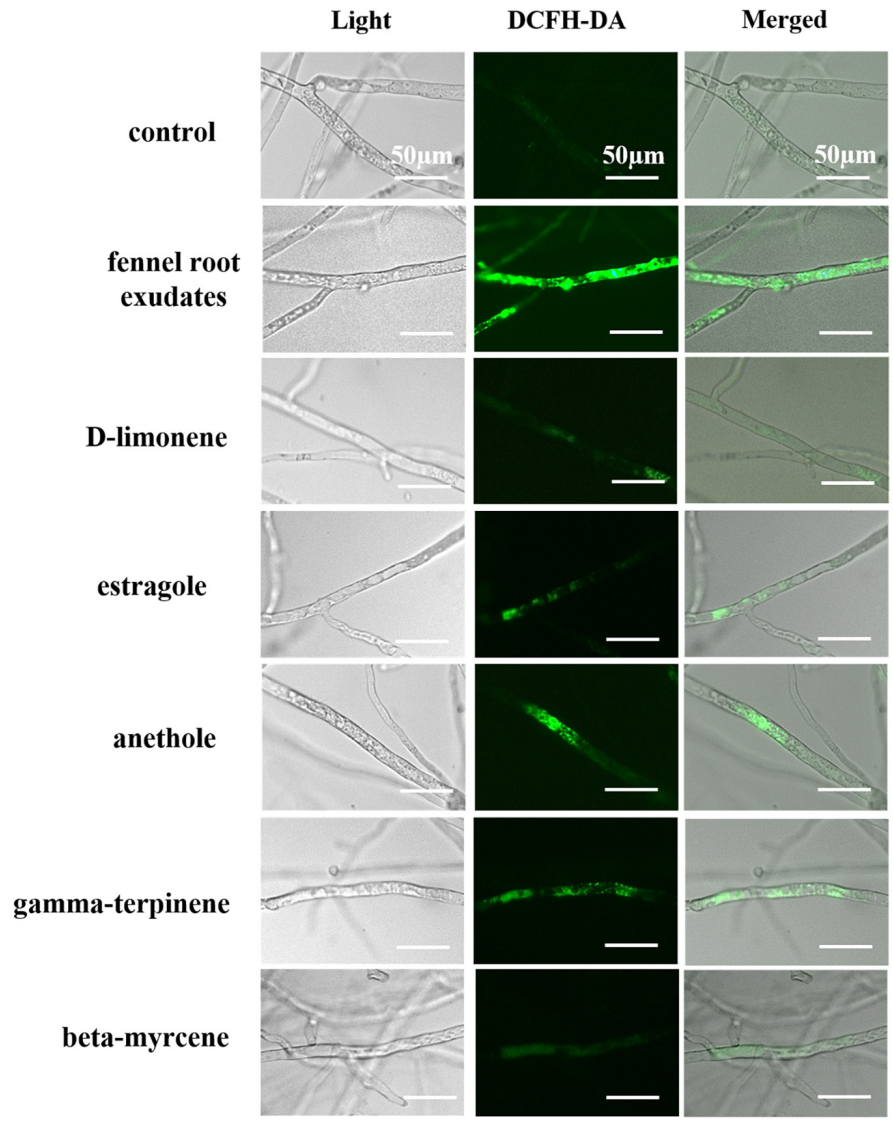

FIGURE 6 | Fennel root exudates and terpene compounds induced ROS accumulation in *P. capsici* hyphae. Bar = 50 μm.

was almost completely cut off by the synergistic interaction of terpenes in the fennel rhizosphere. This synergistic interaction could decrease the minimal effective concentration and increase antimicrobial activity against pathogens (Chung et al., 2011; Berditsch et al., 2015). Hence, the synergistic interaction of terpenes in the root rhizosphere may play a key role in the inhibitory effect of fennel/pepper intercropping on the spread of pepper phytophthora blight.

ROS accumulation has been proposed as the earliest event induced during small molecule substance-pathogen interactions (Camejo et al., 2016). Similarly, terpene compounds could induce ROS accumulation in hyphae, and terpenes with higher antimicrobial activity, such as anethole and gamma-terpinene exhibited a stronger ability to induce ROS accumulation (**Figure 6**), indicating that oxidative stress caused by terpenes may trigger the interference of fennel roots in the infection process of *P. capsici*. Previous studies have shown that allyl isothiocyanate induced ROS accumulation in *Fusarium solani* (Li et al., 2020). Liquiritin inhibited *P. capsici* mycelial growth and sporangial development and significantly induced H_2O_2 accumulation (Liu P. et al., 2021). Hence inducing ROS accumulation may be one of the key mechanisms widely employed by small molecule substances against pathogens.

CONCLUSION

We used fennel roots and *P. capsici* as a model to research the mechanism underlying the inhibitory effect of fennel and pepper intercropping on *P. capsici*. The non-host plant (fennel) roots can attract zoospores of *P. capsici* and then secrete a series of antimicrobial compounds to kill the pathogen. Five antimicrobial

compounds were identified in the fennel rhizosphere, and antimicrobial ability and synergistic interaction play key roles in the interference effect of fennel roots on the infection behavior of *P. capsici*. Moreover, ROS accumulation may be the most important mechanism of the inhibitory effect of key compounds in fennel on *P. capsici*.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YXL and YBL conceived the ideas and designed the methodology. YY, YL, and XM performed the field experiment. YY, JW, and HW performed the GC–MS experiments. JS and YH performed the HS-SPME experiments. YY, YL, and YM performed the biological activity testing of the standards. HH, FD, and SZ collected the data. YY, YL, and XH analyzed the data. YY, YL, YBL, and YXL wrote the manuscript. 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/fpls.2022.890534/full#supplementary-material

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Root-Associated Microbiota Response to Ecological Factors: Role of Soil Acidity in Enhancing Citrus Tolerance to Huanglongbing

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The citrus orchards in southern China are widely threatened by low soil pH and Huanglongbing (HLB) prevalence. Notably, the lime application has been used to optimize soil pH, which is propitious to maintain root health and enhance HLB tolerance of citrus; however, little is known about the interactive effects of soil acidity on the soil properties and root-associated (rhizoplane and endosphere) microbial community of HLB-infected citrus orchard. In this study, the differences in microbial community structures and functions between the acidified and amended soils in the Gannan citrus orchard were investigated, which may represent the response of the host-associated microbiome in diseased roots and rhizoplane to dynamic soil acidity. Our findings demonstrated that the severity of soil acidification and aluminum toxicity was mitigated after soil improvement, accompanied by the increase in root activity and the decrease of HLB pathogen concentration in citrus roots. Additionally, the Illumina sequencing-based community analysis showed that the application of soil amendment enriched functional categories involved in host-microbe interactions and nitrogen and sulfur metabolisms in the HLB-infected citrus rhizoplane; and it also strongly altered root endophytic microbial community diversity and structure, which represented by the enrichment of beneficial microorganisms in diseased roots. These changes in rhizoplane-enriched functional properties and microbial composition may subsequently benefit the plant's health and tolerance to HLB disease. Overall, this study advances our understanding of the important role of root-associated microbiota changes and ecological factors, such as soil acidity, in delaying and alleviating HLB disease.

Keywords: ***Candidatus*** **Liberibacter, soil acidification, metagenomics, endophyte, rhizoplane, defense response, plant-microbe interactions**

INTRODUCTION

Soil acidification has attracted widespread attention due to its negative impacts, including fertility degradation and potential production, and declined root growth of sensitive species. The acidification caused by excessive addition of nitrogen accelerates the loss of base cations, such as calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na), which leads to soil fertility degradation (Zhu et al., 2018). The availability of these base cations is significantly limited because they are replaced by protons and subsequently leached from the root zone accompanied by nitrate in acidic soils (Yang et al., 2013). Therefore, soil pH probably affects plant growth and vitality by limiting nutrient bioavailability. On the other hand, the solubility of toxic aluminum (Al) and manganese (Mn) in highly acidic soil is greatly elevated, which may weaken the productivity of plants by stunting and injuring plant roots (Arunakumara et al., 2012; Riaz et al., 2018). According to previous studies, more than 70% of acidic soils exhibit Al-toxicity and lack of Mg and Ca in tropical America (George et al., 2011). The above indications indicate that the acidified soils can act as a constraint on plant health and disease resistance/tolerance.

Soil is the largest known microbial diversity pool (Torsvik et al., 2002), and rhizoplane are the primary site for plant-microbe interactions. Normally, soil acidity is considered to exert a powerful environmental filter on the soil microbial community assembly and control the activities of soil microbes in surface soils (Schlatter et al., 2020). Meanwhile, the soil microbes, especially bacteria, are key components of the underground ecosystem, which contribute to plant health and defense by affecting carbon and nutrient cycling, plant growth promotion, pesticide degradation, and protecting plants from pathogen infection (Zhu et al., 2014; Trivedi et al., 2020). However, soil acidification weakens and even ceases the activity and survival of beneficial soil organisms, such as nitrogen fixers, decomposers, and nutrient recyclers (Jacoby et al., 2017). On the other hand, the successful colonization and invasion of some soil-borne pathogens are closely related to the ambient pH levels, which regulate the synthesis of pathogenesis factors and the expression of virulence and survival-related genes (Manteau et al., 2003). In South China, soil acidification aggravates the occurrence of bacterial wilt in tobacco plants, because acidic conditions (pH 4.5–5.5) favored the growth of the pathogen *Ralstonia solanacearum*, but inhibited the growth of antagonistic bacteria of *Pseudomonas fluorescens* and *Bacillus cereus* (Li et al., 2017). Altogether, many studies have reported that decreased pH has a direct influence on microbial structure and leads to the imbalance in the topsoil micro-ecosystem and even the accumulation of soil-borne pathogens in acidified soil. However, up to now, researchers have paid limited attention to the correlation between soil acidification and systemic diseases with root symptoms caused by non-soil-borne pathogens, and the impact of soil acidification on the structure and function of the soil microbial community of plants infected with systemic diseases.

Huanglongbing (HLB) is a systemic disease of citrus caused by phloem-limited and nonculturable bacteria "*Candidatus* Liberibacter" spp., which is the greatest threat to citrus production worldwide. In south China, HLB is associated with Asian strain "*Ca*. L. asiaticus" (CLas) is transmitted by citrus psyllids to all commercial cultivars of citrus., the above-ground symptoms of HLB in orchards include blotchy mottle, and yellow shoots, and asymmetric fruits (Li et al., 2020). Inexplicably, the discrepancy between the slow development of foliar symptoms and the rapid yield losses of HLB-infected citrus trees, indicates that unobserved underground damage, such as root decline, has occurred before the emergence of foliar symptoms (Johnson et al., 2014). Meanwhile, the root damage and lower fibrous root density of the infected trees could restrict the absorption of water and soil nutrients (Atta et al., 2020). Additionally, HLB invasion has a non-negligible effect on the remodeling of citrus root-associated microbes, which causes enrichment and reduction of specific disease-induced root-associated microbial taxa in the early and late phases of HLB disease (Trivedi et al., 2010, 2012; Zhang et al., 2017; Ginnan et al., 2020). As one of the top three fruit crops in the world, the citrus industry has developed rapidly in China in recent years. Unfortunately, HLB has emerged in 10 of China's 11 major citrus-growing provinces, but there was no definite and sustainable cure insight (Zhou, 2020). The global citrus industry is facing the huge challenge of HLB invasion, and the prevention and control situation is still grim.

Another challenge of citrus planting in China is soil acidification and nutrient deficiencies. It is well known that citrus cannot thrive in acidic soils, especially when the soil pH is 5.0 or lower, serious problems may arise, which make citrus more susceptible to abiotic and biotic stresses (Long et al., 2017). Regrettably, most of the citrus orchards in China have moderately or strongly acidic soils. Fan et al. (2015) reported that the pH values of over 1,400 soil samples from 477 orchards in 18 counties of Ganzhou, Jiangxi Province was 4.6, of which 80.0% of orchard soils with a pH value lower than 5.0. Citrus plants are perennials, and their roots are exposed to the same soil during their life cycle. Therefore, it is necessary to maintain proper soil acidity in the root zone to avoid Al-toxicity and provide sufficient calcium and magnesium for citrus growth (Natale et al., 2012). Moreover, the causal agents cannot be cultured, which is a great obstacle to revealing the direct interaction between HLB pathogen and ecological factors. Our previous study showed that the application of lime in the HLB-endemic and soil acidified citrus orchards could efficiently correct soil acidity and delay the progress of HLB, thereby improving the fruit yield and quality of citrus groves (Li et al., 2020). The correction of soil acidity enhanced the tolerance of citrus to HLB disease, which is due to the direct promotion of citrus root metabolic activity and the direct activation of citrus immune defense response. On this basis, we also speculate that this transformation in HLB tolerance may be related to the reshaping of citrus root and rhizoplane microbiome. Therefore, more studies are required to explore how soil acidification affects the microbial community assembly process of the HLB-infected citrus root-associated microbiome.

In this study, we evaluated the effects of lime treatment on the soil properties and the resident microbial community of HLB-infected citrus roots in acidic soil. We combined lime application and microbiological sequencing analysis to investigate the distinct and enriched taxa and functions of citrus root and

rhizoplane, and how soil acidity affects those enriched taxonomic and functional attributes in HLB root-related microbiomes. Our results emphasize the associations, not causation, between HLB pathogen, microbiota, soil acidity, and citrus tolerance.

MATERIALS AND METHODS

Site Description and Experiment Set Up

This study was performed in a citrus orchard in Xunwu Country, Ganzhou City, Jiangxi Province, China (lat 24°94′ N, long 115°44′ E) in 2019. The region is characterized by a subtropical, humid monsoon climate with a mean annual rainfall of 1,600 mm and an average annual temperature of 18.9°C. The soil at this site belongs to clay red soil, which is derived from the argillaceous rock with high soil acidity, and the average pH value is 4.3. The experimental plants were 8-year-old orchard trees of "Newhall" (*Citrus sinensis* Osbeck cv. Newhall) navel orange grafted on Carrizo citrange rootstock [*Citrus sinensis* (L.) Osb × *Poncirus trifoliate* (L.) Raf.], 2–3 m tall, and 1.5–2 m wide, spaced 3 × 4 m apart. In the experiment of lime application in citrus orchards, nine CLas-infected trees with equal disease severity were selected based on quantitative PCR results of root samples and visual foliar symptoms of HLB in <30% of the canopy. Three treatments were included in this study: SCK (soil without lime treatment), SLL (soil treated with low-level lime, 1.25 tons/ha), and SHL (soil treated with high-level lime, 2.5 tons/ha), and each treatment with three replications. The slaked lime used in the study was locally purchased, the calcium hydroxide content was higher than 60%, and more than 90% of the material could pass through an 80-mesh sieve. The slaked lime was sprinkled onto the soil surface in the 4 × 4 m area based on the center of the citrus tree trunk to ensure that the corrective had adequate coverage and then was mixed thoroughly into the soil layer by plowing in January 2019. In addition, a total of 450 g of N, 150 g P_2O_5, and 450 g of K_2O were added per plant per year, which was applied in four portions by digging a shallow furrow under the canopy of each tree. A single application of 5 kg peanut cake fertilizer per plant per year was also made. Meanwhile, irrigation, weed, and pest management in orchards were timely implemented according to the unified standard. Fertilizer inputs (N-P_2O_5-K_2O) occurred in all plots, including the control treatment.

Soil and Plant Sample Collection

Fibrous roots (∼1.5-mm diameter) and corresponding soil samples were collected at a depth of 5–15 cm soil layers 1 week before the harvest of citrus fruits in November 2019. For each tree, the samples were collected from the four locations ∼0.8-m away from the trunk and were, respectively, pooled together as one composite sample. Firstly, the loosely attached soil on the roots was acquired with gentle shaking for soil property analysis and was termed root-zone soil. The roots were subsequently placed in sterilized and pre-cooled PBS buffer, and the rhizosphere soil adhering to the roots was removed by hand-shaking for 2 min, and the rhizoplane soil was extracted by ultra-sonication as described by Zhang et al. (2017). Briefly, the roots from the previous step were sonicated twice with a 60 Hz cold Ultrasonic water bath, each time for 30 s, with an interval of 5 s. Finally, the rhizoplane soil was collected by centrifugation at 12,000 × g for 1 min at 4°C and stored at −80°C until DNA extraction. On the other hand, about 5 g of ultrasonically treated root of each sample was preserved at −80°C after being drained with sterile filter paper for nucleic acid extraction, and another part of 10 g was stored at 4°C to test root activity immediately, and the remaining was air-dried to test the physicochemical properties.

Nucleic Acid Extraction

Total DNA from rhizoplane soils and fibrous root segments was, respectively, extracted using the DNeasy Plant Mini Kit and the DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. DNA quality and quantity were assessed using a TBS-380 Mini-Fluorometer (Turner Biosystems, CA, USA) and a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), respectively.

Detection of HLB Pathogen

The q-PCR analysis for CLas titers of root samples was performed with primers and probe as previously described (Li et al., 2006). Additionally, 1 μl (40 ng) of fine root template DNA was used in a 20 μl qPCR reaction volume on an ABI7500 Real-Time PCR system using TaKaRa Ex Taq HS DNA Polymerase according to the manufacturer's instructions. The standard amplification protocol was 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. All reactions were performed in triplicate. The number of CLas genomes in a reaction was calculated using a recombinant plasmid standard curve containing the target sequence.

Root Activity Measurement

Determination of root activity was done according to the triphenyl tetrazolium chloride (TTC) reduction test. First, 0.5 g fresh root tip samples were immersed in 10 ml of TTC solution (0.04% in phosphate buffer at pH 6.8) for 1 h at 37°C. Then, the reaction was terminated by adding 2 ml 1 M H_2SO_4. The roots were drained and then homogenized in ethyl acetate and centrifuged at 12,000 × g for 5 min. Finally, the liquid phase was adjusted to a volume of 10 ml with ethyl acetate and the absorbance of the extract was recorded at 485 nm.

Soil Property Analysis

All soil samples were ground and sieved before soil analysis. The detailed analytical methods for measuring edaphic properties, including soil pH and the contents of soil organic carbon (SOC), available nitrogen (AN), available phosphorus (AP), available potassium (AK), exchangeable magnesium (Mg^{2+}), calcium (Ca^{2+}), and aluminum (Al^{3+}) are described in previous studies (Kachurina et al., 2000; Nelson and Sommers, 2018; Wan et al., 2020). Briefly, Soil pH was measured at a ratio of soil/water of 1:2.5 (m/v) using a digital pH meter; SOC was measured by loss-on-ignition method, and AN was determined using the alkali hydrolyzation procedure. AP and AK were extracted with 0.5 M sodium bicarbonate and 1 M ammonium acetate, respectively, and then measured with inductively coupled plasma optical

emission spectrometry. The exchangeable cations (Ca^{2+} and Mg^{2+}) were extracted using ion exchange resins and determined by atomic absorption spectrometry. The exchangeable Al^{3+} was extracted with neutral 1 mol L^{-1} KCl at a 1:10 soil/solution ratio and determined by titration with a 0.025 mol L^{-1} NaOH solution.

Next-Generation Sequencing

DNA extracted from citrus roots was selected for 16S amplicon sequencing and DNA extracted from rhizoplane soils was used for shotgun metagenomic sequencing, respectively. The samples were sequenced on the Illumina System by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). For the amplicon sequencing, the amplification of 16S DNA fragments was performed using primer pairs 799F-1391R and 799F-1193R covering the prokaryotic 16S rDNA V5-7 regions. After quality control, quantification, and normalization of the DNA libraries, 300-bp paired-end reads were generated from the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions with modifications for 16S amplicon analyses. Large-scale shotgun metagenome sequencing was performed on the Illumina NovaSeq platform (Illumina Inc., San Diego, CA, USA) using NovaSeq Reagent Kits, and each DNA shotgun sequencing sample contained 6 Gb of sequencing data. All sequencing data associated with this project have been deposited in the NCBI Short Read Archive database (Bioproject accessions: PRJNA835291 and PRJNA827960).

The data were analyzed using the free online platform, the MajorBio i-Sanger cloud platform (www.i-sanger.com). For amplicon data analysis, the poor quality reads were subjected to filter and trim using Trimmomatic software and then merged paired-end reads using FLASH software with the default setting (Magoc and Salzberg, 2011). High-quality remaining sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE (Edgar, 2013). Representative sequences of each OTU were classified by the RDP classifier (Wang et al., 2007), and compared with the SILVA database (Quast et al., 2013). The final output of the taxonomic assignment results reached a confidence level of ∼70% in different taxonomic levels. The resulting raw OTUs table was filtered to remove OTUs with less than two sequences in a single sample, and the filtered OTU table was used as input for further analysis. For metagenomic data analysis, the raw reads were pre-processed to remove poor-quality reads and adaptor sequences. The clean filtered reads from all soil samples were assembled using megahit v1.03 (Li et al., 2015). Then, all sequences with an identity cutoff of 95% were clustered and reduced redundancy to construct the non-redundant gene categories (unigenes) using CD-HIT-est (Fu et al., 2012). To access the taxonomic annotation of the unigenes, the amino acid sequences were aligned against the NCBI microbial non-redundant protein (Nr) database using the DIAMOND software (blastp, E-value cut-off of 1×10^{-5}) (Buchfink et al., 2014). The microbial-metabolic functions of the unigenes were predicted by blasting against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) database. Moreover, the high-quality reads from rhizoplane samples were aligned against the unigenes to calculate the taxonomic and functional abundance by SOAP2 (Li et al., 2009) with the default parameter.

TABLE 1 | Changes in soil properties of HLB-infected citrus root-zone soil with low slaked lime (SLL) and high slaked lime (SHL) applied to the soil surface.

Soil parameters	SCK	SLL	SHL
pH	4.23 ± 0.04a	5.20 ± 0.07b	5.90 ± 0.07c
SOC (g/kg)	11.64 ± 0.92a	11.28 ± 1.82a	12.39 ± 2.01a
AP (mg/kg)	20.49 ± 3.19a	20.82 ± 1.60a	23.92 ± 1.69a
AK (mg/kg)	140.47 ± 8.16a	133.08 ± 11.08a	123.00 ± 4.01a
AN (mg/kg)	104.91 ± 3.03c	90.59 ± 5.16b	70.42 ± 1.12a
Ca^{2+}(mg/kg)	705.74 ± 52.15a	1,409.07 ± 86.18b	1,862.6 ± 76.48c
Mg^{2+}(mg/kg)	93.85 ± 2.48a	101.12 ± 3.87a	98.29 ± 5.05a
Al^{3+}(mg/kg)	240.46 ± 15.53c	146.32 ±15.36b	84.34 ± 9.91a

Different letters denote significant differences between treatments from a Tukey's HSD test ($P < 0.05$).
SCK, without lime application.

Statistical Analyses

Simpson, Shannon, ACE, Chao, and coverage indices were used to compare the α diversity of the bacterial community and they were calculated by Mothur version v.1.30 (http://www.mothur.org/), in R with the VEGAN package. Shannon index based on standardized OTU abundance table was used to evaluate the within-samples diversity (Weiss et al., 2017). Before analysis, all data were normalized by the minimum number of reads per sample. Non-parametric Kruskal-Wallis tests were used to determine the significant differences in α diversity across compartments. The taxonomic dissimilarity analysis between samples was conducted based on the principal coordinate analysis (PCoA) method with Bray-Curtis distances (β diversity) (Lozupone et al., 2011). Variation partitioning analysis (VPA) analysis with two-way PERMANOVA was performed by using VEGAN packages in R software based on OTU relative abundance table. The linear discriminant analysis (LDA) effect size (LEfSe) was used to identify differentially abundant taxa using the non-parametric factorial Kruskal-Wallis H test, A significance alpha of 0.05 and an effect size threshold of 4 were used for all of the biomarkers evaluated.

RESULTS

Characterization of Soil Properties

Soil pH significantly increased after lime application (**Table 1**), from 4.32 to 5.2 and 5.9, respectively, when the different slaked lime was applied to orchard soil. Soil AN content showed a generally declining tendency after lime application (**Table 1**). SOC, AP, AK, and exchangeable magnesium contents exhibited little variation among treatments (**Table 1**). In addition, soil pH was negatively correlated with exchangeable aluminum and positively correlated with exchangeable calcium. These results indicated that some soil properties might be closely correlated with the severity of soil acidification of citrus orchards.

TABLE 2 | Changes in CLas population and root activity of HLB-infected citrus with low slaked lime (SLL) and high slaked lime (SHL) applied to the soil surface.

Treatments	CLas population (gene copies in 40 mg of fibrous root DNA)	Root activity ($\mu g \cdot g^{-1}\ h^{-1}$)
SCK	$(4.37 \pm 1.34) \times 10^5$	57.89 ± 9.01a
SLL	$(9.04 \pm 2.34) \times 10^4$	98.45 ± 12.32b
SHL	$(2.04 \pm 1.28) \times 10^5$	81.34 ± 10.71b

Different letters denote significant differences between treatments from a Tukey's HSD test ($P < 0.05$).
SCK, without lime application.

Populations of CLas in Roots and Root Activity

The presence of CLas in roots from different treatments is shown in **Table 2**. In general, soil amendments reduced the levels of CLas genomes in roots. Compared with the control, the populations of CLas in SLL and SHL treatments significantly decreased by more than 50%, respectively. These results were consistent with those of observation results of HLB symptoms in citrus roots. The severity of HLB disease has been greatly alleviated after lime application, which shows that the difference between fibrous root and biomass could be visually distinguished. Meanwhile, the activity of citrus roots from different types of soils was examined, which was significantly enhanced in the limed soils compared with that in the un-limed soils (**Table 2**), but there were no significant differences between the SLL and SHL treatments.

Effects of Lime Application on Taxonomic Features of Root-Endophyte Bacterial Community

The 16S rRNA gene sequencing was used to detect the responses of the endophytic bacterial community resident inside roots of HLB-infected citrus to three soil systems with different soil acidity. After quality filtering, a total of 216,348 high-quality reads were obtained and clustered into 1,305 OTUs for community analysis (**Supplementary Table S2**). Good's coverage estimators were all above 98.6%, suggesting that the sequencing depths were adequate for the bacterial communities. The number of OTUs and richness indices (Chao1, ACE, and Shannon) in bacterial communities displayed a significant increase in the SLL and SHL samples compared to the SCK samples (**Supplementary Table S1**). Regarding the Simpson index, SCK showed a significantly higher Simpson index than SLL and SHL ($p < 0.05$). The application of limestone had a significant effect on the α diversity of the HLB-infected root microbial community. However, the difference was not significant between bacterial community diversity indices of the RLL and RHL samples (**Supplementary Table S1**).

To determine whether the variations of endophytic bacterial community structure in HLB-infected citrus roots are related to soil acidity, we profiled the overall structural changes in bacterial communities in each treatment using Bray-Curtis distances-based PCoA (β diversity) with OTUs annotated at the genus level. The structure of bacterial communities exhibited more similarity in the SLL and SHL treatments, whereas SCK samples form a distinct cluster that was significantly separated from SLL and SHL samples (**Supplementary Figure S1**). Moreover, the ANOSIM result showed that the differences between groups were greater than those within groups ($R = 0.88$, $p = 0.001$). These results indicated that different rates of limestone application affected the structure of the original bacterial community in HLB-infected citrus roots from highly acidified soils.

Based on the diversity analysis of the bacterial community, qualified sequences were assigned to 26 phyla, 51 classes, 147 orders, 280 families, and 869 genera across all samples. At the phylum level, Actinobacteria (56.17–62.89%), Proteobacteria (27.85–34.26%), Firmicutes (3.32–8.14%), Chloroflexi (0.06–0.11%) and Dependentiae (0.05–0.11%) were top 5 dominant bacteria and the composition was similar in all treatments, accounting for over 97.25% of the total OTUs (**Figure 1A**). However, Firmicutes and Chloroflexi were relatively more abundant in RLL and RHL roots than in the control, and the relative abundance of Actinobacteria in RCK was higher than that in SLL and SHL. Whereas, no significant difference was detected among the SLL and SHL treatments.

At the order level, such as *Streptomycetales* (45.36%), *Catenulisporales* (1.12%) of *Actinobacteria*, and *Xanthomonadales* (18.16%) of *Gammaproteobacteria*, were present at a higher relative abundance in RCK, whereas multiple orders, such as *Rhizobiales* (9.07–9.49%) of *Alphaproteobacteria*, *Pseudonocardiales* (4.93–8.56%) of *Actinobacteria*, and *Bacillales* (4.67–7.71%) of *Firmicutes*, were significantly enriched in SLL and SHL (**Figure 1B**). At lower (genus and OTUs) taxonomic ranks, we first selected the genera with the top 50 average relative abundance for heatmap analysis (**Figure 2**). The result showed that *Streptomyces* (45.38%), *Nocardia* (16.12%), and *Rhodanobacter* (17.82%) were the predominant bacterial genera in RCK, which gradually decreased in the limestone application group. As expected, the relative abundance of *Candidatus Liberibacter* in RCK was much higher than that in RLL and RHL, which was consistent with the changing trend of absolute abundance of HLB pathogen root samples determined by qPCR. On the other hand, most other genera, such as *Bacillus*, *Bradyrhizobium*, *Paenibacillus, Pseudomonas,* and *Burkholderia-Caballeronia-Paraburkholderia*, are generally considered to contain many plant-beneficial bacteria, were more abundant in SLL and SHL compared to SCK (**Figure 2**).

Also, Kruskal Wallis H test results ($p < 0.05$) indicated that 13 of the top 15 dominant bacteria from SCK at genus level and *C. Liberibacter* had significant abundant differences in response to different soil acidity (**Figure 3**). Subsequently, the LEfSe results used to examine taxa differences from phylum to genus between the treatment (SLL or SHL) and control groups (SCK) were represented in a multi-level species hierarchy tree diagram (**Supplementary Figure S2**) and a linear discriminant analysis table (**Table 3**) (logarithmic LDA score > 4). In the SCK sample, 8 bacterial taxa (Group 1) belong to two ordres (*Streptomycetales* and *Xanthomonadales*), three families

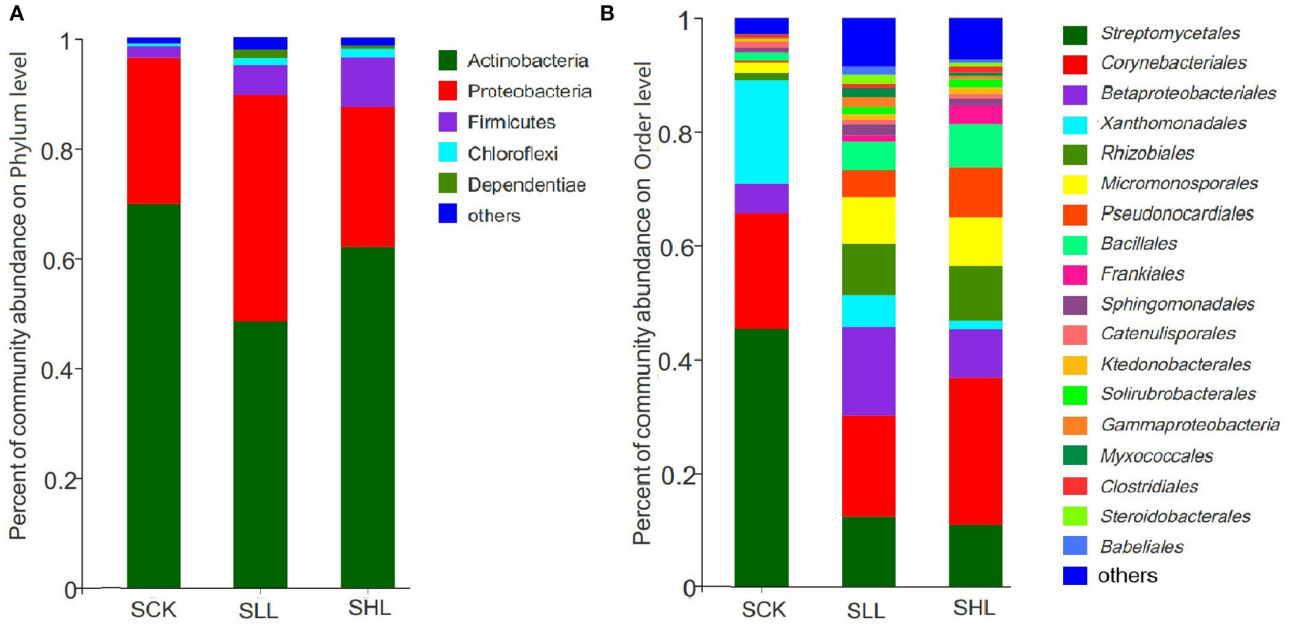

FIGURE 1 | Taxonomic distribution at the phylum **(A)** and Order **(B)** level in HLB-infected citrus root samples based on 16S amplicon. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime. Taxa with <1% of reads were combined together as "others".

(*Nocardiaceae*, *Streptomycetaceae,* and *Rhodanobacteraceae*) and three genera (*Nocardia, Streptomyces,* and *Rhodanobacter*) were significantly more abundant in pairwise comparisons of treatments. For samples from amended soils, SLL and SHL treatments shared 16 core bacterial taxa (Group 2) taken as potential taxon indicators for response to soil acidity of HLB-infected roots. Among those taxa, phylum Firmicutes had significantly higher relative abundance in SLL and SHL treatments. At the genus level, SHL treatment exclusively and significantly increased the relative abundance of 5 additional genera (Group 4), including *Bacillus*, *Acidothermus*, *Micromonospora*, *Amycolatopsis*, and so on.

Effects of Lime Application on Taxonomic Features of Rhizoplane Microbial Community

We collected the rhizoplane soil samples from HLB-diseased citrus trees under different soil acidity for metagenomic sequencing analysis. More than 420.6 million clean reads were generated for the 9 samples, yielding a total of 3,139,960 contigs after assembly. The metagenomic sequences were chosen to define the change of core rhizoplane microbiome from highly acidified to lime-amended soils duo this method could provide more comprehensive taxonomic information given the community compositions. A total of 12,358 species were obtained after taxonomic annotations, 90.37% of which were prokaryotic (bacteria and archaea), and a small fraction of species were annotated as eukaryotes (including fungi, protozoa, algae, and plants) and viruses (**Supplementary Table S2**).

We then investigated the taxonomic distinctiveness of the HLB-infected citrus rhizoplane microbiome under different soil acidity. No significant difference in α diversity between the treatments at the species level was seen, suggesting that limestone application alphas did not significantly alter the overall richness (represented by the Chao index) and diversity (represented by the Shannon index) of the root-associated microbiome. However, PCoA based on Bray_Curtis distance (β diversity) revealed that the community composition of the rhizoplane at the species level in SLL and SHL differed from that in SCK (**Figure 4A**). We compared the relative abundances of microbial communities in all the samples at both high (phylum) and low (genus) taxonomic ranks to identify those community members differing in abundance in citrus rhizoplane soil from acidified soils and amended soils (**Figures 4B–D**). The dominant prokaryotic phyla found in the HLB-infected citrus rhizoplane from different soil types, including Proteobacteria, Actinobacteria, Acidobacteria, and Firmicutes. The results revealed that the relative abundance of Proteobacteria was significantly enriched with the application of limestone, whereas multiple bacterial phyla, such as Actinobacteria, Acidobacteria, and Firmicutes of HLB-infected citrus rhizoplanic microbes, were depleted in amended soils than that in acidified soils (**Figure 4B**). A more detailed multiple comparisons at the genus level between the SCK, SLL, and SHL microbial communities were performed. *Pseudomonas*, *Bradyrhizobium,* and *Burkholderia* were ranked as the top three dominant genera in all samples. Among the 21 genera (relative abundance $\geq$ 1%) identified in all samples, 3 genera *Pseudomonas*, *Streptomyces,* and *Dyella*, exhibited significantly increased relative abundance in the rhizoplane soil with the increased application rate of limestone, while the

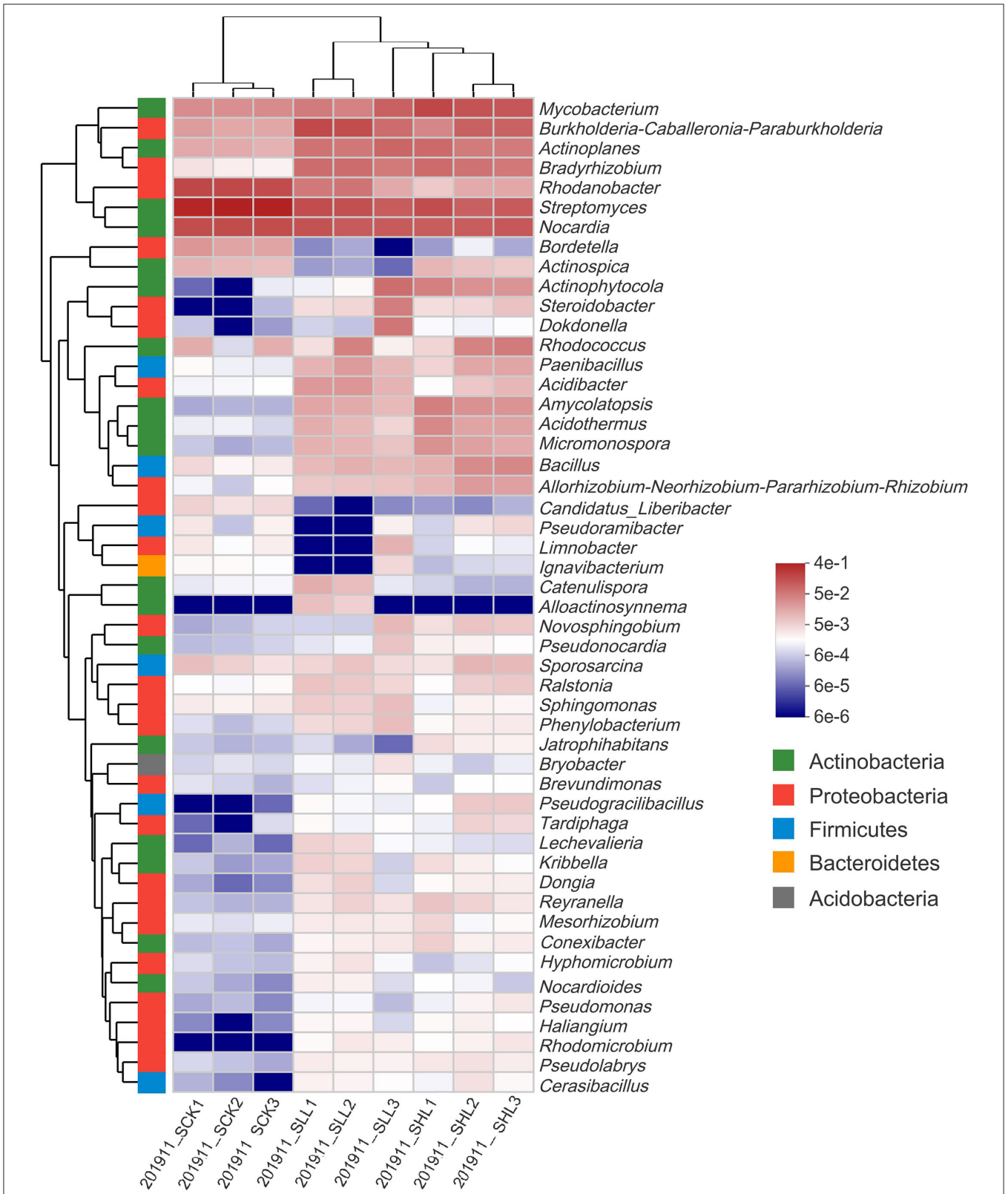

FIGURE 2 | Heatmap for the relative abundance of the 50 most abundant core genera in HLB-infected citrus root samples based on 16S amplicon sequencing. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime.

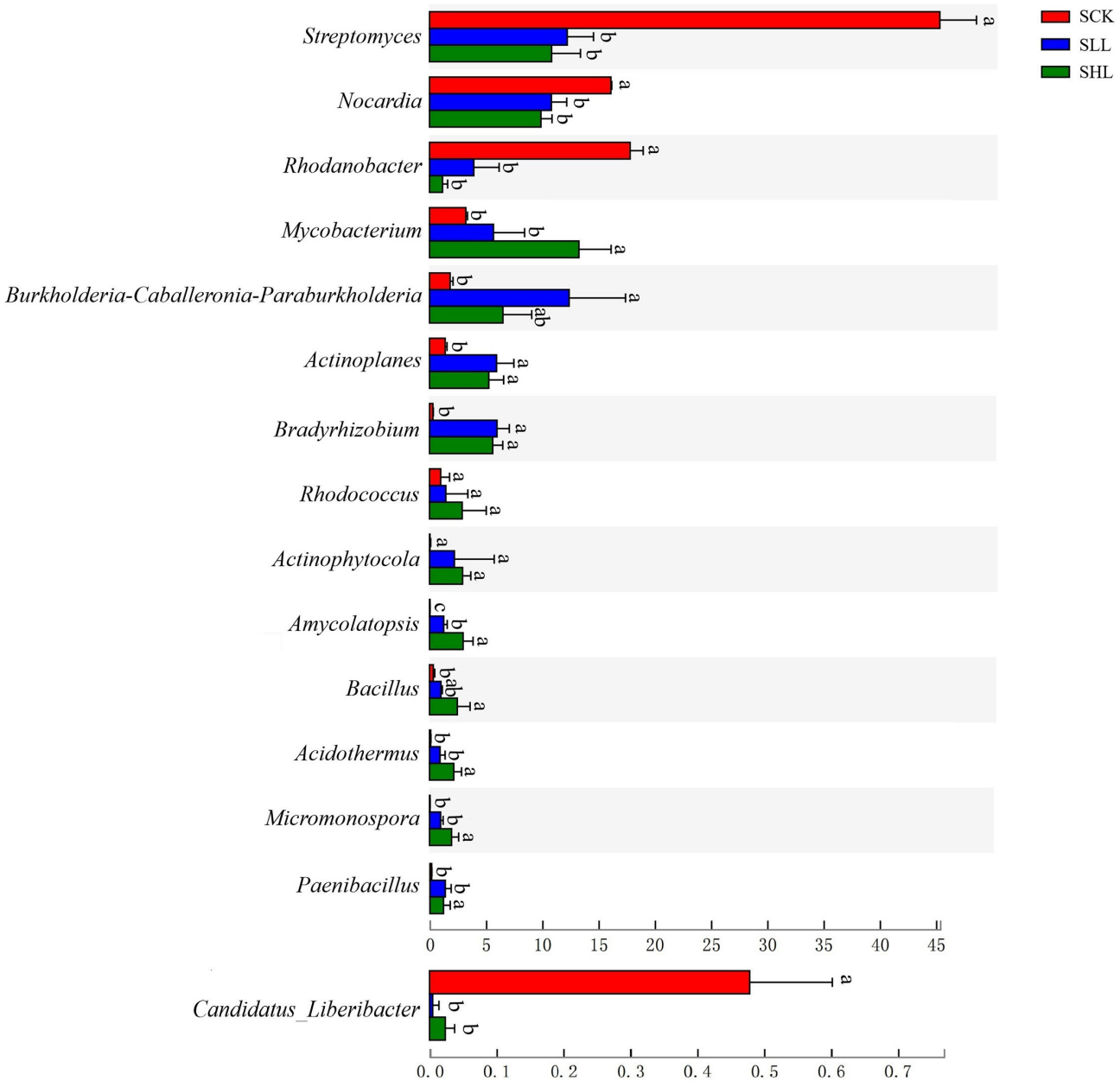

FIGURE 3 | Relative abundances of bacterial taxa at the genus level showed significant differences among HLB-infected root samples from SCK, SLL, and SHL soils. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime. Differences were considered significant at $p < 0.05$ by the Kruskal Wallis H test and respective significant differences were marked using different letters among indicated groups.

relative abundance of 9 genera showed significantly depleted in SLL and SHL compared in SCK, including *Bradyrhizobium*, *Paraburkholderia*, *Mycobacterium*, *Arthrobacter,* and *Candidatus Solibacter*. The change in relative abundance of the other 7 genera is contradictory under different soil acidity conditions, for example, the relative abundance of *Burkholderia,* a key taxon in the root microbiome of healthy citrus (Zhang et al., 2017), decreased slightly in SLL and increased significantly relative abundance in SHL ($p < 0.05$) compared to SCK (**Figure 4C**). Specifically, five of the top 10 species with the relative abundance were significantly higher in the amended soils than in the control soils, including unclassified_g__Pseudomonas, *Pseudomonas fluorescens*, *Pseudomonas umsongensis*, *Burkholderia ambifaria,* and *Pseudomonas sp.* GM55. Meanwhile, the other two

TABLE 3 | Taxonomic biomarkers of bacterial communities in response to the lime application ($p < 0.05$, logarithmic LDA logarithmic score ≥ 4).

Taxonomic biomarkers					LDA value (log10)	
Phylum	**Class**	**Order**	**Family**	**Genus**	**SCK and SLL**	**SCK and SLL**
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*		4.34	4.36
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Nocardia*	4.35	4.51
Actinobacteria	*Actinobacteria*	*Streptomycetales*			5.22	5.23
Actinobacteria	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*		5.18	5.21
Actinobacteria	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*	5.23	5.18
Actinobacteria	*Gammaproteobacteria*	*Xanthomonadales*			4.84	4.93
Actinobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*		4.78	4.91
Actinobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*	*Rhodanobacter*	4.83	4.92
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*		4.17	4.71
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycobacterium*	4.14	4.73
Actinobacteria	*Actinobacteria*	*Micromonosporales*			4.52	4.55
Actinobacteria	*Actinobacteria*	*Micromonosporales*	*Micromonosporaceae*		4.52	4.54
Actinobacteria	*Actinobacteria*	*Micromonosporales*	*Micromonosporaceae*	*Actinoplanes*	4.37	4.33
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*			4.34	4.63
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*		4.38	4.63
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Actinophytocola*	4.08	4.18
Firmicutes					4.20	4.52
Firmicutes	*Bacilli*				4.18	4.49
Firmicutes	*Bacilli*	*Bacillales*			4.14	4.46
Proteobacteria	*Alphaproteobacteria*				4.74	4.72
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			4.56	4.60
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*		4.52	4.51
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*	4.47	4.43
Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Burkholderia-Caballeronia-Paraburkholderia*	4.71	4.33
Proteobacteria					4.89	
Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*			4.71	
Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*		4.70	
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*		4.02
Actinobacteria	*Actinobacteria*	*Frankiales*	*Acidothermaceae*	*Acidothermus*		4.01
Actinobacteria	*Actinobacteria*	*Micromonosporales*	*Micromonosporaceae*	*Micromonospora*		4.01
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Amycolatopsis*		4.16
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*unclassified_Pseudonocardiaceae*		4.03

SLL, soil treated with low-level lime; SHL, treated with high-level lime.

species *Burkholderia pyrrocinia* and *Burkholderia cepacia* are significantly enriched in SLL than SCK, while it is lower in SHL (**Figure 4D**).

In addition, RDA was performed to elucidate relationship among environmental factors and microbial community structure at genus level (**Figure 6A**). Among these soil environmental factors, significant influences of pH (r^2 = 0.975, p-value = 0.002), AN (r^2 = 0.901, p-value = 0.001), Ca2+ (r^2 = 0.962, p-value = 0.002), and Al3+ (r^2 = 0.943, p-value = 0.001) were observed in microbial community structure, indicating that soil pH, exchangeable cations (Ca^{2+} and Al^{3+}) and AN were significant factors regulating the microbial composition. In particular, the relative abundance of *Pseudomonas* and *Burkholderia* was positively correlated with soil pH in SLL and SHL samples.

Effects of Lime Application on Functional Features of Rhizoplane Microbial Community

The metagenomic sequences also provided functional information about a given taxon. Therefore, apart from phylogenic insights, the metagenomic analysis also provided an opportunity to assess the functional potentials associated with the soil microbial community. Functional annotation was performed for non-redundant genes by blasting against the KEGG Orthology (KO). A total of 7,712 KOs were identified from all metagenomes, and they were mainly involved in 6, 45, and 399 KEGG pathways at three levels, respectively. The majority of sequences were functionally associated with metabolism (66.72–68.77%), environmental information processing (9.58–11.66%), cellular processes (7.58–8.35%), genetic information processing

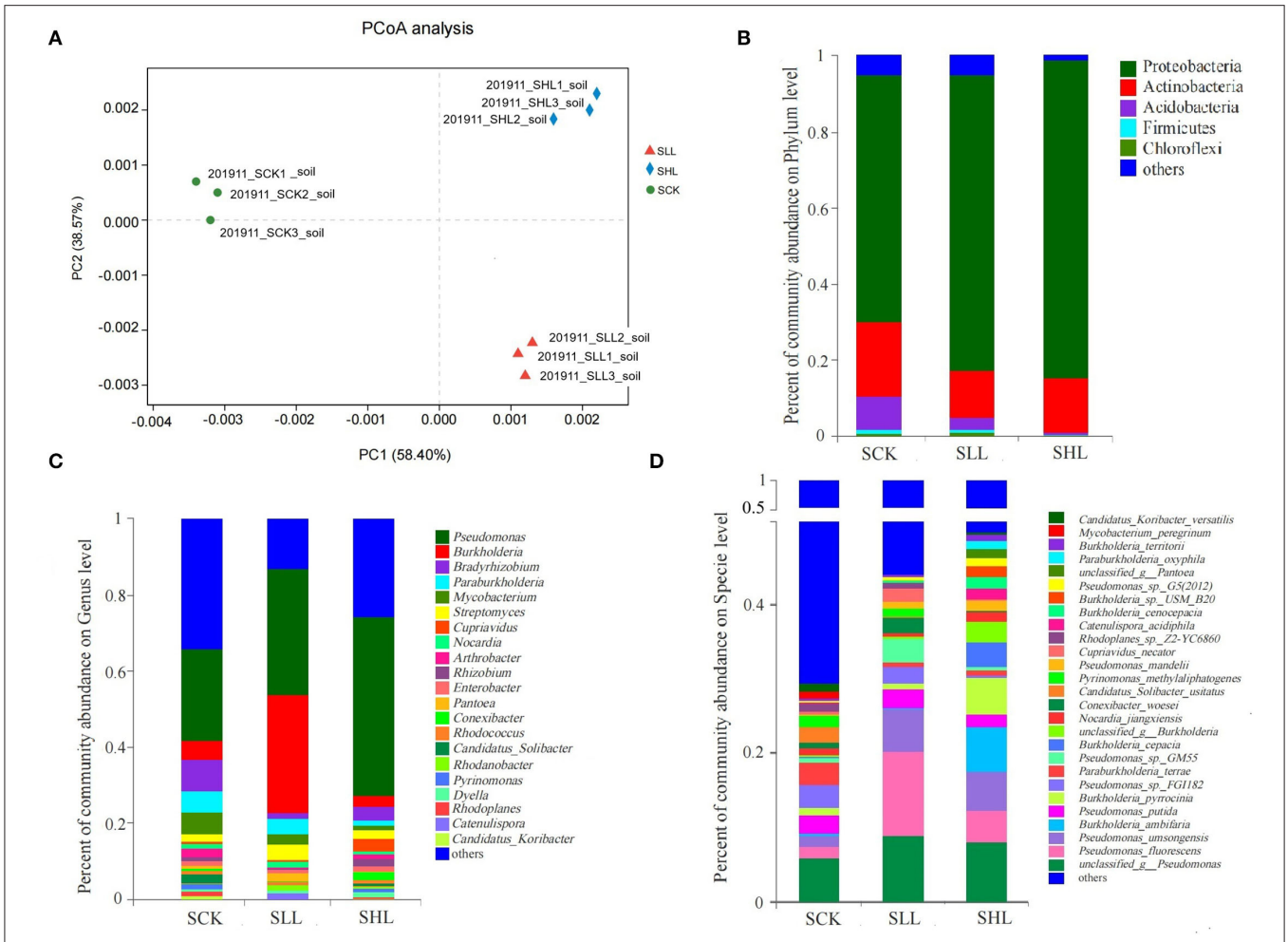

FIGURE 4 | Taxonomic composition of HLB-infected citrus rhizoplane microbiome. **(A)** Principal-coordinate analysis (PCoA) based on bray_curtis distances for the rhizoplane microbiome in acidified (SCK) or amended soils (SLL and SHL) at the specie level. Taxonomic distribution at the Phylum **(B)**, Genus **(C)**, and Specie **(D)** level in HLB-infected citrus rhizoplane soil samples. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime. Taxa with <1% of reads were combined together as "others".

(5.83–6.82%), human diseases (4.57–5.27%), and organismal systems (2.17–2.67%) at KEGG level 1 pathways. The results of the Kruskal Wallis H test indicated that the relative abundance of predicted genes involved in environmental information processing was significantly higher in amended soil samples (SLL and SHL) than in the acidified soil sample (SCK) (**Figure 5A**).

Furthermore, we also investigated the differences of 21 and 30 dominant categories (relative abundance > 1% of the total observed pathways) at KEGG levels 2 and 3 pathways between the treatments and control samples, respectively. Application of limestone significantly enriched the relative abundance of membrane transport, cellular community-prokaryotes, signal transduction, metabolism of cofactors and vitamins, xenobiotics biodegradation and metabolism, cell motility, antimicrobial drug resistance, glycan biosynthesis, and metabolism, and infectious bacterial diseases in SLL and SCK than that in SCK samples (all $p < 0.05$), while the rest 10 categories, except for the KEGG pathways amino acid metabolism and biosynthesis of other secondary metabolites, were depleted in SLL and SHL compared with SCK (**Figure 5B**) at level 2 pathways. Among KEGG level 3 pathways, the relative abundance of the ABC transporters, two-component system, bacterial secretion system, arginine and proline metabolism, bacterial chemotaxis, biofilm formation, flagellar assembly, and phenylalanine metabolism, which belong to environmental information processing and cellular processes, increased after limestone application, and these functions are very critical for microorganisms to adapt to the plant rhizoplane environment (**Figure 5C**).

The relationship between environmental parameters and microbial community functional properties (**Figure 6B**) was further confirmed by PCA based on the abundance of the KEGG (level 2) module. There are four soil environmental parameters that have significant effects on the functional pathways of rhizoplane microbiome, including pH ($r^2 = 0.979$, p-value = 0.002), AN ($r^2 = 0.892$, p-value = 0.002), Ca^{2+} ($r^2 = 0.965$, p-value = 0.002), and Al^{3+} ($r^2 = 0.935$, p-value = 0.001)

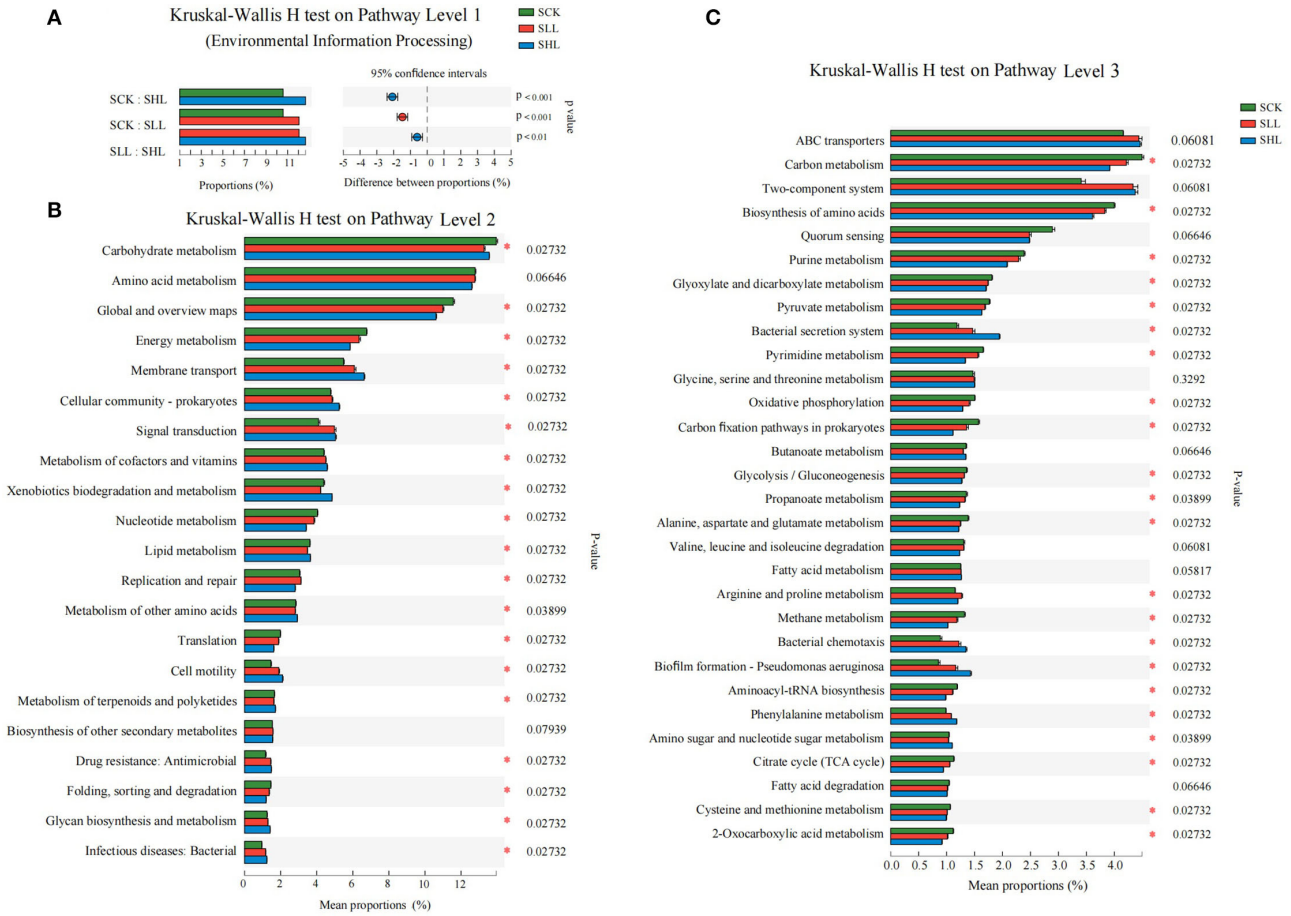

FIGURE 5 | Relative abundance of predicted genes related to KEGG pathways at levels 1 **(A)**, 2 **(B)**, and 3 **(C)** showed significant differences among HLB-infected citrus rhizoplane soil samples. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime. Differences were considered significant at $p < 0.05$ by the Kruskal Wallis H test.

(**Figure 6B**). Especially, it is found that there is a strong positive correlation between soil pH and several functional pathways, such as signal transduction, membrane transport, cellular community and Xenobiotics biodegradation, and metabolism in amended soil samples.

Changes in Nitrogen and Sulfur Metabolism With Neutralization of Soil Acidity

In particular, the functional categories nitrogen metabolism (KO00910) and sulfur metabolism (KO00920) were significantly enriched from acidified soils to improved soils (**Figure 7**). The results revealed that a variety of enzymes encoded by corresponding functional genes were involved in nitrogen and sulfur cycling and influenced by soil acidity in HLB-infected citrus rhizoplane soil (**Supplementary Figure S3A**). In the nitrogen cycle, the nitrite reductase (*nirB* and *nirD*) and the nitrate reductase (*narG, narH, and narI*) are involved in dissimilatory nitrate reduction to ammonium (DNRA) and were more abundant in the SLL and SHL groups. While ferredoxin-nitrite reductase (*nirA*) and glutamate synthase (*GLT1*) were more abundant in the SCK groups. Moreover, two genes *arcC* and *hcp* encoding carbamate kinase and hydroxylamine reductase, respectively, were generally increased in abundance from SCK to SHL. In the sulfur cycle, the assimilation sulfate reduction pathway is important to incorporate sulfide into organic compounds. In this study, a majority of these genes that participated in the assimilatory sulfate reduction pathway were more abundant in the SLL and SHL groups rather than in the SCK (**Supplementary Figure S3B**), such as adenylyl-sulfate kinase (*cysC*) and assimilatory sulfite reductase (*cysI* and *cysJ*). Only assimilatory sulfite reductase (*sir*) showed a slight decline. Meanwhile, the relative abundance of genes *cysE*, *metA*, and *metB* related to cysteine and methionine metabolism decreased after the application of limestone. Our findings indicated that the relative abundance of genes related to nitrogen and sulfur cycling presented diverse and intricate changes during the soil improvement because, besides soil acidity, there are many other soil physicochemical factors that can cooperatively influence the

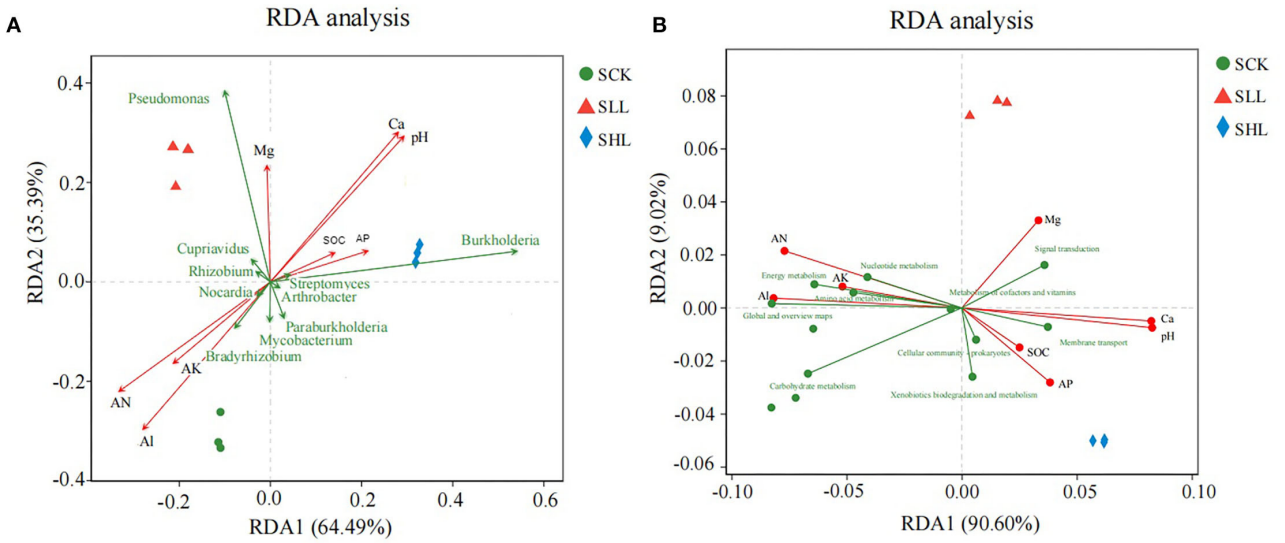

FIGURE 6 | Redundancy analysis plots of the correlation between the structure **(A)** and function **(B)** of the microbial community in HLB-infected citrus rhizoplane and environmental factors during the neutralization of soil acidity.

microbe-driven metabolism cycle to regulate the availability and quantity of different forms of nitrogen and sulfur in soils.

Linking the Rhizoplane Taxonomic and Functional Properties to Different Soil Types

To visualize the association between rhizoplane taxonomic and functional properties during the acid soil improvement, we determined the taxonomic origin of rhizoplane-enriched functional attributes for all samples. The contribution of the top 20 genera in relative abundance to nine clustered categories enriched KOs at KEGG pathway level 3 were chosen and analyzed (**Figure 8**). These functional categories involved in soil nutrient status cycling, such as nitrogen and sulfur metabolism, they also related to host-microbe interactions, including ABC transporters, two-component system, bacterial secretion system, quorum sensing, biofilm formation, bacterial chemotaxis, and flagellar assembly. The genera *Pseudomonas* and *Streptomyces* was the main contributor to these functions and contributed significantly more to the SLL and SHL samples (paired *t*-test for each selected functional category, all $p < 0.01$). Especially, the normalized total relative contribution of *Pseudomonas* for the nine functional categories was 43.01 ± 1.59% and 31.66 ± 0.46% for SLL and SHL samples, respectively, while was only 21.98 ± 1.12% for SCK samples. A reduced contribution of *Bradyrhizobium* and *Mycobacterium* for the SLL and SHL samples was also observed (two taxa together accounting for 14.17 ± 0.94% of the normalized total relative contribution for the SCK samples and 5.52 ± 0.4%, 4.28 ± 0.24% for SLL and SHL samples, respectively). The relative contribution of *Burkholderia* to these functional categories for the SLL and SHL samples ranged from 2.39 to 4.44% and 27.04 to 43.67%, respectively. Compared with SCK samples, the relative contribution of *Burkholderia* to the SHL sample significantly increased but decreased in SLL samples (paired *t*-test, all $p < 005$).

DISCUSSION

In this study, the remediation effects of limestone application on root physiological traits, soil physiochemical properties, and microbial community structure and function in highly acidified and HLB-infected citrus orchards were investigated. Our data demonstrated that the application of slacked lime improved the root activities of HLB-infected citrus, a finding that is with previous studies on ryegrass (Du et al., 2021), wheat (Haling et al., 2010), clovers (Brauer et al., 2002), Sitka spruce (Kakei and Clifford, 2002) and citrus (Li et al., 2020), and reduced the population of HLB pathogen in the roots. In addition, due to the red soil of subtropical China has inherent fragile properties, including low pH, low organic matter and low fertility, the overall soil quality of citrus orchards in south China belongs to the lower middle level according to the SQI grades (Cheng et al., 2016). These environmental factors can have a positive correlation with the infection risk, propagation, and development of plant diseases caused by poor plant vigor (Larkin, 2015). Previous studies have shown the application of limestone can improve soil nutrient status, reduce soil acidification and neutralize toxic aluminum in both annual and perennial planting systems (Natale et al., 2012; Corrêa et al., 2018; Li et al., 2019; Lauricella et al., 2021). Combined with our research results, we believe that amending soil acidification can help optimize the soil nutrient status of citrus orchards and mitigate HLB disease progress.

Plant root loss caused by pathogen infestation and extreme soil environment could limit the water and nutrient uptake of trees, making them more vulnerable to biotic and abiotic stress (Ghimire et al., 2020). The root is the earliest and most common

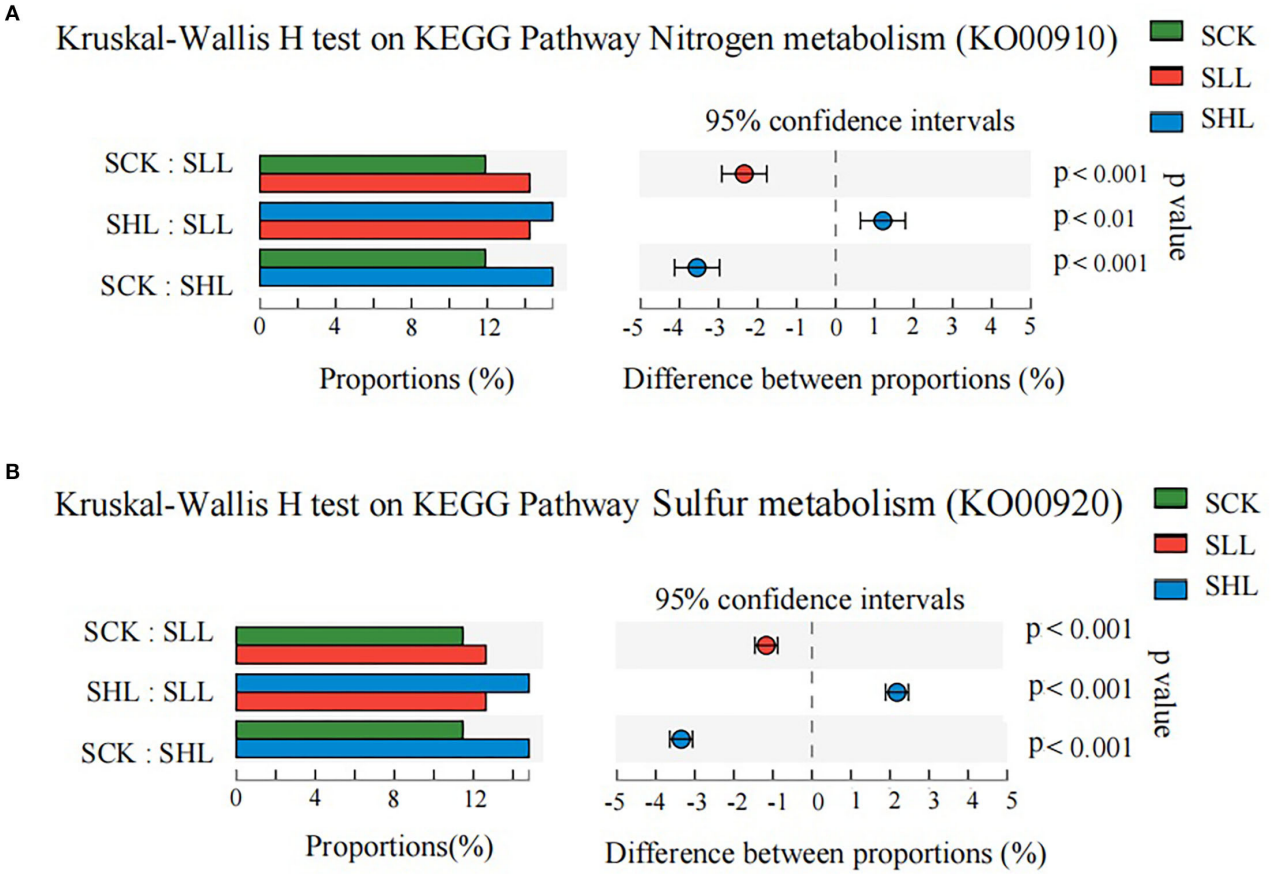

FIGURE 7 | The relative abundance of predicted genes related to nitrogen metabolism (KO00910) **(A)** and sulfur metabolism (KO00920) **(B)** showed significant differences among HLB-infected citrus rhizoplane soil samples. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime. Differences were considered significant at $p < 0.05$ by the Kruskal Wallis H test.

site for CLas colonization and proliferation, a significant damage to feeder roots has occurred on presymptomatic HLB-affected trees (Johnson et al., 2014), which means that CLas infestation could directly cause the loss of roots rather than the carbohydrate starvation caused by phloem plugging of symptomatic HLB affected citrus. The loss of fibrous root function may also cause intolerance to extreme environments and other plant diseases. Recent evidence indicates that the HLB infection was actually accompanied by other citrus pests and diseases under filed conditions (Duan et al., 2021). Prior CLas infection on roots accelerates *Phytophthora* spp. infection and further damage to the fibrous roots, which is due to the fact that the HLB-infected roots are more attractive to swimming zoospores and have lower resistance to root invasion (Graham et al., 2013). In this study, the soil improvement of acidified citrus orchard partially reduced the damage of roots caused by CLas infection. As a result, the CLas pathogen content in the infected roots decreased significantly and the activity of the infected roots increased significantly after soil improvement. Therefore, the restoration of affected fibrous root function by optimizing soil pH, which is an essential cultural practice for sustainable plant root vigor and a fundamental component of plant disease control and tolerance.

Another contributing factor to this tolerance to HLB probably is the intense reshaping of microbial community diversity and structure in citrus roots after soil improvement. A stable correlation between plants and the endophytic microbial community is very important to maintaining the health and productivity of hosts (Blaustein et al., 2017). In this study, the diversity of root bacterial communities in the amended soils is strongly increased compared with the control soils. Previous studies generally believed that the diversity of the citrus endophytic microbiota was negatively associated with HLB disease severity (Trivedi et al., 2010). Meanwhile, the microbiota of roots was significantly disrupted undergoing rapid HLB progression and lacked resilience under pathogen or disease stress (Ginnan et al., 2020). When examining the root (endo-) bacterial communities in this study, the most dominant bacterial phyla Firmicutes and Chloroflexi were at increased levels in trees with higher vigor after soil improvement, but the levels of Actinobacteria were opposite. As the severity of HLB decreases, Ginnan et al. (2020) also confirmed that the relative abundance of

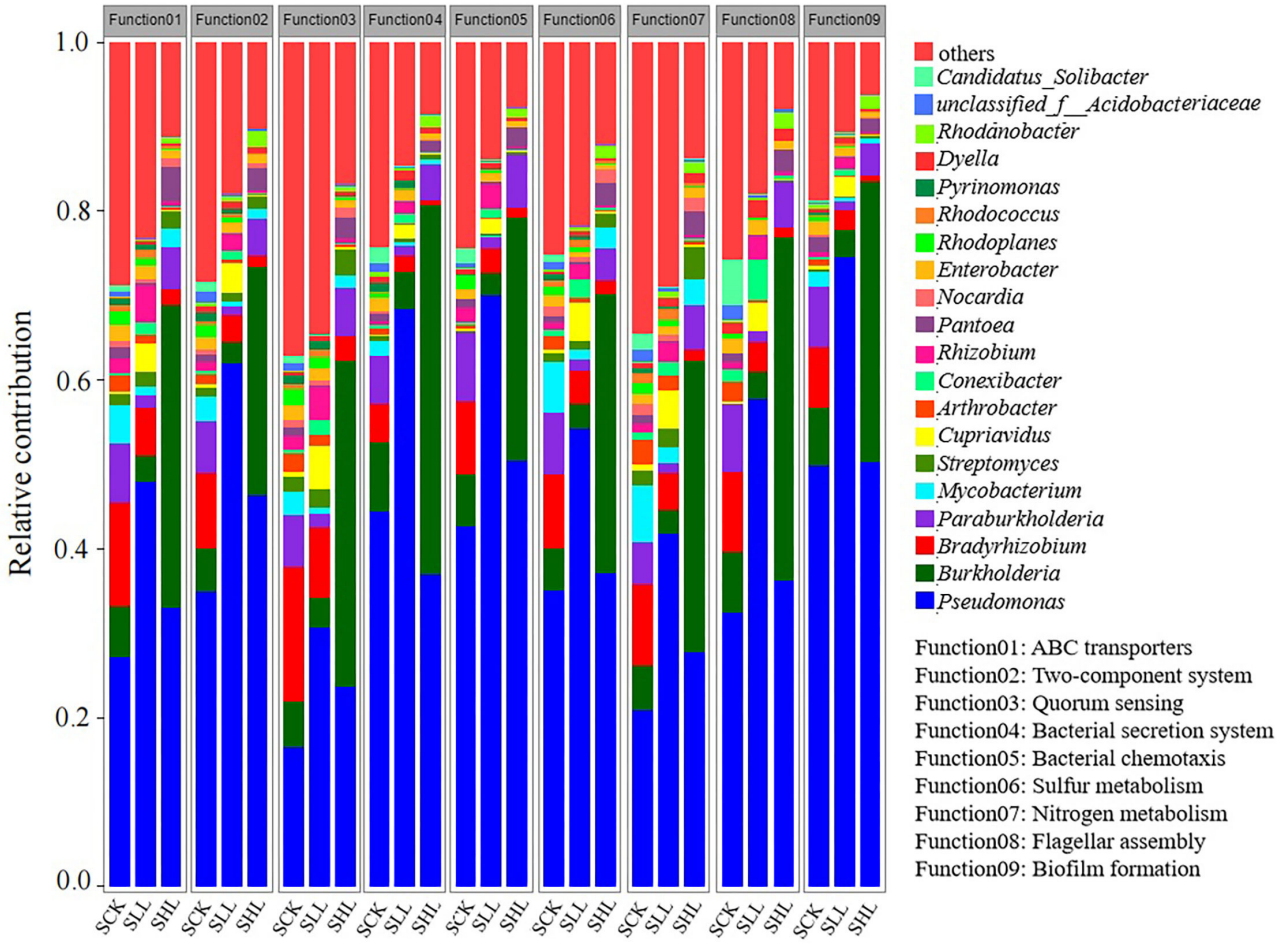

FIGURE 8 | The relative contribution of different taxa (the genus with more than 1% relative abundance) to identified rhizosphere functional attributes in different soil samples. SCK, soil without lime treatment; SLL, soil treated with low-level lime; SHL, treated with high-level lime.

phylum Actinobacteria decreased in HLB-infected trees, which was largely represented by *Streptomyces* spp. known as prolific producers of antibiotics. However, this HLB disease-induced enrichment of *Streptomyces* in the roots is not synchronized with the phase of CLas colonization and is insufficient to mitigate HLB symptoms in infected plants (Ginnan et al., 2020). Notably, there was a sharp increase in the relative abundance of some bacterial genera considered beneficial in the roots of diseased trees after soil improvement, which contributed to the vitality of the whole roots and plants and was critical for inducing plant resistance (Compant et al., 2005; Trivedi et al., 2010). Therefore, we speculate that the restoration and bolstering of potentially beneficial bacteria in roots is one of the main contributors to the more vibrant root observed in HLB trees from amended soils.

The infection of CLas not only restricts phloem transportation of photoassimilates and induces root decline (Wang et al., 2017a), but also negatively modifies the structure and functional diversity of microbial communities in the citrus rhizosphere (Trivedi et al., 2012; Zhang et al., 2017). The relative abundances of the main phylum Proteobacteria in infected rhizospheric microbes was nearly five times less compared with that of healthy citrus, while the other dominant bacteria belonging to Actinobacteria, Acidobacteria, and Firmicutes phyla were significantly enriched in HLB-infected samples (Trivedi et al., 2012). As a result, HLB caused the roots to acidify the soil more than non-HLB controls (Ebel et al., 2019), this phenomenon is probably due to the change of microbial community structure in the citrus rhizosphere. And this adverse effect of CLas infection makes citrus more susceptible to other extreme environments and secondary diseases than healthy trees (Aritua et al., 2013; Johnson et al., 2014). A complex plant root-associated microbial community is considered the second genome of the plant because stable and robust soil microbes are essential and play a key role in the availability and circulation of soil nutrients and even can improve stress tolerance or inhibit pathogens (Pang et al., 2021). Therefore, the improvement of acidified soil in citrus orchards where HLB disease is prevalent in this study does not only help to improve the chemical properties of soil, including supplementing and increasing calcium content and alleviating aluminum toxicity in acidic soils, which is beneficial

to the growth and vigor of citrus trees but also restore the seriously damaged soil microbial community associated with CLas infection to a certain extent in a pH-driven way. Specifically, the relative abundances of phyla Actinobacteria, Acidobacteria, and Firmicutes, which are positively related to the occurrence of HLB (Trivedi et al., 2012), decreased significantly after soil improvement; while the relative abundance of Proteobacteria increased significantly. Members of phylum Proteobacteria were shown to have disease-suppressive activity governed by non-ribosomal peptide synthetases (Mendes et al., 2011).

Especially, the relative abundance of most of the top 10 dominant species in citrus rhizoplane increased significantly after soil improvement. These species are affiliated with the genus *Pseudomonas* and *Burkholderia*, which were generally considered to contain a variety of beneficial microorganisms associated with promoting plant growth and inducing plant resistance (Ganeshan and Kumar, 2005; Stopnisek et al., 2014). For instance, among these enriched species, *Pseudomonas fluorescens*, *Burkholderia ambifaria*, *Burkholderia pyrrocinia,* and *Burkholderia cepaci* are naturally beneficial bacteria with proven biocontrol properties (Ren et al., 2011; Chapalain et al., 2013; Jung et al., 2018; Mullins et al., 2019). Riera et al. (2017) also reported that *Burkholderia metallica* strain A53, *Burkholderia territorii* strain A63, *Pseudomonas granadensis* strain 100, and *Pseudomonas geniculata* strain 95 from healthy-looking citrus rhizosphere in severely HLB-diseased citrus grove showing antibacterial activity against *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* closely related to CLas pathogens. Although the HLB escape plants have the same genotype as the symptomatic trees, the difference in microbial community composition enriched in beneficial traits may result in the promotion of stress tolerance and disease inhibition of escape trees by competing resources or providing plant growth-promoting factors (Riera et al., 2017; Wang et al., 2017b). Furthermore, our research and previous studies have consistently shown that soil pH was the imperative factor influencing community composition, which may be related to the relationship between soil acidity and relative abundances of dominant and core bacteria in diseased citrus root or rhizoplane. Therefore, we speculated that acidic soil improvement can recruit and enrich specific beneficial microbes from root-associated microbiota in a soil pH-driven microbial redistribution pattern, which can promote tolerance to HLB and other stress.

The composition of microbial community and the relative abundance of core bacteria groups in diseased citrus rhizoplane vary with soil acidity, thus, we thought that the functions of rhizoplane microbes might be different between the acidic soils and the amended soils. Beneficial plant-microbe associations modified the metabolic pathways of rhizoplane microbes responding to the external environment, which may play critical roles in plant health and disease resistance. Our results indicated that there is a strong correlation between the environmental factor pH and microbial functions. These functional categories related to host-microbe interactions and soil nutrient status cycling, including two-component system, biofilm formation, bacterial chemotaxis, flagellar assembly, bacterial secretion system, and nitrogen and sulfur metabolism, were obviously enriched in diseased citrus rhizoplane with the neutralization of soil acidity. Firstly, two-component regulatory systems help the rhizobacteria recognize and adapt to environmental changes (Heeb and Haas, 2001). In addition, recent studies highlighted the importance of biofilm formation in initiating and maintaining contact with the host, which enables bacterial populations adhere to plant surfaces, thus affecting the colonization tendency of beneficial bacteria (Ramey et al., 2004; Farrar et al., 2014). Meanwhile, bacterial chemotaxis provides a competitive advantage for motile flagellated bacteria, especially beneficial plant-associated bacteria, in response to root exudates and colonization of plant root surfaces (Scharf et al., 2016). The above functions are the intrinsic components of plant-microbe interaction and are also the prerequisites for establishing stable and beneficial associations. After successful colonization of plant roots, the effectiveness of beneficial rhizobacteria usually depends on the secretion system to help the host to obtain nutrition from the soil, improve plant adaptability, and inhibit pathogen colonization (Lucke et al., 2020). These viewpoints combined with our findings suggested that acidic soil improvement could increase the vitality and motility in soil microbial communities and assist HLB-infected roots to recruit beneficial soil microbes, which play an important role in the secretion of antibiotics and the activation of the plant immune system.

In addition, soil microbes can utilize various forms of inorganic nitrogen to synthesize the organic biomass, which plays an important role in the soil's total nitrogen cycle (Thus, 2005). DNRA consumes nitrogen oxide pool and preserves bioavailable nitrogen in the soil system, which produces soluble ammonium rather than unreactive N_2 or N_2O (Marchant et al., 2014). The enhanced DNRA of soil microbes in the amended soils might promote the recycling of nitrogen, which is very important for the level of soil fertility. However, not all nitrogen metabolism is related to the synthesis of biomass in organic form, because some bacteria need to utilize the energy released by nitrogen reactions to maintain their life activities (Thus, 2005). As in the case of nitrogen, sulfur is required by bacteria as an essential nutrient for cellular biosynthesis and the transformation of sulfur in the environment mainly depends on the activities of microorganisms (Klotz et al., 2011). Normally, most microorganisms acquire sulfur and synthesize the organic sulfur compounds through assimilatory sulfate reduction (Kushkevych et al., 2020). In this process, sulfate is reduced to hydrogen sulfide and then participates in sulfur-containing amino acids biosynthesis. In this study, the abundance of most genes (*cysC*, *cysI*, and *cysJ*) related to the sulfate reduction pathway was increased, meanwhile, the abundance of genes (*cysE*) related to the feedback inhibition of cysteine synthesis was decreased in amended soils compared with that of acidic soils. Therefore, the change of relative abundance of functional genes in the nitrogen and sulfur cycle indicated that the shift of microbial community structure and function in HLB-infected citrus rhizoplane may have a forceful impact on soil microbial vitality and soil fertility reflected in the growth of the plant eventually.

The disease resistance and tolerance of plants are normally determined by heredity, but they could be modified by environmental factors, especially soil conditions and

microorganisms (Trivedi et al., 2020; Thakur et al., 2021). Likewise, the native plant-associated microbial communities are synergistically affected by severe soil acidification and CLas infection in most citrus orchards in south China. This means that it is reasonable and feasible to bolster the stable and healthy plant-microbe associations to a certain extent by neutralizing the acidified soil around the roots of diseased citrus, thus promoting the root nutrient uptake and disease tolerance of citrus. Our research revealed that this practice can temporarily reduce the content of CLas pathogen in roots and boost diseased root vigor. Similar studies showed that applications of Ca, Mg, and B could alleviate the acidification of the soil, alter the phyllosphere and rhizosphere bacterial microbiome and reduce the HLB incidence in Gannan Navel Orange (Zhou et al., 2021). On the other hand, HLB-affected trees benefit from the additional application of essential nutrients in terms of growth and productivity; however, this effect is often inconclusive and unenduring (Morgan et al., 2016; da Silva et al., 2020; Shahzad et al., 2020; Atta et al., 2021). Thus, the CLas pathogen cannot be absolutely eliminated by improving the soil conditions, that is to say, the influence of HLB disease on citrus root-related microbial communities will not completely disappear.

The improvement of soil conditions seems to be beneficial to support the health of the whole citrus trees and might be able to slow HLB disease progression by influencing the resident microbiota, but it is unlikely that a single measure can prevent the occurrence and development of HLB disease. Therefore, many factors, such as disease severity, soil conditions, and community density of citrus psyllids, should be considered comprehensively when formulating control measures for HLB disease. It may be more effective to improve the effectiveness and sustainability of HLB disease management by integrating the approaches of acidic soil improvement, foliar and ground-applied essential nutrients, and inoculating beneficial plant microorganisms with insecticidal controls. On the horizon, there are prospects for utilizing pathogen-free citrus seedlings and breeding tolerant/resistant cultivars to combat HLB disease, because prevention is always a better choice than treatment afterward.

CONCLUSION

The present study investigated the variations in soil properties and HLB-infected citrus root-associated microbial community structure and function in response to the lime application. The results indicated that liming is an effective managerial practice to mitigate soil acidity of citrus orchards in south China, which consequentially improved the vitality of citrus roots and reduced pathogen CLas relative concentration in roots. Additionally, this treatment strongly altered root endophytic microbial community diversity and structure, which is represented by the enrichment of beneficial microorganisms in roots; and it also recruited more functional genes that are involved in host-microbe interactions and nitrogen and sulfur metabolisms in the HLB-infected citrus rhizoplane, which are important in the microbiome-inhabiting plant root surfaces and the utilization of soil nutrients, respectively. These rhizoplane-enriched functional properties may subsequently benefit the plant's health and tolerance to HLB disease. However, this study concentrated only on the short-term responses of soil pH and plant-associated microbial communities after the application of soil amendments in acidic and HLB-infected soils. Whether liming has a persistent effect on soil pH and soil microbial communities or the likely duration of that effect would need further investigation. Overall, our study provides a novel insight for understanding the relationship between neutralization of acidified soil, citrus root-associated microbiota, and citrus tolerance to HLB.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DQ and ShuaW conceived and designed the experiments. BL performed the experiment and data analysis. FF and ShutW gave their constructive criticism of an earlier version of the manuscript. YW and TH helped to revise and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The 1-aminocyclopropane-1-carboxylic acid deaminase-producing *Streptomyces violaceoruber* UAE1 can provide protection from sudden decline syndrome on date palm

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In the United Arab Emirates (UAE), sudden decline syndrome (SDS) is one of the major fungal diseases caused by *Fusarium solani* affecting date palm plantations. To minimize the impact of the causal agent of SDS on date palm, native actinobacterial strains isolated from rhizosphere soils of healthy date palm plants were characterized according to their antifungal activities against *F. solani* DSM 106836 (*Fs*). Based on their *in vitro* abilities, two promising biocontrol agents (BCAs), namely *Streptomyces tendae* UAE1 (*St*) and *Streptomyces violaceoruber* UAE1 (*Sv*), were selected for the production of antifungal compounds and cell wall degrading enzymes (CWDEs), albeit their variations in synthesizing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD). Although both isolates showed antagonism when applied 7 days before the pathogen in the greenhouse experiments, the ACCD-producing *Sv* was relatively superior in its efficacy against SDS over the non-ACCD-producing *St*. This was evident from the symptoms of SDS in diseased date palm seedlings which were greatly reduced by *Sv* compared to *St*. On a scale of 5.0, the estimated disease severity indices in *Fs*-diseased seedlings were significantly ($P < 0.05$) reduced from 4.8 to 1.5 and 0.5 by *St* and *Sv*, respectively. Thus, the number of conidia of *Fs* recovered from plants pre-treated with both BCAs was comparable, but significantly ($P < 0.05$) reduced compared to plants without any BCA treatment. In addition, a significant ($P < 0.05$) decrease in ACC levels of both the root and shoot tissues was detected in *Sv* + *Fs* seedlings to almost similar levels of healthy seedlings. However, *in planta* ACC levels highly increased in seedlings

grown in soils infested with the pathogen alone or amended with *St* prior to *F. solani* infestation (*St* + *Fs*). This suggests a major role of ACCD production in relieving the stress of date palm seedlings infected with *F. solani*, thus supporting the integrated preventive disease management programs against this pathogen. This is the first report of effective rhizosphere actinobacterial BCAs to provide protection against SDS on date palm, and to help increase agricultural productivity in a more sustainable manner in the UAE and the other arid regions.

KEYWORDS

actinobacteria, biocontrol, date palm, *Fusarium solani*, rhizosphere, plant–microbe interaction, sudden decline syndrome

Introduction

Date palm (*Phoenix dactylifera* L.) is an important traditional tree cultivated in the arid region. Like other countries in the Arabian Gulf, the United Arab Emirates (UAE) consider dates as a significant source of food production that play a vital role in the food security of the region (Mahmoudi et al., 2008). Due to its existence in such harsh and competitive environments, date palm constantly interacts with pathogenic microorganisms present in its niche. Several fungi have been recorded as causal pathogens of diseases on date palms. The fungal pathogens, *Thielaviopsis punctulata*, *Thielaviopsis paradoxa*, *Omphalia pigmentata*, *Omphalia tralucida*, *Mycosphaerella tassiana*, and *Graphiola phoenicis* can cause foliar diseases, root diseases, and false smut on date palm in Oman, Qatar, Saudi Arabia, and the UAE (Al-Sadi et al., 2012; Al-Naemi et al., 2014; Saeed et al., 2016; Alhudaib et al., 2022). Sudden decline syndrome (SDS), also known as *Fusarium* wilt disease, of date palm is also caused by different fungal species that belong to *Fusarium* (Al-Hammadi et al., 2019). Bayoud disease, caused by the soil-borne fungal pathogen, *Fusarium oxysporum* f. sp. *albedinis*, is undoubtedly the most destructive disease affecting date palm in North Africa (Sedra, 2013). *Fusarium solani* has previously been identified as the causal agent of SDS on date palms in the UAE (Alwahshi et al., 2019).

In general, SDS causes severe damages in date palm plants that live in warm and dry regions (Shabani and Kumar, 2013). Plants growing in cool and wet areas may also show symptoms but at slower rates. *F. solani* is a soil-borne vascular fungal pathogen that attacks plants through the roots (Mansoori and Kord, 2006). The life cycle of this species can be divided into dormant, parasitic, and saprophytic phases (Okungbowa and Shittu, 2012). In the dormant phase, the germination of the fungal resting structures present in the soil is inhibited. Thus, this can be overcome by the released carbon and nitrogen (N) from root exudates in the rhizosphere of host plants. *Fusarium* spp. enter the parasitic stage by penetrating the roots through the root tip or at the sites of lateral root formation. This fungal pathogen can colonize the vascular (xylem) tissues after crossing the endodermis, spreads to the neighboring xylem elements, and sporulates until it colonizes the xylem tissues of the whole plant; thus, resulting in the accumulation of fungal biomass (Mansoori and Kord, 2006). At the saprophytic stage, *F. solani* can produce its conidia on infected plant tissues that may be dispersed and start another cycle of disease (Shabani and Kumar, 2013). In general, this fungus can persist in soil for several years without a host. The opportunistic fungus can attack old, weak, or injured plants, with similar symptoms of SDS that can be recognized on most date palm cultivars (Bokhary, 2010). Disease symptoms on date palm can be first observed on the lowest outer leaves of the middle crown, followed by yellowing at the base of the leaves/fronds, and move upward (Maitlo et al., 2016). As the disease progresses, leaves gradually turn white on one side before developing on the other side. Finally, the diseased plants are crinkled, leaves are dried, and eventually die from few days to several weeks after infection.

Today, farmers are primarily dependent on the application of chemical fungicides to inhibit the growth or prevent the spread of fungal pathogens and their spores, which in turn, protects crop plants (Youssef et al., 2019). In the UAE, Score® (difenoconazole) and Cidely® Top (difenoconazole and cyflufenamid) are effective chemical treatments for black scorch disease and SDS on date palm (Saeed et al., 2016, 2017b; Alwahshi et al., 2019), dieback on mango (Kamil et al., 2018), and stem canker on royal poinciana (Al Raish et al., 2020). However, the extensive use of chemicals has resulted in the emergence of fungicide-resistant pathogens and other concerns of the residual effects on the environment and human health. Biocontrol agents (BCAs) against phytopathogenic fungi (Al Hamad et al., 2021; Al Raish et al., 2021), botanical and microbial fungicides (Yoon et al., 2013; Moreno-Gavíra et al., 2021), agronanotechnology (Atiq et al., 2020), and deactivation and evacuation of fungal cells (El-Baky and Amara, 2021) can be employed as "green" strategies to control plant fungal diseases.

Integrated disease management (IDM) combining BCAs with fungicides can be another option to reduce the impact of fungicides as well as to effectively manage plant diseases (Saeed et al., 2017b; Kamil et al., 2018; Zapata-Sarmiento et al., 2020). In that sense, actinobacteria have been constantly proposed as possible candidates to replace chemical fungicides (Saeed et al., 2017b; Kamil et al., 2018; Alblooshi et al., 2022); thus the disease management capacity of BCAs heavily relies on uncontrollable environmental conditions (Ons et al., 2020).

Actinobacteria are a phylum of Gram-positive bacteria, which can be terrestrial or aquatic (Locci and Sharples, 1984; Lewin et al., 2016). The genus *Streptomyces* is a unique subgroup of actinobacteria that are known as prolific producers of antibiotics and bioactive secondary metabolites (Goodfellow and Williams, 1983; Barka et al., 2016; Lewin et al., 2016). Many species of *Streptomyces* exhibit biological control potential against phytopathogenic fungi through multiple mechanisms, including the production of antibiotics, hyperparasitism, and induction of plant resistance response (Tamreihao et al., 2016; Saeed et al., 2017a; Al Hamad et al., 2021; Alblooshi et al., 2022). Such an environmentally-friendly strategy has recently received renewed attention to control plant diseases and increase crop production.

Although several reports have used beneficial fungi (e.g., *Trichoderma* and *Chaetomium* species) as BCAs to control date palm pathogens (Soytong et al., 2005; Sánchez et al., 2007; Ammar, 2011; Nishad and Ahmed, 2020), no rhizosphere actinobacterial isolates have been identified against SDS. The endophytic *Streptomyces coeruleoprunus*, on the other hand, has been considered a biocontrol potential against *F. solani* on date palm trees in the UAE (Alblooshi et al., 2022). To avoid the worst impacts of black scorch disease, the rhizosphere isolate, *Streptomyces globosus* UAE1 has been successfully tested to control *T. punctulata* on date palm (Saeed et al., 2017a).

Previous studies showed that actinobacteria are important microorganisms in rhizosphere soils of the UAE (El-Tarabily et al., 2008; Mathew et al., 2020), and demonstrated an antagonism to phytopathogenic fungi including *T. punctulata* (Saeed et al., 2017a). The objectives of the present study were to: (i) isolate actinobacteria and test their abilities *in vitro* to produce antifungal metabolites and cell wall degrading enzymes (CWDEs) to inhibit *F. solani* DSM 106836; (ii) select the most promising isolate(s) possessing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD) activities; and (iii) evaluate the antifungal activities of the identified ACCD-producing and non-ACCD-producing actinobacterial isolates against SDS on date palm seedlings in the greenhouse. Our results demonstrated that the ACCD-producing *Streptomyces violaceoruber* UAE1 can lessen the disease impact compared to the ACCD-non-producing isolate *Streptomyces tendae* UAE1 (*St*), thus, enhancing the resistance of date palm to *F. solani*. Our aim was to discover microbial resources (e.g., actinobacteria) from extreme environments to provide plant protection for sustainable agriculture.

Materials and methods

Cultivation of pathogen and isolation of actinobacteria

The fungus *F. solani* (DSM 106836), previously identified by Alwahshi et al. (2019), was subcultured on fresh potato dextrose agar (PDA; Lab M Limited, Lancashire, United Kingdom) plates (pH 6.0) and the plates were incubated at 28°C.

In order to isolate actinobacteria, five rhizosphere soil samples were randomly collected from healthy date palm trees (depth of ∼25 cm) of Mutaredh Oasis (24.22°N, 55.74°E) in Al-Ain city, UAE. The rhizosphere soils were air-dried for 4 days at 28°C (Williams et al., 1972). The soil dilution plate method (Johnson and Curl, 1972) using inorganic salt starch agar (ISSA; Küster, 1959) with specific soil pre-treatments (Hayakawa and Nonomura, 1987) was used to isolate streptomycete actinobacteria (SA). According to Nonomura and Ohara (1969), soil suspension was treated with a solution containing 6% of yeast extract (YE) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 0.05% of sodium dodecyl sulfate (SDS) (Sigma-Aldrich) at 40°C for 20 min, and subsequently diluted in water. This step was executed to increase actinobacterial populations and reduce the number of non-actinobacterial isolates.

To isolate non-streptomycete actinobacteria (NSA) from the rhizosphere, the following methods were implemented: (i) the use of polyvalent *Streptomyces* phages (Kurtböke et al., 1992); and (ii) the soil dry heat method (Nonomura and Ohara, 1969). These methods were used to reduce the abundance of SA and increase the dominance of NSA on isolation plates. For the polyvalent *Streptomyces* phages, the stock phage suspension was prepared by combining high-titer suspensions ($\times 10^{12}$ plaque-forming units ml^{-1}) of two different phages, and the stock suspension was then used to treat soil suspensions (10 g) in dilution tubes (five replicates). Actinobacterial colonies were isolated on ISSA plates which were dried in a laminar flow cabinet for 20 min and incubated at 28°C in dark for 2 weeks. Control treatments are those inoculated plates with soil dilutions that were not treated with the polyvalent phages. For the dry heat method, 10 g of each soil (five replicates) were heated at 120°C for 1 h. One gram of the heat-treated soil was added to 10 ml of water and vortexed for 5 min. A series of 10^{-2}, 10^{-3}, and 10^{-4} dilutions were prepared in sterile distilled water. Treatments consisting of unheated soil were used as a control. On arginine vitamin agar (AVA) plates, colonies of actinobacterial were spread and isolated (Nonomura and Ohara, 1969). AVA plates were

air-dried and incubated at 28°C in dark for 2 weeks to encourage NSA growth.

Colonies of SA and NSA [$\log_{10}$ colony forming units (CFU) g dry soil^{-1}] were purified on oatmeal agar plates (ISP-3 medium) amended with 0.1% of yeast extract (OMYEA; Küster, 1959) and were identified as previously described by Cross (1989). To distinguish between SA and NSA colonies, morphological criteria and the presence/absence of aerial mycelium, spore formation in aerial and substrate (vegetative) mycelia, and the stability of substrate mycelia were determined (Cross, 1989).

In vitro bioassays for antifungal activities of actinobacterial isolates

All SA and NSA isolates were evaluated according to the secretion of diffusible antifungal metabolites against *F. solani* using the cut-plug method (Pridham et al., 1956). Isolates were inoculated on fish meal extract agar (FMEA) plates and incubated at 28°C in dark for 7 days (El-Tarabily et al., 1997). Plugs (11-mm) from cultures growing on FMEA were transferred to PDA plates seeded with *F. solani* that were kept at 28°C in dark for 5 days. The diameters of the inhibition zone (mm) for each isolate (eight plates) were determined.

Cultures of each isolate were also tested for their production of volatile compounds (VCs) on FMEA (Payne et al., 2000) at 28°C in dark for 10 days. FMEA plates were also inoculated with a 5-mm mycelial plug of the pathogen. After the lid removal, plates of *F. solani* were inverted over the actinobacterial plates (eight plates/isolate), taped together using Parafilm, and incubated at 28°C in dark for 7 days. Non-inoculated plates with any isolate served as a control. The colony diameter of *F. solani* (mm) was measured and compared to that of the control.

In addition, all actinobacterial isolates were evaluated for CWDEs synthesis by measuring the clearing zones (mm) around and beneath the actinobacterial colonies using *F. solani* mycelial fragment agar (MFA; Valois et al., 1996). Only highly active CWDEs-producing isolates showing large diameters of clearing zones (>30 mm) were selected, whereas others were discarded. Chitinase activity on colloidal chitin agar (CCA) plates of each isolate (eight plates/isolate) was also determined. An efficient method of preparing colloidal chitin from crab shell chitin (Sigma-Aldrich) was developed (Hsu and Lockwood, 1975). CCA plates were incubated at 28°C in dark for 7 days and the clearing zone (mm) produced by the individual isolates around and beneath the colonies was measured (Gupta et al., 1995).

An isolate was considered a siderophore producer when a yellow-orange halo zone around the colony developed on chrome azurol S (CAS) agar plates inoculated with a particular BCA and incubated at 28°C in dark for 3 days (Schwyn and Neilands, 1987).

The promising isolates were also tested for the synthesis of hydrogen cyanide (HCN) by adopting the method of Bakker and Schippers (1987). Each actinobacterial strain was grown on tryptic soy agar (TSA; Lab M Limited) plates containing 4.4 g l^{-1} of glycine and was incubated at 28°C for 5 days. A Whatman filter paper No. 1 soaked in 2% of sodium carbonate prepared in 0.5% of picric acid solution was placed on the top of the plate and incubated at 28°C for 5 days. The development of an orange-to-red color indicated the formation of HCN.

Only isolates showing strong inhibition against *F. solani* on FMEA plates (diffusible antifungal metabolite- and VC-producers), large clearing zones on MFA and CCA plates (CWDE-and chitinase-producers), and siderophore production were chosen for further analyses. The rest of the isolates showing no or weak inhibition and clearing zones of less than 30 mm as well as no production of siderophore or HCN were not considered.

In vitro determination of ACC utilization and measurement of ACCD activity

The activity of ACCD was initially screened on Dworkin and Foster (DF) salt minimal agar medium with 3 mM of ACC (Sigma-Aldrich) as a sole N source (Dworkin and Foster, 1985). For positive control, the DF minimal medium was supplemented with 0.2% of $(NH_4)_2SO_4$; and for the negative control, DF medium devoid of any N source was used. Growth of isolates on DF medium supplemented with ACC (DF-ACC agar) was assessed after incubation at 28°C in dark for 7 days and compared to controls. The ACCD producers were selected based on growth on DF-ACC plates as an indicator of the efficiency of the selected isolates to utilize ACC.

The potential activities of BCAs were also quantitatively determined by growing them in inorganic salt starch broth (ISSB; Küster, 1959) at 28°C in dark for 5 days. Spores were harvested, inoculated onto DF-ACC broth (eight independent flasks/isolate), and incubated at 28°C in dark for 5 days on a rotary shaker (Model G76, New Brunswick Scientific, NJ, United States) at 250 rpm. Cells were collected, resuspended in 0.1 M Tris-HCl (pH 8.5), followed by three cycles of freeze/thaw (placing in liquid-N for 1 min, and immersing in a warm water bath for 5 min). The activity of ACC deamination was assayed by measuring α-ketobutyrate formed from the cleavage of ACC (Honma and Shimomura, 1978). One unit of enzyme is the forming activity of 1 μmol of product in 1 min at 28°C. Protein concentration was determined using the method of Bradford (1976).

Phylogenetic analysis and morphological identification of the most promising BCAs

According to Locci (1989), BCA isolates #6 and #26 were identified at the species level. For genomic DNA extraction, cultures were grown in 20 ml of TSB in 50-ml Erlenmeyer flasks for 7 days and then centrifuged for 5 min at 12,000 × *g* (Centra 4 model centrifuge; International Equipment Company, Woonsocket, RI, United States). Mycelial pellets were resuspended in 500 μl of 5 M NaCl and transferred to a 2-ml Eppendorf tube. Cells were centrifuged at 12,000 × *g* for 30 s, and the pellet was resuspended in 1 ml of 10 mM Tris-HCl, l mM EDTA (TE; pH 7.5) containing 20 mg ml^{-1} of each lysozyme and RNase A and incubated at 37°C for 1 h. Following incubation, 250 μl of 0.5 M EDTA, 250 μl of TE containing 5 mg ml^{-1} of proteinase K, and 100 μl of 10% SDS were added to each tube and incubated at 37°C for 1 h. Tubes were mixed by inversion after adding 250 μl of 5 M NaCl. Immediately, 200 μl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB + 0.7 M NaCl) was added and tubes were heated in a water bath at 65°C for 10 min. After centrifugation, using an Eppendorf model 5415 centrifuge at 12,000 × *g* for 5 min, the supernatant solution was transferred to a new 2-ml microcentrifuge tube. One-third of the volume of phenol-chloroform was added, and the phases were mixed and centrifuged at 14,000 × *g* for 5 min. The aqueous phase was transferred to a new tube and DNA was precipitated with isopropanol. After centrifugation, the pellet was rinsed with 70% ethanol, dried, and redissolved in 200 μl of TE.

The two isolates were identified based on their 16S rRNA gene sequence analysis. The amplified products of 16S rRNA (∼1,520 bp) from gDNA obtained from bacterial cultures by PCR were sequenced using the primers described by Rainey et al. (1996): 907R (5′-CCGTCAATTCATTTGAGTTT-3′); 803F (5′-ATTAGATACCCTGGTAG-3′) and 357F (5′-TACGGGAGGCAGCAG-3′). The reaction mixture contained ∼50 ng of DNA, *Ex*Taq PCR buffer, 1.5 mM of $MgCl_2$, 10 mM of deoxynucleoside triphosphate mixture, 50 pmol of each primer, and 0.5 U of Ex*Taq* polymerase. PCR conditions consisted of an initial denaturation at 95°C for 3 min; 28 cycles at 95°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension), followed by a final 5-min extension at 72°C. All sequencing reactions and phylogenetic analyses were carried out by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany.

Briefly, pairwise sequence similarity using 16S rRNA gene sequence was determined. For the phylogenetic analyses, the reference strain was selected from the top hits of the determination using GenBank BLAST[1]. To predict the species of isolates, the neighbor-joining method implemented in Molecular Evolutionary Genetics Analysis 7.0 (MEGA7) software (Saitou and Nei, 1987; Kumar et al., 2016) was used. Bootstrap values were calculated with 500 resampled datasets.

Scanning electron microscopy (SEM) was carried out using the Philips XL-30 SEM (FEI Co., Eindhoven, Netherlands) to examine the morphology of spore chains and surfaces. The SEM analysis was determined following the growth on ISP3 medium (Shirling and Gottlieb, 1966) at 28°C for 14 days.

In vivo greenhouse experiments

In vivo bioassays were performed to determine the efficacy of BCAs on date palm seedlings in soil infested with *F. solani*. In greenhouse Experiment (1), date palm seedlings were transplanted in soil colonized with each of the ten potential isolates showing the strongest production of diffusible antifungal metabolites and CWDEs (see Section "*In vitro* Bioassays for Antifungal Activities of Actinobacterial Isolates") of which four were ACCD producers (isolates #26, #43, #46, and #50) and six were not ACCD producers (#6, #7, #33, #40, #42, and #44) (see Section "*In vitro* Determination of ACC Utilization and Measurement of ACCD Activity"). Similarly, BCA1 (isolate #6) and BCA2 (#26) were further tested on date palm seedlings cultivated in *F. solani*-infested soil (Experiment 2).

In both experiments, BCA applications and pathogenicity tests were conducted on 6-month-old date palm (cv. Barhi) seedlings obtained from the Date Palm Development Research Unit, UAE University, UAE. To ensure rhizosphere/root colonization by the BCAs, soils were initially treated with the individual BCA for 7 days prior to *F. solani* inoculation.

To prepare the pathogen inoculum, millet (*Panicum miliaceum* L.) seeds were prepared by soaking 25 g of seeds to 40 ml of distilled water in 250 ml of Erlenmeyer flasks which were autoclaved on three consecutive days at 121°C for 45 min (El-Tarabily et al., 2000). Sterilized-millet seeds were inoculated with 20 agar plugs from the actively growing margins of an *F. solani* colony and incubated at 28°C for 2 weeks in dark. To ensure uniform colonization, the flasks containing 1 × 10^6 spores ml^{-1} of *F. solani* were shaken periodically. The control consisted of colonized and autoclaved millet seeds. Before use, small amounts of the control and colonized millet seeds were plated onto PDA to confirm the absence or presence of *F. solani*.

The antagonist inoculums were prepared by placing 50 g of moist oat bran into 500 ml Erlenmeyer flasks and autoclaving at 121°C for 20 min on three successive occasions as described by El-Tarabily et al. (2000). A 25-ml spore suspension, adjusted to 10^8 cfu ml^{-1} by using a hemocytometer (Agar Scientific

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

Limited, Essex, United Kingdom), of each antagonist was aseptically inoculated into the oat-bran and incubated at 28°C for 3 weeks in dark. To ensure uniform colonization, Erlenmeyer flasks were shaken frequently. Similarly colonized and autoclaved oat bran was used as a control. The colonized oat bran and the control oat bran were both suspended in 50 ml of sterile distilled water prior to use. To confirm the presence or absence of antagonists, an aliquot of the suspension (0.2 ml) was spread onto OMYEA plates which were incubated at 28°C for 7 days weeks in dark.

In Experiment (2), the aim was to test the efficacy of BCA1 and BCA2 prior to *F. solani* infection on date palm. In this experiment, six treatments were applied:

(i) Healthy control (C): seedlings cultivated in the soil without the pathogen, *F. solani*;
(ii) Diseased control (*Fs*): seedlings cultivated in the soil with *F. solani*-colonized millet seeds only;
(iii) *St*: seedlings cultivated in the soil colonized with *S. tendae* UAE1 (BCA1; non-ACCD-producing isolate #6) without *F. solani*;
(iv) *St* + *Fs*: seedlings cultivated in the soil colonized with *S. tendae* UAE1 followed by *F. solani* inoculation;
(v) *Sv*: seedlings cultivated in the soil colonized with *S. violaceoruber* UAE1 (BCA2; ACCD-producing isolate #26) without *F. solani*; and
(vi) *Sv* + *Fs*: seedlings cultivated in the soil colonized with *S. violaceoruber* UAE1 followed by *F. solani* inoculation.

To carry out the pathogenicity test and biological control trials, the soil was collected from the same farm described above (see Section "Cultivation of Pathogen and Isolation of Actinobacteria") and air-dried before being passed through a 3-mm mesh sieve. The antagonist-colonized oat bran inoculum (1% weight of colonized oat bran inoculum/weight of air-dried non-sterile soil) was dispersed through the soil using a cement mixer. The BCA-colonized soil was added into 4 kg of plastic-free draining plant pots and left for 7 days on a bench in an evaporative-cooled greenhouse (15-h day/9-h night; temperature = 25 ± 2°C; relative humidity = 60 ± 5%; photosynthetic photon flux density = 700 μmol m^{-2} s^{-1}). The pathogen inoculum (1% weight of colonized millet seed inoculum/weight of air-dried non-sterile soil) was added to each pot and dispersed through the soil by the same method as that of the BCAs. Date palm seedlings were planted in pots containing all the combinations described above and grown randomly on a bench in the greenhouse, and the pots were watered every 2 days.

For each treatment, eight pots (one seedling pot^{-1}) were arranged in a completely randomized design. Plants were kept in the greenhouse for 35 days post-treatment (dpt) for Experiments 1 and 2. Disease severity index was recorded for SDS symptoms at 35 dpt (Experiment 1) and at 15 and 35 dpt (Experiment 2) using a scale of 0–5: 0 = no apparent symptoms, 1 = 1–10% necrotic or white area in leaves or rotting in roots, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% (Alblooshi et al., 2022). For disease symptoms/recovery of diseased plants, conidial counts of *F. solani* were recorded at 35 dpt (Experiment 2). Conidia were harvested from the affected root tissues of eight seedlings $treatment^{-1}$ in 5 ml of water, and counted using a hemocytometer (Agar Scientific Limited, Essex, United Kingdom) as previously described (Alwahshi et al., 2019). All *in vivo* experiments were independently repeated three times with similar results. At the end of the experiment, the fungus was re-isolated from tissues with disease symptoms attempting to fulfill Koch's Postulates.

Measurements of endogenous ACC in plant tissues with high-pressure liquid chromatography

At the end of greenhouse Experiment (2), endogenous ACC contents were determined from date palm tissues. Pieces of roots and shoots (each was 10 cm in length) were collected. According to Lanneluc-Sanson et al. (1986), derivatization of ACC was carried out by adding phenylisothiocyanate (Sigma-Aldrich), and the subsequent separation and quantification of the resulting phenylthiocarbamyl-ACC by reverse-phase high-pressure liquid chromatography (HPLC). Phenylthiocarbamylation of ACC, and other amino acids, in date palm extracts was completed within 20 min at 25°C. After removing solvents and reagents, the phenylthiocarbamyl derivatives were separated on an octadecyl reverse-phase column, eluted with a mixture of acetonitrile and sodium acetate buffer (pH 4.6), and monitored with a UV-detector set at 254 nm. After removing solvents and reagents, 10 μl of the resulting phenylthiocarbamyl derivatives were separated on an octadecyl 10-μm reverse-phase column (Waters Associates μBondapak C_{18}, 4 mm × 30 cm), eluted with a mixture of acetonitrile and sodium acetate buffer (pH 4.6) in a Waters Associates liquid chromatograph and monitored with a differential 254 nm-UV detector. An analysis of date palm extract was achieved in 25 min and detected quantities as low as 1 pmol. Eight independent replicate samples were analyzed.

The concentration of ACC was obtained by the comparison of the peak area in the unknown sample with that of the corresponding area obtained with the authentic samples of a known concentration (Sigma–Aldrich).

Quantitative production of antifungal compounds and CWDEs

Only the non-ACCD-producing BCA1 and ACCD-producing BCA2 were further assessed for the production of

diffusible antifungal metabolites against *F. solani* using the cup plate method (Bacharach and Cuthbertson, 1948). Erlenmeyer flasks (250 ml) containing 50 ml of sterile fish meal extract broth (FMEB; El-Tarabily et al., 1997) were inoculated with 1 ml of 10% glycerol suspension of each BCA ($\sim 10^8$ cfu ml^{-1}) and incubated at 200 rpm on a gyratory shaker (Model G76, New Brunswick Scientific-Edison, NJ, United States) in dark at 28°C for 5 days. The suspensions from each flask were centrifuged at 8,000 × *g* for 30 min. The crude culture filtrate (supernatant) was filtered using 0.22 μm Millipore membranes (Millipore Corporation, MA, United States) and stored at 4°C. To prepare *F. solani*-seeded PDA plates, inocula were prepared according to the same method as for the cut-plug technique above. A sterilized 11-mm cork borer was used to cut the centers of the freshly seeded PDA plates with *F. solani*. By using a sterilized syringe, aliquots (0.3 ml) of the filter-sterilized crude culture filtrate were injected into the wells. The diameter of inhibition zones was determined for the two selected BCAs after 5 days of incubation in dark at 28°C.

A dialysis membrane (Type 45311; Union Carbide Corporation, IL, United States) overlay technique on FMEA or CCA plates (Gupta et al., 1995) was also performed to assay inhibition of *F. solani* by BCAs as previously recommended (El-Tarabily et al., 1997). Briefly, the membrane surface was inoculated with the BCA by evenly streaking cells/spores of a 7-day old culture of BCAs grown on OMYEA. Then, the FMEA or CCA plates were incubated at 28°C in dark for another 10 days. The membranes of adhering colonies were subsequently removed from the agar plates and the center of the plate was inoculated with a 5-mm disk of *F. solani* culture grown for 7 days on FMEA at 28°C in dark. The diameter of *F. solani* colony (mm) was measured after 8 days and compared to that of FMEA or CCA plates with the growing pathogen without any BCA (control). In case no pathogen grew from the agar plugs, these plugs were transferred to a fresh PDA plate, and incubated at 28°C for 5 days. If the pathogen did not grow from the plug, the diffused metabolites were fungicidal; however, if otherwise, these metabolites were considered fungistatic.

The production of chitinase and β-1,3-glucanase by the BCAs (Singh et al., 1999) was quantitatively determined using the minimal synthetic medium (Tweddell et al., 1994) amended with 2 mg ml^{-1} of either colloidal chitin or laminarin (Sigma-Aldrich), respectively. Chitinase-specific activity was calculated by measuring the release of *N*-acetyl-D-glucosamine (NAGA) from colloidal chitin. One unit (U) of chitinase activity was defined as the amount of enzyme releasing 1 μmol of NAGA mg^{-1} protein h^{-1} (Reissig et al., 1955) under the assay condition. The specific activity of β-1,3-glucanase was determined by measuring the amount of reducing sugars liberated from laminarin using dinitrosalicylic acid (DNS) solution (Miller, 1959). One U of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar mg^{-1} protein h^{-1} (Miller, 1959) under the above conditions. Protein concentration was determined by the Folin phenol reagent method (Lowry et al., 1951), using bovine serum albumin as standard.

Evaluation of crude extracts from culture filtrates of BCAs on *F. solani*

Filter-sterilized crude culture filtrates of BCA1 and BCA2 using FMEB or colloidal chitin broth (CCB) (Gupta et al., 1995) were proportionally poured in PDA plates. The medium was inoculated with a 5-mm diameter agar plug of *F. solani* mycelium that was placed upside-down. The colony diameter (mm) of the pathogen was measured after 10 days at 28°C. Inhibition of mycelial growth was also calculated upon mixing crude culture filtrates with potato dextrose broth (PDB; Lab M) inoculated with a 5-mm diameter agar plug of *F. solani* (Lorito et al., 1993) after 10 days of incubation in dark at 28°C.

To determine the mycelial dry weight, the tested mycelial contents of each culture were placed in a beaker containing boiling distilled water for 4 min as previously described by Suiton Iii and Starzyk (1972). The contents were then filtered through a Buchner funnel by using Whatman no. 114 filter paper. The contents were washed and the resultant mycelial mat on the filter paper with a constant vacuum of 15 psi was maintained throughout the working procedure. After the final wash, the sample was aspirated, and dry weights were obtained by placing the samples in a vacuum desiccator for 6 days. Then, the dry weight of mycelia was recorded for each sample.

Using Nikon-Eclipse 50i light microscope at 40× (Nikon Instruments Inc., NY, United States), the percentage of spore germination and the average germ tube length of *F. solani* after 24 h in PDB (Lorito et al., 1993) were determined. Briefly, tubes containing PBD, actinobacterial isolates, enzymes, and the fungus (treatment) or water (control) were incubated at 25°C for 24 h. The percentage of the first 100 spores seen on the microscope slide was considered as the percentage of germinating conidia, and the length of 20 germ tubes was measured and averaged. Observations with an oil immersion lens (100×) were essential to examine the effects of the crude culture filtrates of BCAs on the hyphal morphology of *F. solani* (Sneh, 1981). For all control treatments, *F. solani* mycelium was incorporated with non-inoculated filter-sterilized FMEB or CCB.

Statistical analysis

In vitro evaluation of BCAs against *F. solani*, data were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test at 5% level of significance. These experiments

were repeated thrice using eight plates treatment^{-1} for each time with similar results.

The two greenhouse experiments were independently repeated twice, and the obtained data were combined and analyzed. Similar results were obtained in each replicate. For the fungal spore counts and DSI against *F. solani*, eight replicates for each experiment were examined. Data represent the mean ± SE from 16 plants. ANOVA and Duncan's multiple range test were used to determine the statistical significance at $P < 0.05$. For all statistical analyses, SAS Software version 9 (SAS Institute Inc., NC, United States) was used.

Results

Isolation and screening of actinobacteria with antifungal properties

From the 54 actinobacteria, 34 SA (62.9%) and 20 NSA (37.1%) strains were isolated on ISSA plates from the rhizosphere soil of healthy date palm trees. Prior to isolation, polyvalent *Streptomyces* phages and dry heat methods were effectively used to introduce diverse rare actinobacterial isolates, i.e., NSA. Consequently, the numbers of SA were significantly ($P < 0.05$) reduced, but the numbers of NSA significantly ($P < 0.05$) increased by 66.8 and 70.3% by polyvalent *Streptomyces* phages and dry heat pretreatment, respectively (**Supplementary Table 1** and **Supplementary Figure 1**). Besides the dominant *Streptomyces*, rare actinobacteria of the genera *Actinomadura*, *Actinoplanes*, *Dactylosporangium*, *Kribbella*, *Microbispora, Micromonospora*, *Microtetraspora, Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus,* and *Streptosporangium* were also isolated.

Next, two *in vitro* screenings were simultaneously carried out to determine the production of diffusible antifungal metabolites and CWDEs. Our results showed that 25 isolates (12 SA and 13 NSA) showed strong production of both diffusible antifungal metabolites and CWDEs against *F. solani* (**Table 1** and **Supplementary Figure 2**). Isolates belonging to SA (#2, #6, #9, #17, #22, #26, #29, #33, #37, #41, #42, and #50) and NSA (#7, #12, #16, #20, #38, #40, #43, #44, #46, #49, #51, #53, and #54) produced large inhibition and clearing zones (>30 mm) on FMEA and MFA, respectively, were further selected for subsequent experiments (**Table 1**). The rest of the isolates showing inhibition zones on FMEA or clearing zones on MFA less than 30 mm were excluded.

Considering the multiple mechanisms of antifungal effects, the above-mentioned isolates were checked if they could produce VC, siderophores, and HCN. Only ten isolates of SA and NSA (#6, #7, #26, #33, #40, #42, #43, #44, #46, and #50) were found to share the production of diffusible antifungal metabolites and CWDEs in addition to the production of VC (**Table 1**) on FMEA and siderophores on CAS agar plate (**Table 1** and **Supplementary Figure 3**). None of the isolates, on the other hand, produced HCN. Isolate #13 (the non-antifungal/CWDE/ACCD producer) was used as a positive control in all *in vitro* tests. These potent ten isolates were further selected for subsequent experiments. Our data suggest that the actinobacteria (SA and NSA) isolated from the rhizosphere soils and showing multiple modes of antagonism against *F. solani* can be effective to manage SDS in the UAE.

Effect of ACCD activities produced by the rhizospheric actinobacterial isolates

In addition to the tested antifungal properties, the enzymatic ACCD activities of the ten potential BCAs on DF-ACC plates were quantitatively determined (**Supplementary Figure 3**). Four isolates (#26, #43, #46, and #50) were considered ACCD producers, albeit the variation in ACCD levels among them (**Table 1**). On the other hand, six isolates (#6, #7, #33, #40, #42, and #44) were non-ACCD producers. These isolates were further investigated under greenhouse conditions.

The *in vivo* Experiment (1) was carried out on date palm seedlings cultivated in soil colonized with *F. solani* using the potential ten antagonists. For that reason, we visually estimated DSI on seedlings to assess their recovery from SDS caused by *F. solani* after the application of BCAs. Date palm seedlings cultivated in soil infested with *F. solani* without any BCA treatment, designated as *Fs*, did not recover from the disease by the end of Experiment 1 (**Figure 1**).

In general, there was a significant ($P < 0.05$) difference in DSI among all treatments on diseased seedlings (**Figure 1**). Thus, seedlings that were treated with ACCD-producing isolates demonstrated lower DSI than those treated with ACCD-non-producing isolates (except of #6) or *Fs* (control) treatment. On the other hand, plants treated with individual ACCD-non-producing isolates for 7 days before inoculation with the pathogen significantly ($P < 0.05$) showed lesser DSI than in control plants. Thus, isolate #6 was the best among the tested ACCD-non-producing isolates, and was termed BCA1.

Similarly, plants grown in soils amended with any of the ACCD-producing isolates infested with *F. solani* were compared for their recovery. Even though the ACCD-producing isolates #43, #46, and #50 showed a comparable reduction of DSI on diseased seedlings, they did not reach to the same level of reduction obtained by isolate #26 at 35 dpt (**Figure 1**). Isolate #26, also labeled as BCA2, was relatively superior over the others; thus, its preventive application dramatically reduced DSI resulting in minimal

disease effect and relatively healthy-grown seedlings. Our preliminary data suggest that ACCD production might be a major mechanism that BCAs utilize to offer promise for the improvement of plant health in response to biotic stresses.

Identification of the promising BCAs

In order to identify BCA1 (isolate #6) and BCA2 (isolate #26) to the species level, the nucleotide sequence of their 16S rRNA gene was determined. The resulting sequences were deposited in NCBI[2] under GenBank accession numbers: OL356342 for BCA1, and OL356341 for BCA2.

In comparison with other 16S rRNA gene sequences obtained from the GenBank database, the 1,520-bp sequence of BCA1 showed 99.9% similarity with that in *Streptomyces tendae* ATCC 19812^T (D63873) and *Streptomyces violaceorubidus* LMG 20319^T (AJ781374; **Supplementary Figure 4**). The rest of the *Streptomyces* spp. showed less similarity with our studied strain. To obtain definitive identification of isolate #6, its pure culture was cultivated on ISP medium 3. After 14 days of cultivation,

2 https://www.ncbi.nlm.nih.gov/nucleotide/

TABLE 1 *In vitro* antagonistic and ACCD enzymatic activities by actinobacterial isolates against *Fusarium solani*.

Taxa	Species	Isolate	Inhibition diameter[a] (mm)	Clearing diameter[b] (mm)	Production of		ACCD activity[c]
					VC	Siderophore	nmol α-ketobutyrate mg^{-1} protein h^{-1}
	F. solani (pathogen)		0.0	0.0	−	−	0.00 a
	#13 (positive control)		0.0	0.0	−	−	0.00 a
SA	*Streptomyces*	#2	56.1 ± 2.4 d	50.3 ± 2.0 c	−	−	ND
		#6	58.3 ± 2.9 d	59.2 ± 3.1 d	+	+	0.00 a
		#9	47.2 ± 1.2 c	57.3 ± 1.8 d	−	+	ND
		#17	45.2 ± 0.9 bc	43.2 ± 1.1 b	−	−	ND
		#22	31.6 ± 0.6 a	44.2 ± 1.3 b	+	−	ND
		#26	58.9 ± 2.2 d	58.0 ± 2.9 d	+	+	548.28 ± 27.8 e
		#29	30.2 ± 0.5 a	30.8 ± 0.7 a	+	−	ND
		#33	41.9 ± 2.3 b	31.4 ± 0.6 a	+	+	0.00 a
		#37	58.7 ± 2.8 d	31.8 ± 0.9 a	−	−	ND
		#41	44.5 ± 1.4 b	57.7 ± 2.3 d	−	+	ND
		#42	43.3 ± 3.4 b	42.4 ± 2.1 b	+	+	0.00 a
		#50	58.2 ± 1.9 a	46.6 ± 2.4 bc	+	+	46.72 ± 11.3 b
NSA	*Actinomadura*	#7	57.8 ± 2.0 d	47.5 ± 1.7 bc	+	+	0.00 a
		#20	30.6 ± 0.4 a	56.8 ± 1.8 d	+	−	ND
	Actinoplanes	#40	33.0 ± 0.4 a	32.7 ± 0.6 a	+	+	0.00 a
		#43	42.6 ± 1.6 b	42.2 ± 0.8 b	+	+	368.66 ± 18.6 d
		#53	33.0 ± 1.2 a	57.7 ± 2.1 d	−	−	ND
	Dactylosporangium	#49	58.0 ± 2.6 d	58.8 ± 1.2 d	−	−	ND
	Microbispora	#12	31.7 ± 0.8 a	44.4 ± 1.4 b	−	+	ND
	Micromonospora	#16	31.5 ± 0.2 a	31.7 ± 0.8 a	+	−	ND
		#38	43.9 ± 1.1 b	31.2 ± 0.8 a	−	−	ND
		#46	57.2 ± 2.2 d	43.3 ± 1.2 b	+	+	141.24 ± 8.8 c
		#51	32.3 ± 0.6 a	31.2 ± 0.5 a	−	+	ND
	Microtetraspora	#44	32.2 ± 0.7 a	42.2 ± 1.0 b	+	+	0.00 a
	Streptosporangium	#54	42.0 ± 0.9 b	45.1 ± 1.3 bc	−	+	ND

[a]Production of diffusible antifungal metabolites active against *F. solani* using the cut-plug method.
[b]Production of CWDEs on MFA.
[c]Production of ACCD on DF-ACC medium for 5 days at 28 ± 2°C.
For all *in vitro* experiments, values are means ± SE of eight replicates. Values within each column, followed by the same letter are not significantly ($P > 0.05$) different according to Duncan's multiple range test.
Isolate #13 is a non- antifungal-, non-CWDE- and non-ACCD-producing positive control. Isolates #6 and #26 represent the ACCD-non-producing *Streptomyces tendae* UAE1 (BCA1) and the ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2), respectively.
SA, streptomycete actinobacteria; NSA, non-streptomycete actinobacteria; VC, volatile compounds; ACC, 1-aminocyclopropane-1-carboxylic acid; ACCD, ACC deaminase; CWDEs, cell-wall-degrading enzymes; MFA, mycelial fragment agar; DF, Dworkin and Foster's salts minimal broth medium; BCA, biological control agent; +, producing; −, not producing; ND, not determined.

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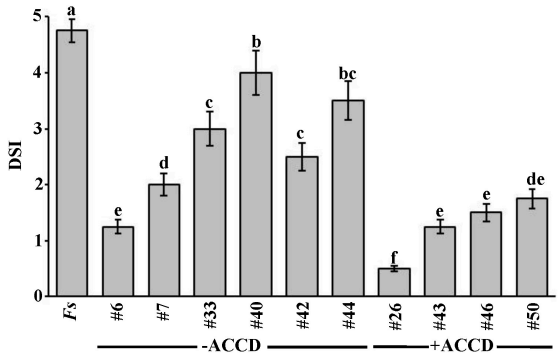

FIGURE 1

In vivo effect of selected BCAs on date palm seedlings inoculated with *Fusarium solani* (Experiment 1). Disease severity index after the recovery of affected date palm seedlings from the fungal pathogen, *F. solani*, at 35 dpt with the BCA under greenhouse conditions. DSI was based on a scale of 5:0 = no infection, 1 = 1–10, 2 = 11–25, 3 = 26–50, 4 = 51–75, and 5 = 76–100% damage including necrosis, white area in leaves or rotting in roots. Values with different letters are significantly different from each other at $P < 0.05$. Bars represent standard errors. Seedlings ($n = 16$) were cultivated in soil colonized with each BCA isolate 7 days prior to soil infestation with *F. solani*. Isolates #6 and #26 represent the ACCD-non-producing *Streptomyces tendae* UAE1 (BCA1) and ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2), respectively. BCA, biological control agent; *Fs*, seedlings cultivated in soil infested with *F. solani* only; dpt, days post treatment; DSI, disease severity index; ACCD, 1-aminocyclopropane-1-carboxylic acid deaminase.

the isolate developed light gray aerial mycelium and yellow to greenish yellow substrate mycelium with the production of yellow pigment on the reverse side of cultures (**Supplementary Figure 4**). The configuration of the spore chains of the isolate was examined using SEM and our results revealed that spore chains belonged to section Spirales (closed spirals), consisting of 10–50 mature spores with smooth surfaces (**Supplementary Figure 4**). Together, this suggests that the outstanding isolate #6 can be identified as *S. tendae* (Ettlinger et al., 1958) strain UAE1.

The 1,519-bp of 16S rRNA of the other antagonist (BCA2) showed 100% similarity with the nucleotide sequence of four *Streptomyces* spp., namely *S. violaceoruber* DSM 40049^T (NR041914), *S. anthocyanicus* NBRC 14892^T (NR041168), *S. tricolor* NBRC 15461^T (NR041189), and *S. coelescens* AS 4.1594^T (NR027222; **Figure 2A**). Consequently, its pure cultures were checked on ISP medium 3. It turned out that aerial mycelia mass color was gray with violet substrate mycelium and with the production of violet pigment on the reverse side of cultures (**Figure 2B**). The formation of spore chains belonged to the Spirales type (closed spirals) that consisted of 10–50 smooth-surfaced spores/chains on the aerial hyphae (**Figure 2C**). BCA2 was assigned as *S. violaceoruber* (Waksman and Curtis, 1916) Pridham, 1970 strain UAE1.

Effects of *S. tendae* or *S. violaceoruber* on date palm seedlings in *F. solani*-infested soils (Experiment 2)

The efficacy of BCA1 and BCA2 against SDS was tested and compared with date palm (cv. Barhi) in a greenhouse pot study. Seedlings transplanted in soils infested with *F. solani* (*Fs*) displayed typical foliar and root symptoms of SDS. At the early stages, leaves developed chlorosis (yellowing), wilting, and mild necrosis, turning to brown, dryness, and death of plants at later stages (**Figure 3A**). Advanced foliar symptoms in plants removed from the soil were associated with a weak, rotted root system when compared to a healthy root system (**Figure 3B**).

Treatments of either *S. tendae* UAE1 (BCA1; ACCD-non-producing isolate) or *S. violaceoruber* UAE1 (BCA2; ACCD-producing isolate) exhibited varying degrees of SDS suppression on the above and underground parts of infected seedlings. We noticed that plants cultivated in soils infested with *F. solani* that were pre-treated with BCA1 (*St* + *Fs*) and BCA2 (*Sv* + *Fs*) started to recover at 15 dpt (**Figure 3A**). At 35 dpt, not only newly fresh leaves in the center of the crown emerged, but also the root system appeared healthy and strong (**Figure 3B**), confirming our *in vitro* results of the inhibitory effect of BCAs against *F. solani*. This is in contrast to *Fs* plants without BCA treatments. In general, the application of *S. violaceoruber* UAE1 was visually more efficient in decreasing SDS symptoms than *S. tendae* UAE1 treatment. It is worth mentioning that the corresponding control seedlings, designated as *St* or *Sv*, looked good and had vigorous root systems.

It is essential to know how the modes of action and production of ACCD by BCAs affect their efficacy on conidial counts and DSI *in planta*. The numbers of conidia recovered in *St* + *Fs* and *Sv* + *Fs* seedlings were about three-fold less than of *Fs* (**Figure 3C**), suggesting that *S. tendae* UAE1 and *S. violaceoruber* UAE1 significantly ($P < 0.05$) reduced conidial counts to the same level. The DSI was also estimated on plants treated with *St* or *Sv* in soils infested with *Fs*. During the period of infection, SDS symptoms progressed on seedlings grown in soil colonized with *F. solani*, which was also reflected on the high

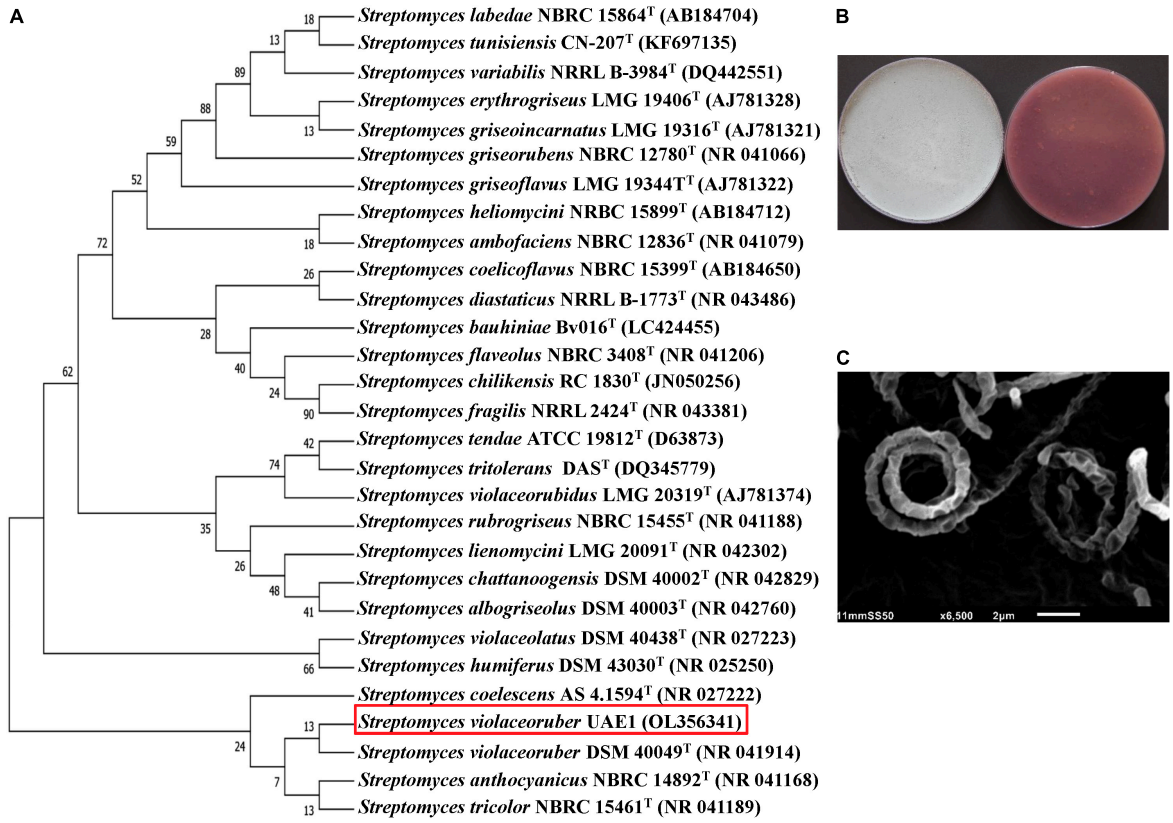

FIGURE 2
Taxonomic identification of the ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2). **(A)** The dendrogram showing the phylogenetic relationships between *S. violaceoruber* UAE1 (isolate #26; 1519 bp; OL356341) and other members of *Streptomyces* spp. on the basis of 16S rRNA sequences. **(B)** Gray aerial (left) and violet substrate mycelia with the production of violet pigment on the reverse side of cultures (right) growing on ISP3 medium supplemented with yeast extract; and **(C)** scanning electron micrograph (6,500×) of the section Spirales spore chains (closed spirals) with 10–50 smooth-surfaced spores/chain of *S. violaceoruber* UAE1. In **(A)**, numbers at nodes indicate percentage levels of bootstrap support based on a neighbor-joining analysis of 500 resampled datasets. GenBank accession numbers are given in parentheses. BCA, biological control agent; ACCD, 1-aminocyclopropane-1-carboxylic acid deaminase.

DSI scores (Figure 3D). It was clear that the two BCAs had a substantial drop in DSI. Thus, the DSI estimates were greatly ($P < 0.05$) reduced in *Sv* + *Fs* seedlings when compared with that of *St* + *Fs* plants at 35 dpt.

To test if ACCD produced by *S. violaceoruber* UAE1 had additive protective effects on diseased seedlings, the ACC levels in the root and shoot tissues were quantitatively determined. Seedlings grown in soil with *St* only showed comparable amounts of ACC in their root and shoot tissues with those when no pathogen was applied (Figure 3E). However, seedlings grown in *Sv*-amended soil significantly ($P < 0.05$) reduced the ACC levels of the root and shoot tissues to the minimum compared to any other treatment. This suggests that seedlings can perform better when treated with the ACCD-producing *S. violaceoruber* UAE1.

Upon infection with *F. solani,* ACC levels significantly ($P < 0.05$) increased *in planta* compared to their corresponding control treatments (Figure 3E). Unlike *S. tendae* UAE1-treated soil, *S. violaceoruber* UAE1 decreased not only the disease severity (Figures 3A–D), but also ACC in diseased plants to almost the same levels as in non-diseased control plants (Figure 3E). The ACC levels were the same in the root and shoot tissues of *St* + *Fs* and *Fs* seedlings. Overall, our data indicate that the most effective BCA (i.e., *S. violaceoruber* UAE1) showing multiple antagonistic mechanisms and relatively high ACCD activities can suppress pathogen development and improve plant performance when exposed to fungal attacks.

In vitro inhibitory effects of the culture filtrates of BCA1 and BCA2 on *F. solani*

Apart from the variation in ACCD production, the antagonistic activities of BCA1 and BCA2 were tested *in vitro*. When the culture filtrate of either BCA1 or BCA2 was applied using the cup plate method, there was a clear zone of inhibition in the growth of *F. solani*, compared to that when isolate #13 (positive control) was used (Figure 4A and Supplementary

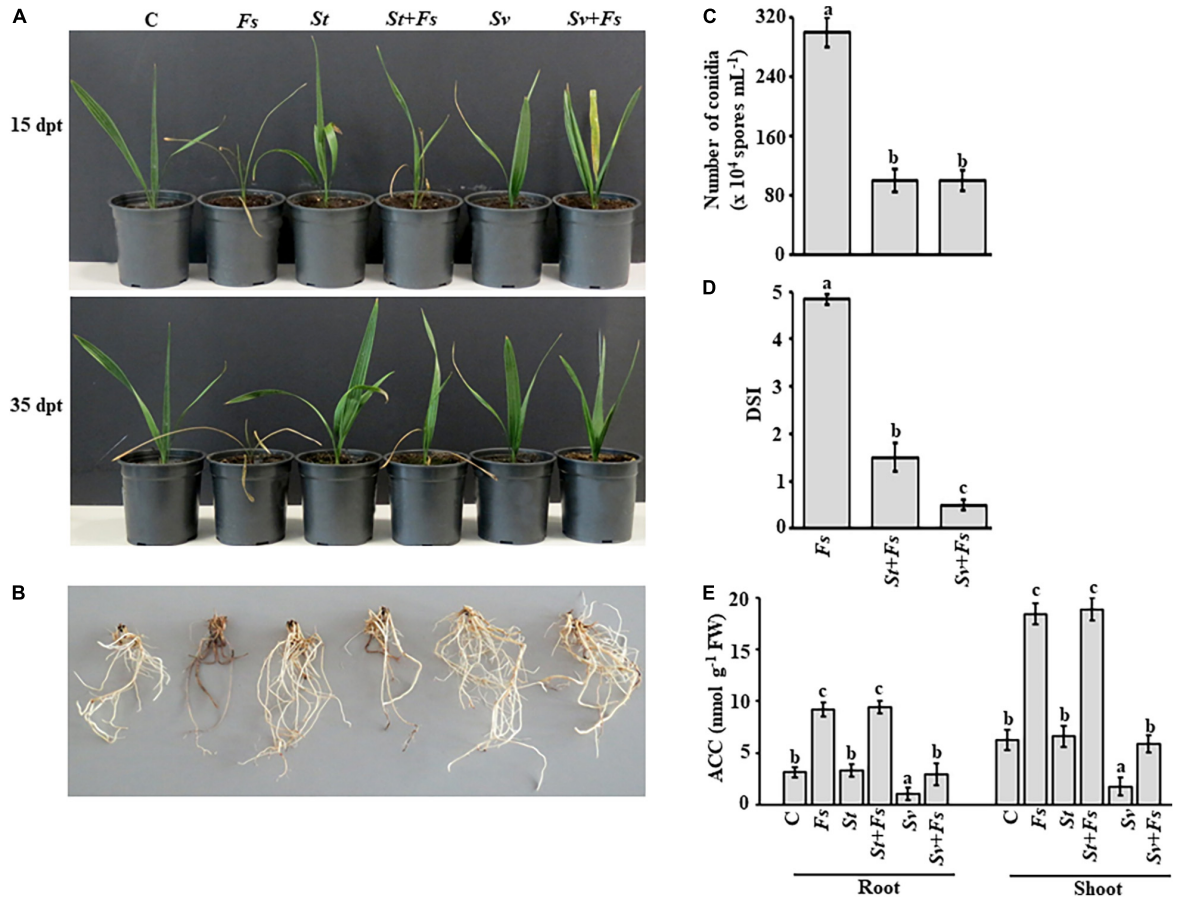

FIGURE 3
Effect of BCA treatments on date palm seedlings in soils infested with *Fusarium solani*. Preventive effect of BCA treatments on **(A)** seedlings and **(B)** roots in soil not-infested or infested with *F. solani* after 15 (upper panel) and 35 dpt (lower panel). Recovery of seedlings (n = 16) from pathogen infection was measured according to **(C)** the number of conidia; **(D)** DSI, **(E)** endogenous contents of ACC in the root and shoot tissues of inoculated date palm seedlings after BCA treatment after 35 dpt under greenhouse conditions. In **(D)**, DSI was based on a scale of 5:0 = no infection, 1 = 1–10, 2 = 11–25, 3 = 26–50, 4 = 51–75, and 5 = 76–100% damage including necrosis, white area in leaves or rotting in roots. In **(C–E)**, values with different letters are significantly different from each other at $P < 0.05$. Bars represent standard errors. C, seedlings grown in non-pathogen colonized soil (control); *Fs*, seedlings grown in soil infested with *F. solani* (diseased control); *St* or *Sv*, seedlings grown in soil colonized with either the non-ACCD-producing *Streptomyces tendae* UAE1 (BCA1; isolate #6) or the ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2; isolate #26), respectively; *St + Fs* or *Sv + Fs*, seedlings grown in soil colonized with either *S. tendae* UAE1 or *S. violaceoruber* UAE1, respectively, 7 days prior to incorporation of *F. solani*-millet seed based inoculum; ACC, 1-aminocyclopropane-1-carboxylic acid; ACCD, ACC deaminase; BCA, biological control agent; FW, fresh weight; dpt, days post treatment; DSI, disease severity index.

Table 2). This inhibition, however, was not significant ($P < 0.05$) on the plates containing the two BCAs and the pathogen, suggesting that the diffused antifungal metabolites of BCA1 and BCA2 were effectively performing almost the same on the growth of *F. solani*. In addition, the results obtained from the dialysis membrane overlay technique using FMEA plates indicated that the antifungal metabolites of BCA1 and BCA2 were fungicidal. This was evident when *F. solani* did not recover from the plugs transferred from BCA1- or BCA2-treated plates, but not isolate #13, to fresh PDA (Figure 4B).

In contrast to isolate #13, experiments obtained from the dialysis membranes using CCA plates of isolates (#6 and #26) showed that these BCAs completely inhibited *F. solani* growth *in vitro* (Figure 4C), suggesting fungicidal activities of their diffused CWDEs on *F. solani*. The enzymatic activities of chitinase and β-1,3-glucanase in both BCAs on media amended with colloidal chitin/*F. solani* cell walls and laminarin/*F. solani* cell walls, respectively, were also detected. No significant ($P > 0.05$) difference was found in the *in vitro* enzymatic activities between the ACCD-producing and ACCD-non-producing isolates, thus significant ($P < 0.05$) from isolate #13 (Supplementary Table 2).

In addition, the filter-sterilized crude culture filtrates of BCA1 and BCA2 were effective against *F. solani*. By increasing the culture filtrates of the BCAs to 50%, this significantly ($P < 0.05$) reduced the colony growth and

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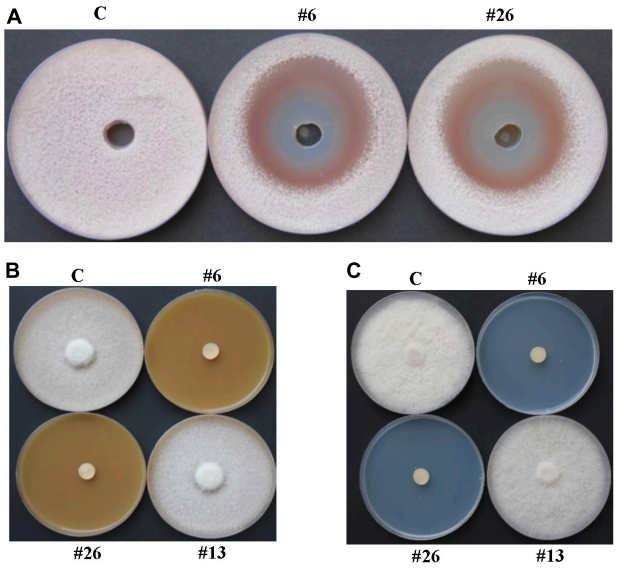

FIGURE 4
In vitro antifungal activities of BCA candidates against *Fusarium solani*. Inhibition of *F. solani* mycelial growth by actinobacterial isolates #6 and #26 using **(A)** cup plate method on PDA; and dialysis membrane overlay technique using **(B)** FMEA or **(C)** CCA plates. In **(A,B)**, inhibition of *F. solani* mycelial growth was observed by the diffusible antifungal metabolite-producing isolates #6 and #26. In **(C)**, inhibition of *F. solani* mycelial growth was observed by the chitinase-producing isolates #6 and #26. In **(A)**, wells are inoculated with either filter-sterilized FMEB **(C)**, or filter-sterilized crude culture filtrates of isolates #16 or #26; while in **(B,C)**, FMEA or CCA plates are either colonized by no BCA, or isolates #6, #13 or #26. BCA, biological control; PDA, potato dextrose agar; FMEA/B, fish meal extract agar/broth; CCA, colloidal chitin agar; C, sterile non-inoculated PDA agar plug/filter-sterilized FMEB (negative control); #13, *Streptomyces* sp. (positive control): #6, *Streptomyces tendae* UAE1 (ACCD-non-producing BCA1); #26, *Streptomyces violaceoruber* UAE1 (ACCD-producing BCA2).

TABLE 2 Effects of the crude culture filtrate of the BCAs on the morphological characteristic of mycelia, conidia, and germ tube of *Fusarium solani*.

Media	Isolate	Culture filtrate (%)	Colony diameter (mm)	Mycelial dry weight (g)	Conidia germination (%)	Germ tube length (μm)
FMEB	#6	0	97.6 ± 0.8 c	72.4 ± 1.0 c	82.4 ± 0.8 c	48.6 ± 2.0 c
		50	15.3 ± 1.2 b	8.1 ± 0.8 b	12.2 ± 1.9 b	10.7 ± 2.6 b
		100	0.0 a	0.0 a	1.1 ± 0.2 a	1.8 ± 0.3 a
	#26	0	97.2 ± 1.3 c	70.9 ± 1.4 c	80.6 ± 1.1 c	46.8 ± 1.5 c
		50	15.7 ± 1.0 b	8.8 ± 0.6 b	13.8 ± 1.5 b	9.9 ± 2.2 b
		100	0.0 a	0.0 a	0.9 ± 0.1 a	1.7 ± 0.4 a
CCB	#6	0	96.6 ± 0.4 c	69.4 ± 1.6 c	85.8 ± 0.8 c	62.3 ± 1.1 c
		50	13.5 ± 1.8 b	12.6 ± 1.0 b	16.7 ± 1.9 b	8.0 ± 0.8 b
		100	0.0 a	0.0 a	0.8 ± 0.1 a	0.0 a
	#26	0	95.8 ± 0.5 c	71.0 ± 1.2 c	84.4 ± 0.7 c	6163 ± 1.2 c
		50	13.6 ± 1.4 b	13.2 ± 0.9 b	15.8 ± 1.3 b	7.8 ± 0.7 b
		100	0.0 a	0.0 a	0.8 ± 0.0 a	0.0 a

Values are means ± SE of eight replicates. Values with the same letter within a column for each BCA are not significantly ($P > 0.05$) different according to Duncan's multiple range test. Isolates #6 and #26 represent the ACCD-non-producing *Streptomyces tendae* UAE1 (BCA1) and ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2), respectively. BCA, biological control agent; FMEB, fish meal extract broth; CCB, colloidal chitin broth.

mycelial biomass of *F. solani* after 5 days of incubation at 28°C on PDA plates, compared to no application of culture filtrates (Table 2). Thus, fungal growth, represented by colony diameter and mycelial dry weight, was completely retarded when 100% of the culture filtrate of BCA1 or BCA2 was applied. Although the reduction in conidial germination and germ tube elongation clearly occurred in *F. solani*, the fungal response did not differ due to the culture filtrates of BCA1 and BCA2 (Table 2).

When *F. solani* was treated with the filter-sterilized crude culture filtrate of any of the two BCAs, there were remarkable abnormalities in hyphal formation. This was clear from the aseptate (coenocytic) and branch-forming hyphae and coagulating cytoplasm of *F. solani*-treated with the culture

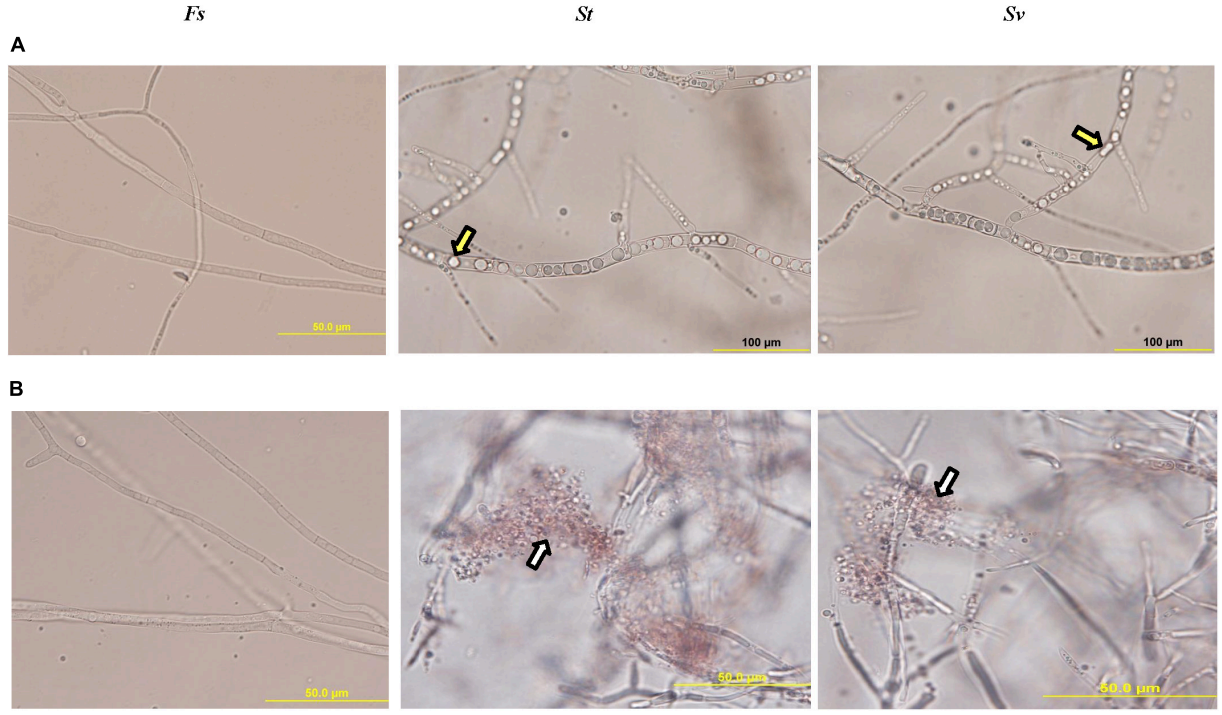

FIGURE 5
Inhibitory effect of the metabolites of BCA candidates on *Fusarium solani*. Abnormalities evident in the hyphal morphology and cytoplasmic contents of *F. solani*, following the treatment with filter-sterilized crude culture filtrate on **(A)** FMEB or **(B)** CCB of *St* (middle panel) and *Sv* (right panel) compared to control (left panel). Yellow arrows point to hyphal non-septate formation and cytoplasmic coagulation, while white arrows point to cytoplasmic lysis; respectively. *St*, the ACCD-non-producing *Streptomyces tendae* UAE1 (BCA1; isolates #6); *Sv*, the ACCD-producing *Streptomyces violaceoruber* UAE1 (BCA2; isolate #26); BCA, biological control agent; FMEB, fish meal extract broth; CCB, colloidal chitin broth. Light microscopy images were taken at 1,000 times total magnification.

filtrate of BCA1 or BCA2 on FMEB (**Figure 5A**). On CCB, lysis of fungal hyphae was observed in any of the BCA culture filtrate-amended flasks (**Figure 5B**). On the other hand, light microscopy detected healthy, unimpaired mycelial mats of *F. solani* in flasks that did not contain a BCA (control). Regardless of ACCD synthesis, *in vitro* BCA1 or BCA2 treatment had adverse effects on mycelial growth, spore germination, and germ tube elongation, ultimately causing membrane damage and outflow of cytoplasmic contents in *F. solani*.

Discussion

Date palm diseases, such as SDS, associated with fungal pathogens are an emerging economical threat to the production of dates in the Arabian Peninsula, including the UAE. Microbial antagonists, also known as BCAs, for plant diseases prevent infection or establishment of the pathogen in host plants to control phytopathogens (Thambugala et al., 2020). Compared to other microorganisms, actinobacteria appear to withstand harsh conditions such as high temperature, salinity, and aridity (Dion, 2008; Qin et al., 2019; Xie and Pathom-aree, 2021). Therefore, we hypothesize that rhizospheric actinobacteria secreting ACCD along with a broad spectrum of antagonistic mechanisms against *F. solani* can substantially reduce the detrimental effect of SDS on date palm.

For that reason, several *in vitro* assays were carried out to give us a hint about the isolates showing multiple biocontrol properties to *F. solani* and possessing ACCD activities, even though this might not allow for drawing definite conclusions about them as effective BCAs *in vivo*. First, 54 actinobacteria, including 34 SA and 20 NSA, were isolated from rhizosphere soils of healthy date palm trees in the UAE. In general, *Streptomyces* species were found highly abundant, in alignment with other reports (Jose and Jebakumar, 2013; Palaniyandi et al., 2013). In addition, a series of *in vitro* assays were performed to determine the production of diffusible antifungal metabolites, CWDEs, VC, siderophores, and HCN by the isolated actinobacteria. According to Köhl et al. (2019), *in vitro* screening of microbial BCAs with multiple modes of action can potentially minimize the number of isolates to be used in the bioassay detection on plants or plant tissues. In the current study, ten (five from each SA and NSA) isolates with multiple modes of action were *in vitro* active against *F. solani* (**Table 1**), confirming the importance of actinobacteria as

potential BCAs against plant pathogens (Saeed et al., 2017a; Kamil et al., 2018; Al Raish et al., 2021). It has been reported that inoculation of plants with ACCD-producing microorganisms can ameliorate the effect of environmental (biotic and abiotic) stresses in plants (Glick, 2014; Al Hamad et al., 2021; Glick and Nascimento, 2021; Jiménez-Mejía et al., 2022). In our study, the enzymatic activities of ACCD in these isolates were also measured. With four producing and six ACCD-non-producing isolates, preliminary studies for *in vivo* biological control under greenhouse conditions were evaluated.

According to their efficacy to SDS on date palm seedlings, two potential BCAs (isolates #6 and #26) were chosen. When tested for further biocontrol characteristics, both isolates confirmed their comparable production of diffusible antifungal metabolites and CWDEs (chitinase and β-1,3-glucanase) *in vitro*. Their culture extracts inhibited mycelial growth, conidial germination, and germ tube elongation of *F. solani* in a dose-dependent manner. Investigation of *F. solani* hyphae in the crude culture filtrate of BCA1 or BCA2 demonstrated that the hyphae showed abnormal growth and loss of internal content, resulting in a significant rise in the cellular leakage of *F. solani* mycelium. This suggests that the synthesis of secondary metabolites (i.e., diffusible and volatile antifungal substances) and the production of siderophores by the tested BCAs are involved in the inhibition of the growth of *F. solani*. Furthermore, the cell walls of *F. solani* which consist of chitin and glucan, are affected by chitinase and β-1,3-glucan enzymes produced by these BCAs. Except for ACCD production, almost similar *in vitro* biochemical results were obtained by *S. tendae* UAE1 (BCA1; isolate #6) and *S. violaceoruber* UAE1 (BCA2; isolate #26). Thus, the most effective BCAs studied to-date appear to antagonize pathogens using multiple mechanisms (Barka et al., 2016; Al Hamad et al., 2021; Alblooshi et al., 2022).

Genome sequencing of *S. tendae* UTMC 3329 has revealed important biological features of this actinobacterium tolerant to abiotic stresses (Eftekharivash and Hamedi, 2020), thus anticipating a future biotechnological and agricultural research. The siderophores-secreted *S. tendae* F4 has promoted the growth and improved the cadmium uptake in sunflower (Dimkpa et al., 2008). Activities of the chitinase and β-1,3-glucanase in ATCC 31160 and the expression of chitin-binding AFP1 protein from Tü901 have been attributed to the key role of nikkomycin-producing *S. tendae* as a bio-fungicide and bio-insecticide (Engel, 1987; Bormann et al., 1999). Thus, the current study is the first to report the isolation and characterization of *S. tendae* UAE1 as a BCA to manage SDS on date palm in the UAE.

Researchers tend to apply a combination of isolates possessing multiple functions to maximize the effect to control phytopathogens. A mixture of actinobacterial, bacterial, or fungal isolates showing multiple modes of action has been reported effective in the suppression of diseases associated with soil-borne pathogens in plants (El-Tarabily et al., 2009; Thangavelu and Gopi, 2015a,b). In contrast, *S. tendae* UAE1 was used solely in the present study to enhance resistance in date palm against *F. solani*. Similarly, royal poinciana seedlings pre-inoculated with the endophytic *Streptomyces wuyuanensis* resulted in disease protection against *Neoscytalidium dimidiatum* (Al Hamad et al., 2021). This could be attributed to antibiosis and the production of CWDEs and other antagonistic substances (e.g., siderophores and/or VC) in *S. tendae* and *S. wuyuanensis*. Screening trials for a potential isolate with multiple mechanisms to serve as a BCA have identified *S. samsunensis* and *S. antibioticus* to manage dieback disease on mango and stem canker disease on royal poinciana, respectively (Kamil et al., 2018; Al Raish et al., 2021). Thus, these BCAs were more effective than other *Streptomyces* spp. possessing single modes of action.

Since *S. violaceoruber* UAE1 (BCA2) was relatively superior to *S. tendae* UAE1 (BCA1) as a BCA to manage SDS, we checked if the ACCD activity in the former *Streptomyces* species had additional effects on *F. solani*–date palm interaction (**Figure 3**). Previously, *S. violaceoruber* has been identified for its secondary metabolite products (Pham et al., 2005) and antibiotic properties (Duangmal et al., 2005). In addition, β-glucanase and phospholipases used as food additives (US FDA, 2007, 2015; Harazono et al., 2020), and cellulase and xylanase used in biological delignification (Abou-Dobara and Omar, 2014) are *S. violaceoruber* related-enzymes. This confirms the role of *S. violaceoruber* in many medical, industrial, and biotechnological applications (Duangmal et al., 2005). Yet, the inclusion of *S. violaceoruber* as a BCA in agricultural practices has not been reported.

Plant-emitted ethylene (ET), as a stress hormone, has received a considerable attention in the role it plays in resistance/tolerance to biotic/abiotic stresses at low concentrations. This can be achieved *via* the hydrolysis of ACC (the immediate precursor of ET) by the enzyme ACCD (Glick and Nascimento, 2021). It is widely reported that ACCD can degrade ACC to ammonia and α-ketobutyrate, thus reducing the level of ET *in planta* (Glick, 2014; Raghuwanshi and Prasad, 2018; Glick and Nascimento, 2021). Along with its biocontrol properties, *S. violaceoruber* UAE1 had additive effects of the suppression of SDS on seedlings in the greenhouse pot experiments. Similar to our results, Al Hamad et al. (2021) have demonstrated that the ACCD-producing *Streptomyces griseorubens* UAE2 had augmentative BCA effects on royal poinciana plants inoculated with *N. dimidiatum* compared to *S. wuyuanensis* UAE1 that did not have ACCD activity. This supports previously published studies reporting that plants inoculated with BCAs possessing ACCD activity can make the plant more resistant to environmental stresses (Wang et al., 2000; El-Tarabily et al., 2019, 2021; Mathew et al., 2020; Glick and Nascimento, 2021; Jiménez-Mejía et al., 2022).

Similar observations have been reported in non-actinobacterial isolates containing ACCD. Tomato plants

treated with the ACCD-producing *Methylobacterium* spp. significantly reduced wilt symptoms of *Ralstonia solanacearum* and lowered the ET emission (Yim et al., 2013). Inhibition of crown gall tumors on tomato infected by pathogenic *Agrobacterium* strains was also associated with the application of ACCD-producing bacteria (Toklikishvili et al., 2010). In the current study, we claim that *S. violaceoruber* UAE1 is a more efficient BCA than *S. tendae* on date palm trees to control *F. solani*. The present study is the first report of an actinobacterial isolate producing ACCD and showing multiple antifungal characteristics to obtain *F. solani*-resistant date palm trees. This could potentially implement *S. violaceoruber* UAE1 in IDM programs. In addition to field studies evaluating *S. violaceoruber* UAE1 as a natural enemy to date palms infested with *F. solani*, omics-approaches (AbuQamar et al., 2016) predicting the outcomes of the molecular BCA-pathogen-plant interactions are on the top of our priorities.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

SFA and KE-T designed and conceived the research and supervised the study. KA, GP, ES, HA, SFA, and KE-T performed *in vitro* experiments. KA, GP, ES, SFA, and KE-T performed *in vivo* greenhouse experiments. GP and SFA developed the phylogenetic analysis. KA, GP, SFA, and KE-T analyzed the data. KA, HA, and SJA assisted with experiments and/or data evaluation. SFA and KE-T wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.904166/full#supplementary-material

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Synergism of endophytic *Bacillus subtilis* and *Klebsiella aerogenes* modulates plant growth and bacoside biosynthesis in *Bacopa monnieri*

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Bacopa monnieri is the main source of pharmaceutically important bacosides; however, the low content of these molecules *in planta* remains a limiting factor for fulfilling the industrial requirement. The accumulation of secondary metabolites can be enhanced in plants upon inoculation with endophytes. In this study, we isolated and analyzed the culturable endophytes associated with different plant parts. By analyzing their impact on plant growth parameters (*in vitro* and *in vivo*) and Bacoside A content, we found few candidates which increased bacoside accumulation significantly. Finally, two promising endophytes namely *Bacillus subtilis* (OK070745) and *Klebsiella aerogenes* (OK070774) were co-cultivated with *B. monnieri* cuttings singly and in combination mode to clarify their effect on bacoside biosynthesis and their accumulation in *B. monnieri* shoot. Consortium-inoculated plants significantly enhanced the plant biomass and Bacoside A content with respect to single inoculation. The results of real-time quantitative (RT-PCR) revealed significant accumulation of bacoside biosynthetic pathway transcripts (*HMGCR, PMVK, FDPS, SQS,* and β-*AS)* in the case of plants inoculated with microbial combination, while the single inoculation of *B. subtilis* diverted the plant's machinery toward the synthesis of phenylpropanoid genes like *CCR, CAD, CHS,* and *HST.* In addition, higher expression of *MYB 2* and *WRKY 1* transcription factors in combinational treatment points out their probable role in better physiological and developmental processes. Altogether, this is the first study on *B. monnieri*-endophyte interaction showing improvement in the accumulation of bacoside A by modulating various genes of metabolic pathway and thus suggests an effective "green approach" for augmenting *in planta* production of pharmaceutically important bacosides.

KEYWORDS

***Bacopa monnieri*, bacosides, endophytes, mevalonate pathway, phenylpropanoid pathway, lignin**

Introduction

Bacopa monnieri (L.) Pennell (Family: Scrophulariaceae), an economically important medicinal plant, is used in Ayurvedic System of medicine since 5000 BC for improving memory, attenuating mental deficits, treating epilepsy, and reducing anxiety (Vishnupriya and Padma, 2017). The plant contains an extensive range of secondary metabolites ranging from alkaloids and herpestine to triterpenoid saponins. However, the major pharmacological actions of this plant are primarily due to the presence of bacosides which are therapeutically important dammarane class of triterpenoid saponins. Among the reported 12 analogs of bacosides; Bacoside A which is a mix of bacoside A_3, bacopasaponin C, bacopaside II, and a jujubogenin isomer of bacopasaponin C is the major bioactive molecule reported to have preventive activity against Alzheimer's diseases, chemically induced liver and cerebral toxicity, antidepressant, wound healing, etc. and is also the main reason for the pharmacological importance of this plant (Sukumaran et al., 2019).

The plant has two independent pathways for synthesizing the saponins of interest: mevalonate (MVA) pathway, which operates in cytosol, and the methyl-D-erythritol 4-phosphate (MEP) pathway, which operates in plastid. The product of both the pathways namely 3,3-dimethylallyl diphosphate and isopentenyl diphosphate acts as important mediators for producing the useful saponins in *B. monnieri* (Miziorko, 2011; Gupta et al., 2017a). The production of useful saponins through the abovementioned pathway is a result of action of several enzymes which bring about alterations in the structure of intermediates through oxidation, glycosylation, and substitution. In the MVA pathway, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) intermediate formed is converted to MVA *via* 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) using NADPH as the reducing agent. Later on in the same pathway, 5-phosphomevalonate kinase (*PMVK*) converts mevalonate 5-phosphate to mevalonate 5-diphosphate, the fifth step in the MVA pathway of isoprenoid biosynthesis. Further downstream the pathway, farnesyl diphosphate synthase gene (*FDPS*) initiates the catalysis of geranyl pyrophosphate and farnesyl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Ultimately, squalene synthase (*SQS*) and beta-amyrin synthase (β-*AS)* genes play a key role in synthesizing saponins of interest in the plants (Jeena et al., 2017).

Both plant yield and production of secondary metabolites are under the governance of multiple genes. For crop improvement, genetic manipulation, overexpression of key regulatory genes, and development of transgenic are a preferred choice. However, high production cost, acceptability by the society, and sustainability act as a roadblock for the implementation of such techniques (Kumar et al., 2020; Bate et al., 2021). Furthermore, to fulfill the industrial requirement of bacosides, the wild natural population of *B. monnieri* is being injudiciously used which has brought it in the category of threatened plant list. As an alternative to the above options, the application of microbes is being looked upon as a sustainable approach for enhancing crop and secondary metabolite yield in various medicinal and aromatic plants (MAPs) (Pandey et al., 2016, 2018; Gupta et al., 2019; Mastan et al., 2019; Ray et al., 2019). Most of the studies have focused on the application of plant growth-promoting microorganisms (PGPMs). Our group has also elucidated the role of microbes in modulating the secondary metabolite pathway in different MAPs (Singh et al., 2016, 2019; Gupta et al., 2019). Since last few years, the attention has shifted toward harnessing the benefits of endosymbionts known as endophytes. They are reported to invade the plant system through natural openings like stomata or root hairs which, later on, disperse systemically, thereby colonizing the intercellular or intracellular spaces (Wu et al., 2021). The endophytes help the plants by helping in nutrient acquisition, block the entry of pathogenic microorganisms, provide stress tolerance, and enhance the secondary metabolites of pharmaceutical importance (Omomowo and Babalola, 2019; Wu et al., 2021). In addition, endophytic microbes produce therapeutically important bioactive molecules similar to the host plant like Taxol, azadirachtin, camptothecin, and podophyllotoxin (Gupta et al., 2020; Slama et al., 2021). However, their large-scale production through fermentation has numerous hurdles ranging from instability in gene expression to losing the trait during repeated sub-culturing (Kusari et al., 2014). Therefore, use of endophytes to increase the production of secondary metabolites in plants is being considered as a better approach than independent *in vitro* cultures.

In case of MAPs, the secondary metabolite content holds an important place, and the role of endophytes in the modulation of secondary metabolite biosynthetic in the host plant has been previously established in medicinally important crops (Pandey et al., 2016; Mastan et al., 2019; Ray et al., 2019). Recently, *Echinacea purpurea* interaction with an endophyte was shown to enhance the secondary metabolites of therapeutic properties in this medicinal plant (Maggini et al., 2017). Likewise, tanshinone biosynthesis was enhanced in roots of *Salvia miltiorrhiza* when treated with an endophytic fungus *Chaetomium globosum* D38 and *Mucor circinelloides* DF20 (Zhai et al., 2018; Chen et al., 2021). Apart from single endophyte inoculation, co-inoculation studies involving different groups of microorganisms have also shown promising leads in benefiting the plants (Ray et al., 2019; Singh et al., 2021). In a study, a consortium of *Trichoderma viride* and *Glomus mosesae* showed significant enhancement in photosynthetic parameters and phosphorus content in *Rauvolfia serpentina* (L.) Benth (Kaushish et al., 2012). Recently, an amalgamation of four endophytes comprising of *Bacillus subtilis, Burkholderia* sp., *Bacillus*

licheniformis, and *Acinetobacter pittii* significantly enhanced the yield of artemisinin than the individual microbes (Tripathi et al., 2020).

Considering the availability of scanty information available on endophytes-*B. monnieri* interaction efforts were made to identify, characterize, and study the impact of promising endophytic monocultures, as well as their synergistic combinations on plant productivity, *in planta* Bacoside A content. In addition, genes of bacoside and phenylpropanoid biosynthetic pathways were also targeted to validate the information obtained through biochemical studies.

Materials and methods

Sample collection and isolation of the endophytes

The plants of *Bacopa monnieri* "CIM-Jagriti" variety were collected from the experimental farm of CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. The fresh plants were cleaned with water for removing any adhered epiphytes, soil debris, or dust particles on the plant surface. Different tissues of the plant parts (roots, stems, leaves, and buds) were dissected, and surface sterilization was done using 3% (v/v) sodium hypochlorite for 3 min followed by washing with 70% ethanol for 1 min. Finally, the plant material was washed 3 to 4 times with sterile distilled water, and the surface moisture was removed using sterilized tissue paper. For isolation of bacterial and fungal endophytes, each sterilized plant tissue was homogenized in a sterile mortar and pestle and serially diluted (up to 10^{-6}) with 100 μl of the homogenate. Each dilution prepared was spread on nutrient agar (NA) and potato dextrose agar (PDA), respectively, for isolation of bacterial and fungal cultures. The NA plates were kept at 28 ± 1°C for 48–72 h, while the PDA plates were incubated at 28 ± 1°C for 5–10 days. Pure endophytic cultures were obtained by doing single colony isolation for bacteria on NA plates, while mycelia from fungal cultures were placed on PDA plates. After obtaining pure culture, bacterial isolates were classified based on their on-plate phenotypes which included colony color, shape, size, opaqueness, texture, surface, edge, and height. Likewise, fungal cultures were classified on the basis of color and texture of the fungal mat. In addition, sterilization check of surface-sterilized tissue was done by pipetting out 100 μl aliquot from the final wash buffer and inoculating on NA and PDA plates. Samples were rejected if any microbial growth was observed on check plates, and accordingly, new tissue samples were recollected. All the purified cultures were preserved at −80°C in 50% glycerol stock.

Biochemical characterization of endophytes for plant growth-promoting traits

Each of the bacterial and fungal endophytic isolates was screened for their biochemical and plant growth-promoting activities by using standardized procedures and selective growth medium. The indole acetic acid production by the bacterial and fungal isolates was determined as per the protocol of Gordon and Weber (1951) using 1% L-tryptophan as a substrate. To estimate the phosphate solubilization ability of the endophytes, Pikovskaya's agar medium was used (Pikovskaya, 1948). The inoculated plates were kept for 2–7 days at 28 ± 2°C. The appearance of clear zone around the bacterial and fungal colonies formed due to the solubilization of phosphate confirmed the phosphate solubilization ability of the culture/s.

Nitrate reduction ability of the endophytes was detected as per the protocol of Buxton (2011). Likewise, nitrogen fixation ability of the isolates was confirmed by the luxuriant growth on nitrogen-free medium Jensen's medium (Jensen, 1965). Hydrogen cyanide and ammonia production ability was tested following the standard procedures described by Lorck (1948) and Cappuccino and Sherman (2005). Zinc solubilization was estimated as per the protocol of Vazquez et al. (2000). For rapid screening of cellulase producers, the isolates were cultured on the agar medium containing 0.5% (w/v) carboxymethyl cellulose (CMC) (Ruegger and Tauk-Tornisielo, 2004). The ACC deaminase activity of the bacterial and fungal isolates was screened as per the method of Dworkin and Foster (1958) using DF (Dworkin and Foster) minimal salts medium, while siderophore production was studied by the method described by Schwyn and Neilands (1987).

Preparation of bacterial and fungal inocula for greenhouse experiment

Preparation for bacterial inoculum was done by culturing the bacterial isolates in 100 ml Nutrient broth (NB) at 28°C for 24 h under shaking condition at 120 rpm, while in case of fungal endophytes, the cultures were grown in PDA plates for 5–8 days and kept in BOD incubator at 28°C. The bacterial cultures were harvested by centrifugation at 7000 rpm carried out at 4°C for 10 min. The obtained culture pellet was washed with sterile distilled water and then dissolved in 0.85% saline. The concentration of the suspension was measured to A_{600} = 1.0 using spectrophotometer. Prior to the application of the inoculum to the plants, 1 × 10^8 colony-forming unit (CFU) per ml was maintained for bacterial suspension. For fungal spore suspension preparation, the selected fungi were allowed to grow for 5–8 days till they were fully sporulating. The spores were scraped with the help of spore scraper and suspended in distilled water. Before inoculating

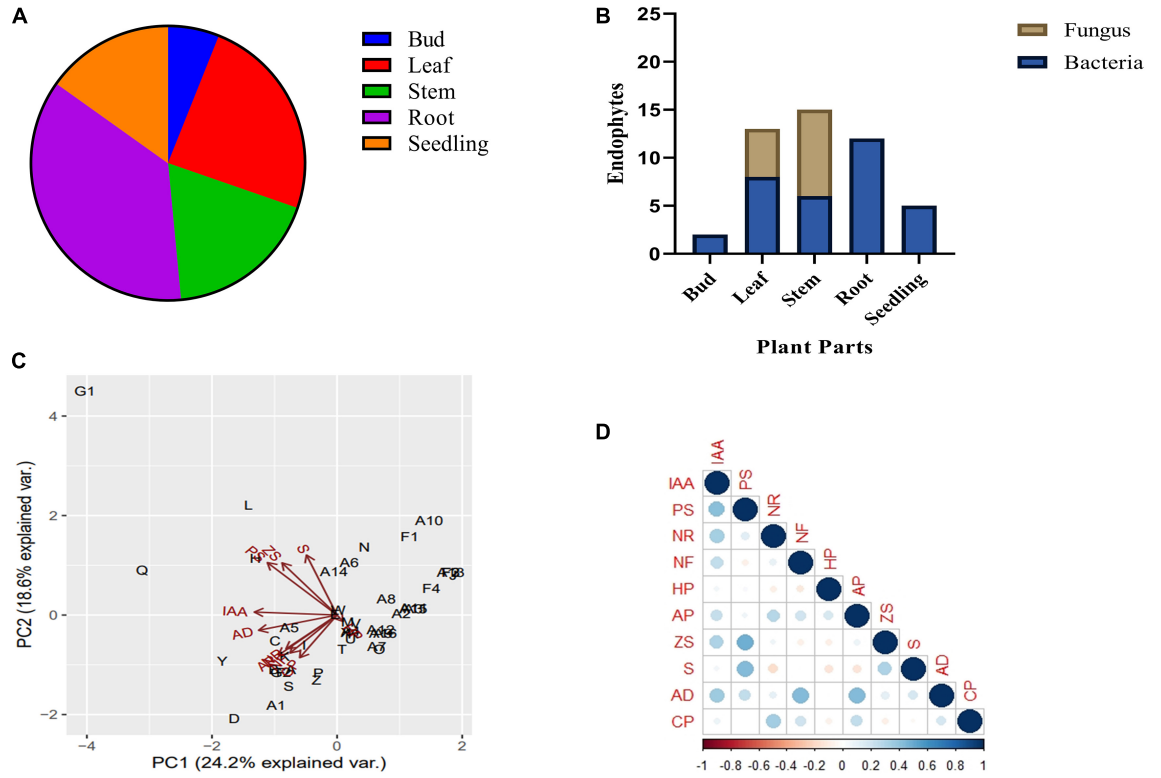

FIGURE 1
Endophytic bacterial and fungal diversity associated with different plant parts of *Bacopa monnieri* **(A)**; percentage distribution of bacterial and fungal endophytes associated with different plant parts **(B)**. Bi-plot ordination diagram of principal component analysis describing plant growth-promoting traits and functional activities of endophytes isolated from *B. monnieri*. PS, phosphate solubilization; NR, nitrate reduction; NF, nitrogen fixation; HP, hydrogen cyanide; AP, ammonia production; ZS, zinc solubilization; CP, cellulase production; AD, ACC deaminase activity; and S, siderophore production **(C)**. Pearson's correlation matrix showing significant positive and negative correlations between different plant growth parameters. Blue circles represent positive correlation, red circles denote negative correlation, and the color intensity is proportional to the correlation coefficients **(D)**.

in the plants, the concentration of spores was maintained to 1 × 10^8 spores/ml. The greenhouse pot trial was carried out in the glasshouse. Earthen pots (4cm × 30cm × 25cm) were filled with 2:1 v/v mixture of autoclaved soil and vermicompost and watered with distilled water from time to time. The soil used in the study was sandy loamy with pH 7.2, 0.41 EC dS m^{-1}, organic carbon 4.40 g kg^{-1}, 120.6 kg ha^{-1} N, 11.2 kg ha^{-1} available P, and 96.8 kg ha^{-1} available K. Before filling the above potting mixture in pots, it was previously sterilized by autoclaving at 15 lbs for 20 min at 121°C.

For microbial inoculation, the surface-sterilized terminal cuttings of *B. monnieri* (L.) Pennell cv. "CIM-Jagriti" variety were collected from the National Gene Bank for Medicinal Aromatic Plants located at CSIR-CIMAP, Lucknow, India. The cuttings were dipped in individual endophyte bacterial/fungal spore suspension in the abovementioned concentrations and kept in the same state for 2 h before transferring them in pots. The cuttings dipped in only sterile 0.85% saline were taken as control. Pots were maintained at 28°C ± 2°C in a glasshouse with natural photoperiod and light intensity, and watering with distilled water was done as per the requirement. For ensuring the presence of sufficient number of endophytes in the soil, the inoculation with the previously mentioned CFU was carried out again at 20th day after the first inoculation. The experiment was set up in randomized block design (triplicates for each treatment) and to minimize experimental errors plant sampling for all analyses was done at the same stage and same position of the leaves.

Estimation of photosynthetic pigments and plant growth parameters

Chlorophyll a, chlorophyll b, and total chlorophyll content of the fully expanded leaves was measured by crushing 500 mg leaf tissue in 10 ml of 80% acetone at 4°C and quantified as per the protocol of Arnon (1949). The absorbance of the supernatant was recorded at 480, 663, and 645 nm against

the solvent (acetone) blank. The content of chlorophyll a and chlorophyll b was calculated by the given formulas:

$$\textbf{Chlorophyll a } (\mu g/ml)\ [Chll_A] = (12.7 \times A_{663}) - (2.59 \times A_{645})$$

$$\textbf{Chlorophyll b } (\mu g/ml)\ [Chll_B] = (22.9 \times A_{645}) - (4.7 \times A_{663})$$

$$\textbf{Total chlorophyll } (\mu g/ml) = 20.2(A645) + 8.02(A663)$$

The sampling was done 90 days after transplanting, and the plants were harvested for recording fresh weight (FW) followed by shade drying of the same material for 3–4 days before recording the dry weight (DW).

Evaluation of the impact of endophytes on Bacoside A content

Hundred milligrams of shade-dried leaf samples was pulverized for preparing the methanolic extracts. The extraction was repeated three times, and the pooled extracts were vacuum-concentrated before determining the Bacoside A concentration using high-performance liquid chromatography (HPLC) system (Shimadzu Prominence-i LC 2030C 3D Plus) (Gupta et al., 1998). Bacoside A (1 mg/ml) was used as reference standard, and the identification of Bacoside A content of *B. monnieri* plants was determined by comparing the retention times with reference standard at 205 nm.

Compatibility testing and development of microbial combination from two promising endophytes

The compatibility among two endophytic cultures (Y and A5) enhancing the bacoside content was determined *in vitro* by the cross-streak method. The bacterial inocula were prepared by culturing the bacterial isolates in 100 ml NB at 28°C for 24 h under shaking condition at 120 rpm. The final concentration of each bacterium was adjusted to 10^8 CFU/ml. About 100 μl of a single cell suspension was spread on NA plates and was left for drying for 30 min at room temperature. Other bacterial culture was streaked on previously dried NA plate in such a way that both the cultures crossed each other at a single place on the plate. The plates were then incubated at 28°C for 24 to 48 h for checking the compatibility. No zone formation at the juncture was considered as positive compatibility. For consortium development, bacterial isolates were individually grown in NB at 28°C for 24 h under shaking conditions at 120 rpm. The bacterial cultures were harvested at 7000 rpm for 10 min at 4°C by centrifugation. The obtained pellet was washed with sterile distilled water and then dissolved in 0.85% saline. The concentration of the suspension was measured to A_{600} = 1.0 using a spectrophotometer. In case of consortium, an equal amount of each bacterial suspension was added and mixed well before inoculating in plants as described previously.

Morphological and molecular characterization of the two potential endophytes

Morphological features of the two endophytes (Y and A5) were determined using light microscopy (Leica, Germany) and scanning electron microscopy (SEM). For SEM analysis, 1 ml of each bacterial endophyte grown in NB broth was fixed in 7% formaldehyde and kept overnight at 4°C. To the above samples, 9 ml of phosphate buffer saline (PBS; pH 7.0) was added in each vial followed by filtration through 0.2-μm Millipore filter and washing with PBS. Later on, dehydration of bacterial cells till critical point with 25, 50, 70, and 100% ethanol solutions was done three times for 10 min each. The bacterial endophytic cells were mounted on SEM stubs, sputter-coated with gold, and viewed on a FEI Quanta 250 SEM, Netherland (Nowell and Parules, 1980).

The bacterial genomic DNA of both the endophytic cultures was isolated by CTAB procedure as per the protocol of Sambrook (1989). The quantity and quality of extracted DNA were also checked spectrophotometrically using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Polymerase Chain Reaction (PCR) reaction mixture of 25 μl consisted of 1.25 U Taq polymerase, 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 2.5 mM $MgCl_2$, 10 pmol of each primer, 2.5 μl of 10 × reaction buffer, and 1 μg of template DNA and sterile Milli-Q water. 16S rRNA gene amplification was done by setting the thermocycling conditions as denaturation for 5 min at 94°C, followed by 30 cycles at 94°C for 45 sec, 57.4°C for 45 sec, 72°C for 2 min, and a final extension step at 72°C for 5 min. The PCR product was visualized on 1% (w/v) agarose gel amended with 10 μg ml^{-1} ethidium bromide which was also purified through Nucleo-pore PCR Clean-up Gel Extraction kit (Genetix Biotech Asia Pvt. Ltd., India) as per manufacturer's instructions for carrying out Sanger's sequencing. Finally, the identity of the two selected endophytes was done by doing Sanger sequencing using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, United States) on a Genetic Analyzer 3130 9 L (Applied Biosystems, United States). The 16S rRNA partial sequence data were deposited in the GenBank public sequence repository at the National Center for Biotechnology Information (NCBI) for getting the accession numbers. Phylogenetic tree construction was done using the bootstrap neighbor-joining tree approach using Clustal W alignment program.

TABLE 1 Plant growth-promoting properties of the endophytic isolates of *Bacopa monnieri*.

S. No.	Isolate		IAA	Phosphate solubilization	Nitrate Reduction	Nitrogen Fixation	HCN Production	Ammonia Production	Zn Solubilization	Sidero phore	ACC Deaminase	Cellulase Production
1	Control		–	–	–	–	–	–	–	–	–	–
2	A	Bacteria	+	–	+	+ +	–	+	–	–	+	+
3	B	Bacteria	+	–	+++	++	–	++	–	–	+	–
4	C	Bacteria	+ +	–	+ + +	–	+	++	–	–	+	–
5	D	Bacteria	+	–	+++	+++	–	++	–	–	+	+
6	E	Bacteria	+	–	–	–	–	+ +	–	–	+	–
7	F	Bacteria	+	–	+++	–	–	++	–	–	+	+
8	G1	Bacteria	+ +	+	+ +	–	–	+ +	+	+	+	–
9	G2	Bacteria	+	–	+++	–	–	++	–	–	+	+
10	H	Fungus	+	+	+ + +	–	–	+	–	–	+	–
11	I	Bacteria	+	–	–	++	–	++	–	–	+	–
12	J	Fungus	–	–	–	–	+	–	–	–	–	–
13	K	Fungus	+	–	+ + +	–	–	+ ++	–	–	+	–
14	L	Bacteria	+	+	–	–	++	++	–	+	+	–
15	M	Bacteria	+	–	–	+	+++	+	–	–	+	–
16	N	Bacteria	–	–	–	–	–	+	–	+	+	–
17	O	Bacteria	–	–	+ ++	–	+	+	–	–	–	+
18	P	Bacteria	+	–	++	–	+	+++	–	–	–	+
19	Q	Bacteria	+ +	+	+ + +	+	–	+ +	–	+	+	–
20	R	Bacteria	–	–	–	+	+	+ +	–	–	+	–
21	S	Bacteria	–	–	+++	+++	–	++	–	–	+	–
22	T	Bacteria	–	–	–	–	–	+ +	–	–	+	+
23	U	Bacteria	–	–	–	–	++	+++	–	–	+	–
24	V	Fungus	–	–	+ ++	–	–	+	–	–	+	–
25	W	Fungus	++	–	+++	–	–	+	–	–	–	–
26	X	Fungus	–	–	–	–	–	+ +	–	–	+	–
27	Y	Bacteria	+++	–	++	++	+	++	–	–	+	–
28	Z	Bacteria	–	–	+ ++	–	+ +	+ +	–	–	+	+
29	A1	Bacteria	–	–	+++	+	–	+++	–	–	+	+
30	A2	Bacteria	–	–	+	–	–	+ +	–	–	–	–
31	A3	Bacteria	–	–	–	–	+	++	–	–	–	–
32	A4	Bacteria	–	–	–	+	–	+ +	–	–	+	–
33	A5	Bacteria	–	–	–	++	–	++	–	+	+	+
34	A6	Bacteria	–	–	–	+	–	+	–	+	+	–

(Continued)

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TABLE 1 (Continued)

S. No.	Isolate		IAA	Phosphate solubilization	Nitrate Reduction	Nitrogen Fixation	HCN Production	Ammonia Production	Zn Solubilization	Sidero_phore	ACC Deaminase	Cellulase Production
35	A7	Bacteria	–	–	+++	–	–	+	–	–	–	+
36	A8	Bacteria	–	–	–	–	–	+	–	–	+	–
37	A9	Bacteria	–	–	+++	–	+	++	–	–	–	–
38	A10	Fungus	–	–	–	–	–	–	–	+	–	–
39	A11	Bacteria	–	–	++	–	+	+	–	–	–	–
40	A12	Fungus	–	–	+++	–	–	++	–	–	–	–
41	A13	Fungus	–	–	–	–	–	–	–	–	–	–
42	A14	Bacteria	–	–	+++	–	–	+	–	+	+	–
43	A15	Fungus	–	–	–	–	+	++	–	–	–	–
44	A16	Bacteria	–	–	+++	–	+	++	–	–	–	–
45	F1	Fungus	–	–	–	–	–	+	–	+	–	–
46	F2	Fungus	–	–	–	–	–	–	–	–	–	–
47	F3	Fungus	–	–	–	–	–	–	–	–	–	–
48	F4	Fungus	–	–	–	–	–	+	–	–	–	–

+++ = Best performance; ++ = Average performance; + = Least performance, – = Absent.

Effect of single and co-inoculation on the foliar nutrient uptake

Total nitrogen (N) content in the aerial part was quantified 60 days post-microbial treatment through Kjeldahl method by digesting the tissue with H_2SO_4 + H_2O_2. The content of potassium (K) and phosphorus (P) in the air-dried ground shoot samples was assessed after wet digestion with HNO_3 + H_2O_2 following the protocol of Singh et al. (2009).

Effect of single and co-inoculation on phytochemicals and antioxidant profile

Leaves from each treatment were randomly plucked 60 days post-microbial treatment and were immediately frozen using liquid nitrogen. Catalase activity in the leaves of *B. monnieri* was estimated as per the protocol of Aebi (1984). Superoxide dismutase (SOD) enzyme activity was calculated by observing the changes in absorbance at 560 nm (Nishikimi et al., 1972).

Total phenolic content (TPC) was estimated using the Folin–Ciocalteu method based on the reduction of phosphotungstate–phosphomolybdate complex by phenolics to a blue reaction product (Zheng and Shetty, 2000). Free radical-scavenging activity was estimated using the method of Brand-Williams et al. (1995). The scavenging potential of the extract was estimated according to the following equation:

$$\text{Scavenging potential (\%)} = [(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})/\text{absorbance}_{\text{control}}]^{*}100$$

Effect of single and co-inoculation on the lignin deposition

Transverse sections of stem were cut, mounted in glycerol, and visualized under a fluorescent microscope EVOS FL Cell imaging system (Thermo Fisher). Lignin-deposited cells autofluoresced as blue-colored cells under excitation by UV range light (Singh et al., 2016).

Quantitative real time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from the leaves of inoculated and control plants using TRIzol reagent (Invitrogen). RNAse free DNase1 (Takara) was used to remove DNA impurity, and the concentration of RNA was determined using Nanodrop 1000 spectrophotometer (Thermo Fisher

TABLE 2 Effect of selected endophytes on chlorophyll a and chlorophyll b content in *Bacopa monnieri* plants with respect to control set plants. Results represent mean ± standard error of three replicates.

S.No.	Treatments	Chlorophyll a (μg/ml)	Chlorophyll b (μg/ml)	Total chlorophyll (μg/ml)
1	Control	1.08 ± 0.13	2.12 ± 0.10	3.59 ± 0.77
2	C	3.26 ± 0.39	5.18 ± 0.39	9.48 ± 0.81
3	D	2.93 ± 0.27	4.14 ± 0.15	7.94 ± 0.90
4	F	2.08 ± 0.13	4.07 ± 0.28	6.89 ± 0.66
5	H	4.74 ± 0.98	4.43 ± 0.17	10.31 ± 0.86
6	G1	4.05 ± 0.28	7.01 ± 0.29	12.42 ± 0.82
7	L	2.93 ± 0.94	4.14 ± 0.37	7.94 ± 0.88
8	M	4.67 ± 0.79	6.2 ± 0.12	12.21 ± 1.13
9	N	3.31 ± 0.23	5.74 ± 0.57	10.16 ± 0.54
10	Q	4.18 ± 0.29	4.64 ± 0.84	9.91 ± 0.77
11	S	3.74 ± 0.72	5.78 ± 0.40	10.68 ± 1.28
12	W	1.21 ± 0.12	3.04 ± 0.38	4.76 ± 0.69
13	Y	5.20 ± 0.27	7.36 ± 1.01	14.11 ± 0.81
14	A5	2.63 ± 0.13	2.89 ± 0.13	6.21 ± 0.49
15	A6	3.47 ± 0.44	5.29 ± 0.13	9.83 ± 0.60
16	A7	4.39 ± 0.14	6.88 ± 0.73	12.66 ± 0.33
17	A14	2.22 ± 0.12	2.66 ± 0.22	5.48 ± 0.99
18	A16	5.29 ± 0.98	6.64 ± 0.34	12.54 ± 0.93

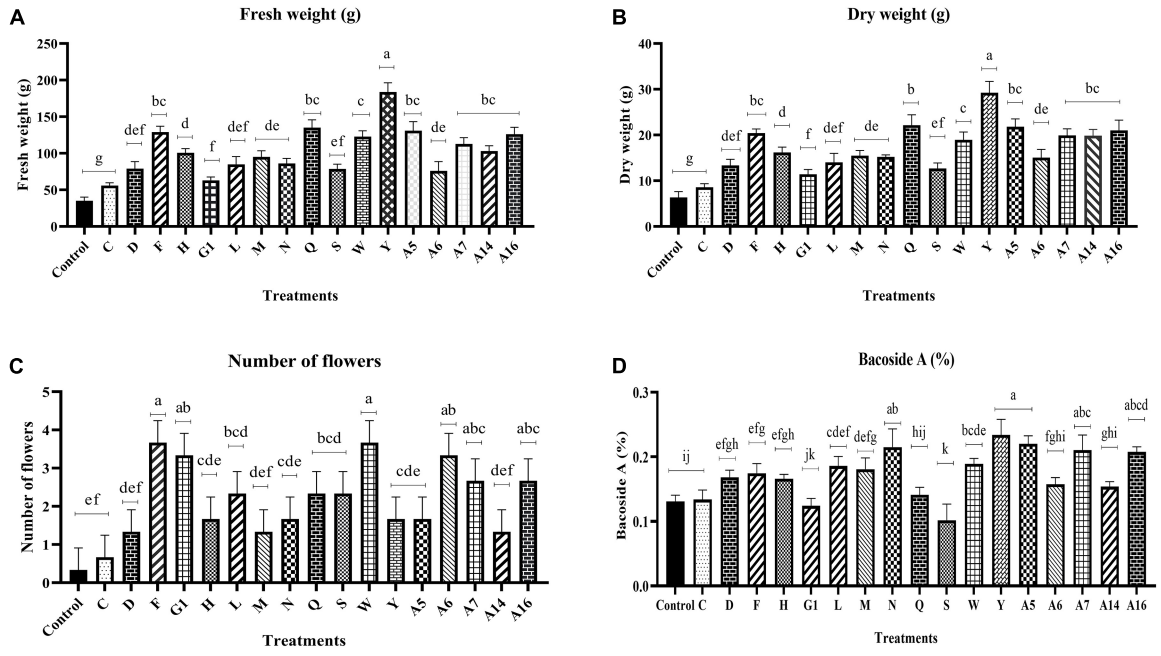

FIGURE 2
Effect of endophyte inoculation on fresh weight **(A)**, dry weight **(B)**, number of flowers **(C)**, and Bacoside A content **(D)** in 90 days old *Bacopa monnieri* plants. Non-inoculated plants were used as control. The results are expressed as means of three independent biological replicates. Different Duncan's letters denote significant difference at $P < 0.05$.

Scientific) followed by synthesis of cDNA using RevertAid First-Strand cDNA Synthesis Kit (Puregene) as per manufacturer's protocol. Transcripts of 14 genes involved in bacoside and phenylpropanoid biosynthetic pathways were quantified with gene-specific primers (**Supplementary Table 1**) picked from a transcriptomic study based on *B. monnieri* (Jeena et al., 2017). qRT-PCR was performed in triplicates of each biological sample. PCR mixture included 5 μl Power SYBR Premix Ex Mix

(Takara), 0.2 μl ROX Reference Dye, 1 μl of 10 times diluted cDNA template, and 300 nM primers (forward and reverse) in a total reaction volume of 10 μl. qRT-PCR conditions were set as initial denaturation at 95°C for 10 min, 40 cycles of denaturation for 15 s at 95°C, and final extension step at 60°C for 1 min each. Fluorescent signals were recorded and analyzed on an Applied Biosystems Step One PlusTM Real-Time PCR System. The specificity of RT-qPCR was evaluated by using melting curve analysis (Applied Biosystems) of all amplicons. The actin gene was used as reference transcript, and the Ct value of control plants was used as calibrator. The analysis of mean expression of selected/target genes and relative quantification was done by $2^{-\Delta\Delta Ct}$method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the data was done using ANOVA applicable to completely randomized block design (RBD using SPSS version 16). Significant differences among different treatments were carried out using Duncan's multiple range tests (DMRTs) at a significance level of $P \leq 0.05$. Correlations matrix and principal component analysis (PCA) were made using the R software (R v.4.0.2) with corrplot and factoextra packages.

Results

Isolation and biochemical characterization of endophytes for plant growth-promoting microorganisms traits

A total of 47 endophytes (34 bacteria and 13 fungi) were isolated from seedlings, buds, leaves, stems, and roots of *B. monnieri* plant (**Supplementary Table 2**). Out of the above, 10.64% endophytes were obtained from small seedlings, 4.25% from buds, 27.66% endophytes from leaves, 31.92% endophytes from stem, and 25.53% endophytes from the roots (**Figure 1A**). While talking about distribution pattern of endophytes, maximum number of endophytes were observed in stem followed by the leaf tissues (**Figure 1B**). All the bacterial and fungal endophytes were distinguished on the basis of different morphological characteristics of their colony (**Supplementary Tables 3, 4**). The isolated bacterial and fungal endophytes exhibited various biochemical and plant growth-promoting properties, and the compiled results of the study are presented in **Table 1**. Among all the endophytes, 17 isolates were found to be positive for IAA-producing trait while four isolates were found to solubilize phosphate in Pikovskaya's agar with highest solubilization index being recorded for isolate G1 (**Supplementary Table 5**). Nitrate reduction trait was found in thirty-one isolates which was indicated by the formation of red violet color, while thirteen isolates were found to fix atmospheric nitrogen when grown on nitrogen-free Jensen's medium (**Table 1**). In case of volatiles being produced by the endophytes, 15 isolates were found to be efficient HCN producers while forty-two were found to be positive ammonia producers (**Table 1**). Likewise, only one endophyte G1 was found to be zinc solubilizer with zinc solubilization index (ZSI) of 1.66 and solubilization efficiency (ZSE) of 266.67 (**Supplementary Table 6**). Siderophore production, ACC deaminase activity, and cellulase activity were found to be positive in ten, twenty-eight, and eleven isolates, respectively (**Table 1**). Furthermore, the ordination diagram using the above-screened plant growth-promoting traits revealed three major groups occupying two quadrants (**Figure 1C**). The first quadrant comprised of phosphate solubilizers, zinc solubilizers, indole acetic acid, and siderophore producers, while the second quadrant had ACC deaminase producers, nitrate reductase producers, nitrogen fixers, ammonia, and cellulase producers. Using correlation study, a positive correlation was observed between IAA production-phosphate solubilization traits and nitrate reductase producers-nitrogen fixers. In addition, a strong correlation was also found between phosphate solubilization with siderophore and zinc solubilization trait (**Figure 1D**).

Impact of selected endophytes on the photosynthetic pigments

Out of the 47 endophytes isolated from the different parts of *B. monnieri*, seventeen isolates which showed promising *in vitro* plant growth-promoting traits were selected for carrying out the plant tests. Chlorophyll estimation was done in fresh green leaf samples, and the results varied significantly as highest increment in chlorophyll a was observed in plants treated with endophyte A16 followed by Y culture. Likewise, chlorophyll b was maximally enhanced in plants treated with endophyte Y followed by A7 culture (**Table 2**).

Impact of selected endophytes on biomass, number of flowers, and Bacoside A content

The impact of endophyte inoculation on the *B. monnieri* plants was clearly visible in terms of enhancement of plant fresh biomass as significant increase ranging from 59.27 to 422.66% was observed in endophyte inoculated set in comparison with the control plants (**Figure 2A** and **Supplementary Figure 1**). The results followed a similar trend in case of dry weight as maximum significant increment was observed in plants inoculated with culture Y with least increase in plants treated with endophytic culture C (**Figure 2B**). The application of endophytes also had a significant impact on flowering status of

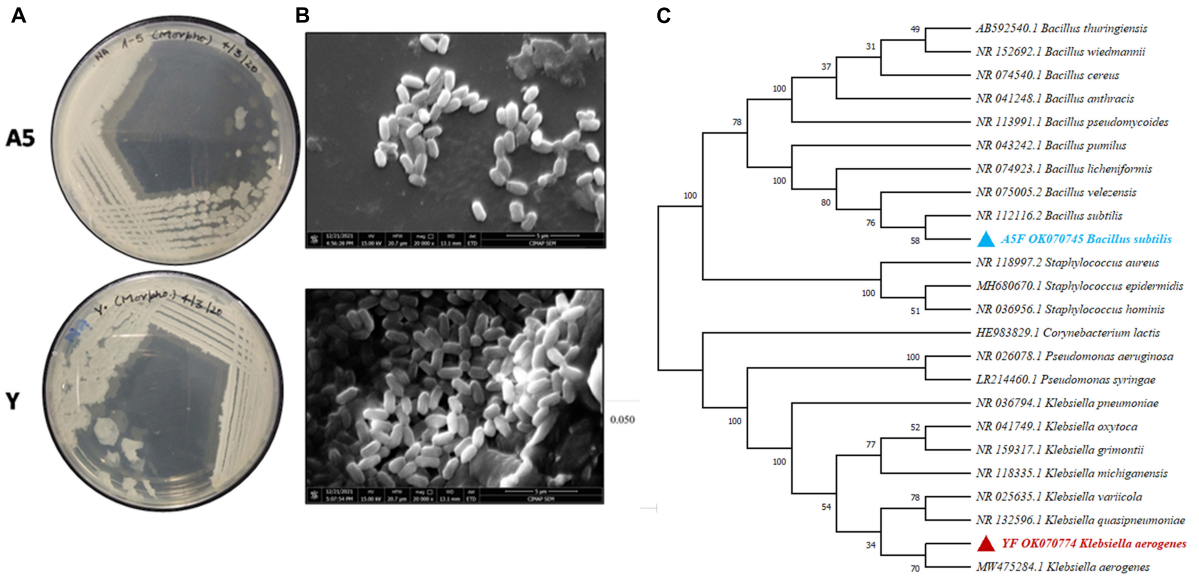

FIGURE 3
Morphological **(A)** and scanning electron micrographs showing the size and shape of bacterial endophytes, *Bacillus subtilis* (A5) and *K. aerogenes* (Y). The image of clusters of bacterial colonies was focused at 5-μm scale **(B)**. The 16S rRNA gene sequences of bacteria A5 and Y, as well as related bacteria in the genera *Bacillus* and *Klebsiella*, were used to create a maximum-likelihood phylogenetic tree with bootstrap values expressed as a percentage of 1000 replicates. *Corynebacterium lactis* strain HE983829.1 was used as an outgroup for making the phylogenetic tree **(C)**.

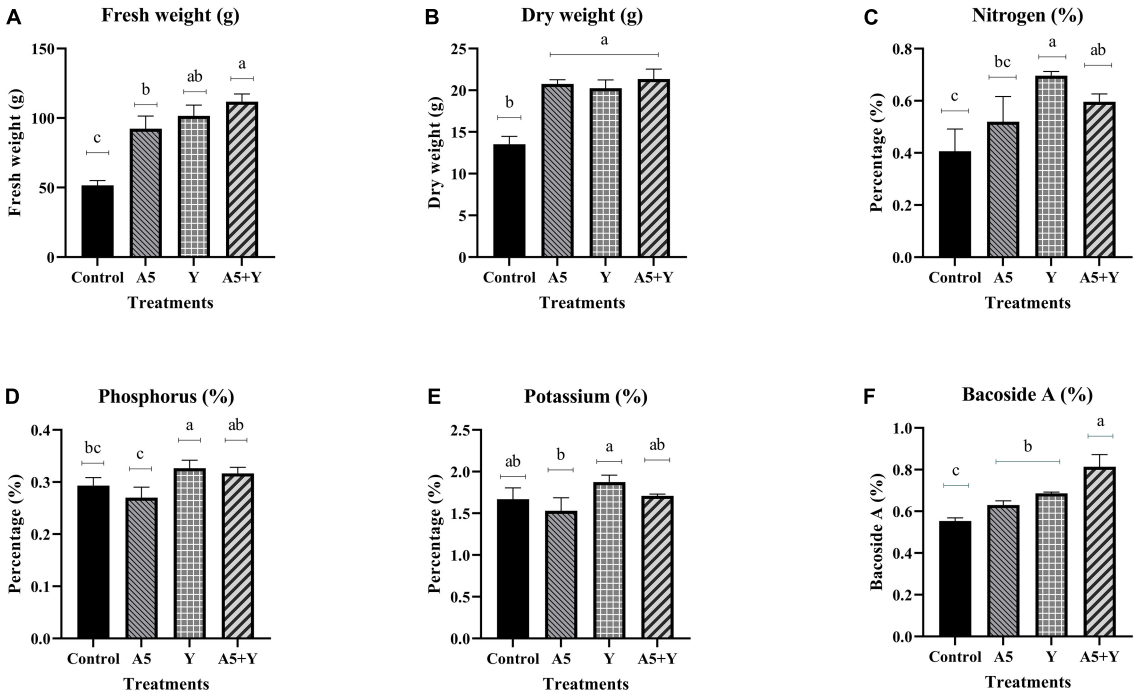

FIGURE 4
Influence of endophyte inoculation on fresh weight **(A)**, dry weight **(B)**, nitrogen content **(C)**, phosphorus content **(D)**, potassium content **(E)**, and Bacoside A **(F)** in 60 days old *Bacopa monnieri* plants inoculated with endophytes A5 and Y individually and in the form of consortium (A5 + Y). Non-inoculated plants were used as control. The results are expressed as means of three independent biological replicates. Different Duncan's letters denote significant difference at $P < 0.05$.

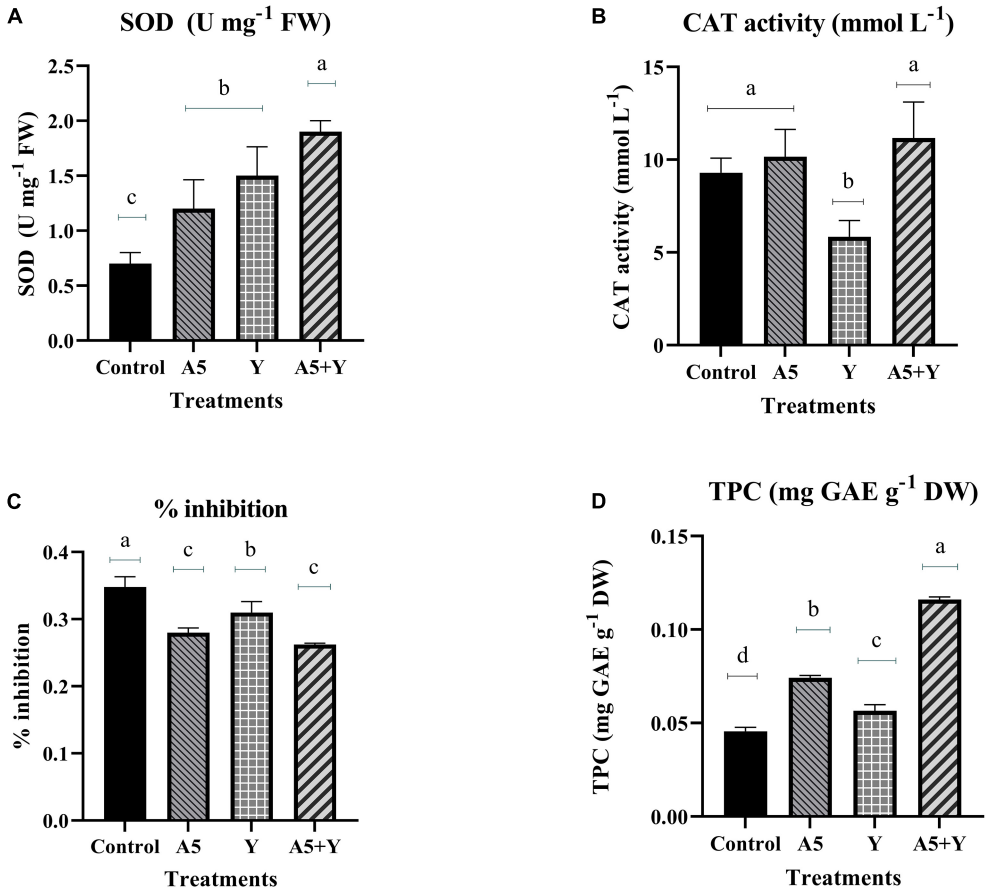

FIGURE 5
Influence of endophyte inoculation on superoxide dismutase enzyme **(A)**, catalase enzyme activity **(B)**, free radical-scavenging activity **(C)**, and total phenolic content **(D)** in 60 days old *Bacopa monnieri* plants inoculated with endophytes A5 and Y individually and in the form of consortium (A5 + Y). Non-inoculated plants were used as control. The results are expressed as means of three independent biological replicates. Different Duncan's letters denote significant difference at $P < 0.05$.

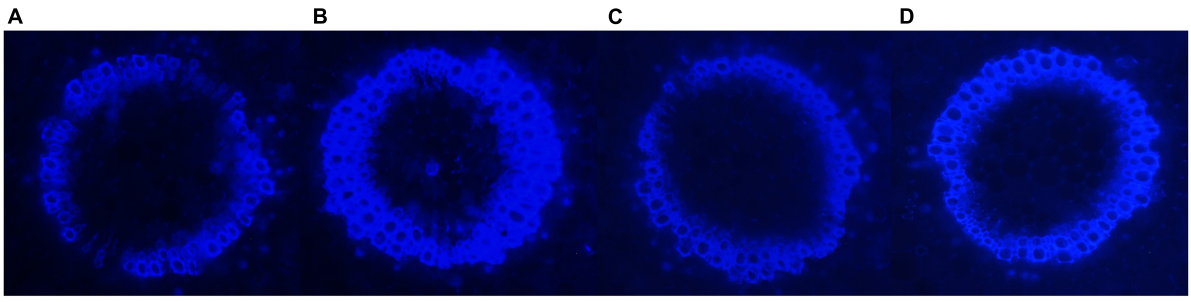

FIGURE 6
Influence of endophyte inoculation on lignification pattern in the stem of control **(A)**; *Bacillus subtilis* (A5) inoculated plants **(B)**; *Klebsiella aerogenes* (Y) inoculated plants **(C)**; and consortium (A5 + Y) inoculated **(D)** *B. monnieri* plants.

the plants as endophytic cultures F, G1, W, A6, A7, and A16 significantly increased the number of flowers with respect to the control plants (Figure 2C). Likewise, promising effect of the endophytes was also observed on the Bacoside A content as maximum significant increase by 78.70% was observed in culture Y followed by A5 (Figure 2D). In addition to the two mentioned cultures, N, A7, and A16 too increased the bacoside significantly. However, looking into the cumulative

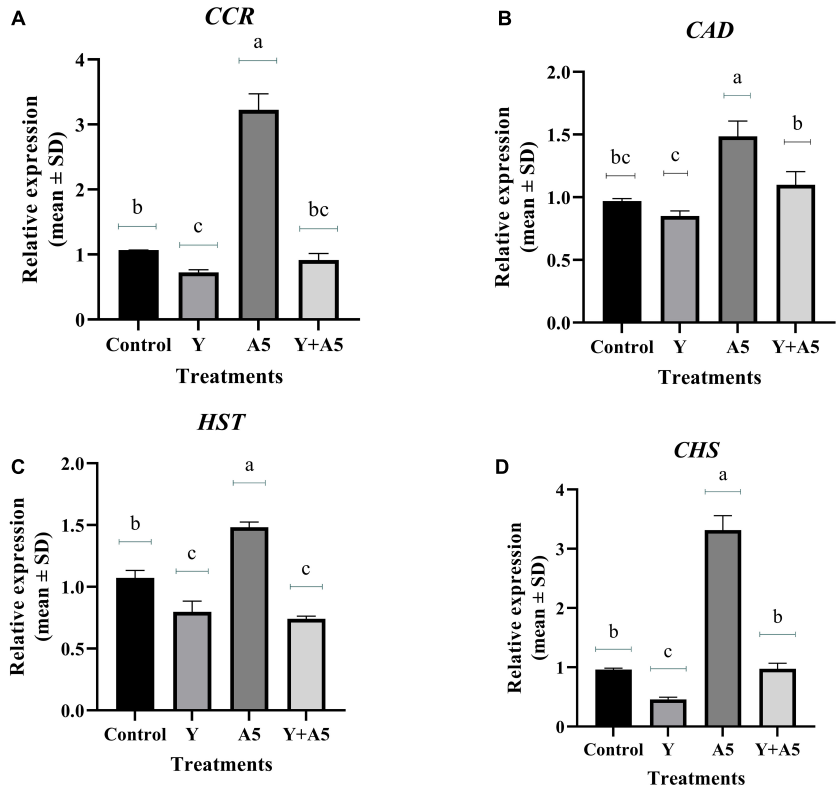

FIGURE 7
Influence of endophyte inoculation on the expression of genes involved in phenylpropanoid metabolism. Total RNA was isolated from the leaves of 60 days old *B. monnieri* plants inoculated with endophytes A5 and Y individually and in the form of consortium (A5 + Y), reverse-transcribed and used for expression analysis by SYBR Green-based RT-qPCR leaves on untreated plants were considered as control. Expression of *CCR* **(A)**, *CAD* **(B)**, *HST* **(C)**, and *CHS* **(D)** was analyzed. Actin was used as a reference transcript. Data are means ± SE (n = 3 biological replicates), and Y-axis represents relative expression which was calculated using $2^{-\Delta\ \Delta\ Ct}$ method. Duncan's letter denotes significant difference at $P < 0.05$.

effect of endophytes on plant growth parameters and Bacoside A content, A5 and Y cultures were taken up for further studies. In addition, to our surprise, one endophytic culture had a negative effect on Bacoside A content as it reduced its content with respect to the control set plants.

Compatibility testing, and morphological and molecular characterization of two potential isolates

Based on the above results, cultures A5 and Y were found to be potential candidates for the development of synergistic combination. The cross-streak method results revealed that both the endophytes grew well together and did not hamper each other's growth.

The morphological appearance recorded through light microscopy and SEM analysis revealed rod-shaped structure of both the bacterial endophytes (**Figures 3A,B**). The 16S rRNA gene sequence NCBI-BLAST result confirmed that bacterial endophyte A5 showed maximum similarity with *Bacillus subtilis* (NCBI accession number OK070745). Likewise, the 16sRNA gene sequence of endophytic culture Y showed maximum homology with *Klebsiella aerogenes* (NCBI Accession Number OK070774). Phylogenetic relationship of A5 and Y was analyzed using related sequences obtained from NCBI (National Centre for Biotechnology Information) GenBank, and the relationship has been depicted in **Figure 3C**.

Effect of selected endophytes singly and in combination on foliar nutrient uptake, plant biomass, and Bacoside A content

The application of endophytic consortium significantly enhanced the fresh weight, dry weight, N, and Bacoside A content in *B. monnieri* plants. Inoculation with a consortium of *B. subtilis* and *K. aerogenes* increased the fresh weight and dry weight of *B. monnierri* plant by 117.08% and 57.63%, respectively, when compared to the control plants

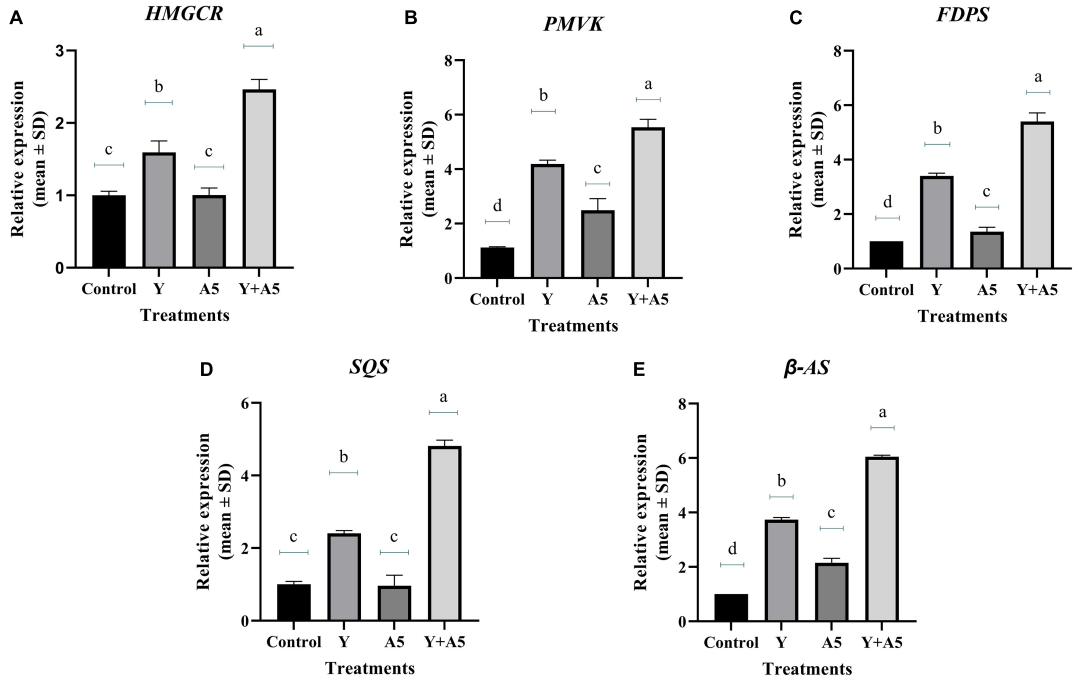

FIGURE 8
Influence of endophyte inoculation on the expression of genes involved in bacoside biosynthesis. Total RNA was isolated from the leaves of 60 days old *B. monnieri* plants inoculated with endophytes A5 and Y individually and in the form of consortium (A5 + Y), reverse-transcribed and used for expression analysis by SYBR Green-based RT-qPCR leaves on untreated plants were considered as control. Expression of *HMGCR* **(A)**, *PMVK* **(B)**, *FDPS* **(C)**, *SQS* **(D)**, and β- *AS* **(E)** was analyzed. Actin was used as a reference transcript. Data are means ± SE (n = 3 biological replicates), and Y-axis represents relative expression which was calculated using $2^{-\Delta\Delta Ct}$ method. Different Duncan's letters denote significant difference at $P < 0.05$.

(Figures 4A,B). Significant increase by 46.63% was observed in case of N content in microbial combination treatment; however, insignificant effect of combination of endophytes was observed on K content (Figures 4C–E). Likewise, significant increment in Bacoside A content by 46.99% was observed in microbial consortium treated plants than that of control set plants (Figure 4F).

Effect of selected endophytes singly and in combination on antioxidant status and lignification pattern

To study the impact of selected individual endophytes and their combination on antioxidant status of *B. monnieri* plants, the activity of SOD and CAT enzyme was assessed (Figure 5). To our observation, there was a significant increase in SOD enzyme activity in all the microbial treatments; however, maximum increment by 171.42% was observed in endophytic consortium treatment (Figure 5A). In case of catalase (CAT) enzyme, insignificant effect was observed in all the microbial treatments with respect to the control plants (Figure 5B). The free radical-scavenging activity and TPC content in *B. monnieri* plants were significantly influenced by both monocultures and microbial combination. The highest significant% inhibition in free radicals was observed in microbial consortium treatment followed by A5 culture (Figure 5C). Likewise, TPC was also significantly enhanced in microbial consortium treatment (92.20%) with respect to control set plants. Among the single individual inoculations, *B. subtilis* performance was better than *K. aerogenes* and control plants (Figure 5D).

To envisage the effect of selected endophytes individually and their combination on the lignin deposition, stem sections of *B. monnieri* plants were visualized under fluorescent microscope (Figure 6). The maximum and uniform lignification pattern visible as a blue ring was observed in A5 followed by combination and Y treatment alone. However, stem sections of control plants had thinner and interrupted lignified cells which pointed out toward the role of microbes in strengthening the defense barrier of the plant system (Figure 6A).

Quantitative real-time-PCR analysis of transcription factors, bacoside, and phenylpropanoid pathway genes

To understand the mode of action of endophytes, the expression of several key genes of biosynthetic and

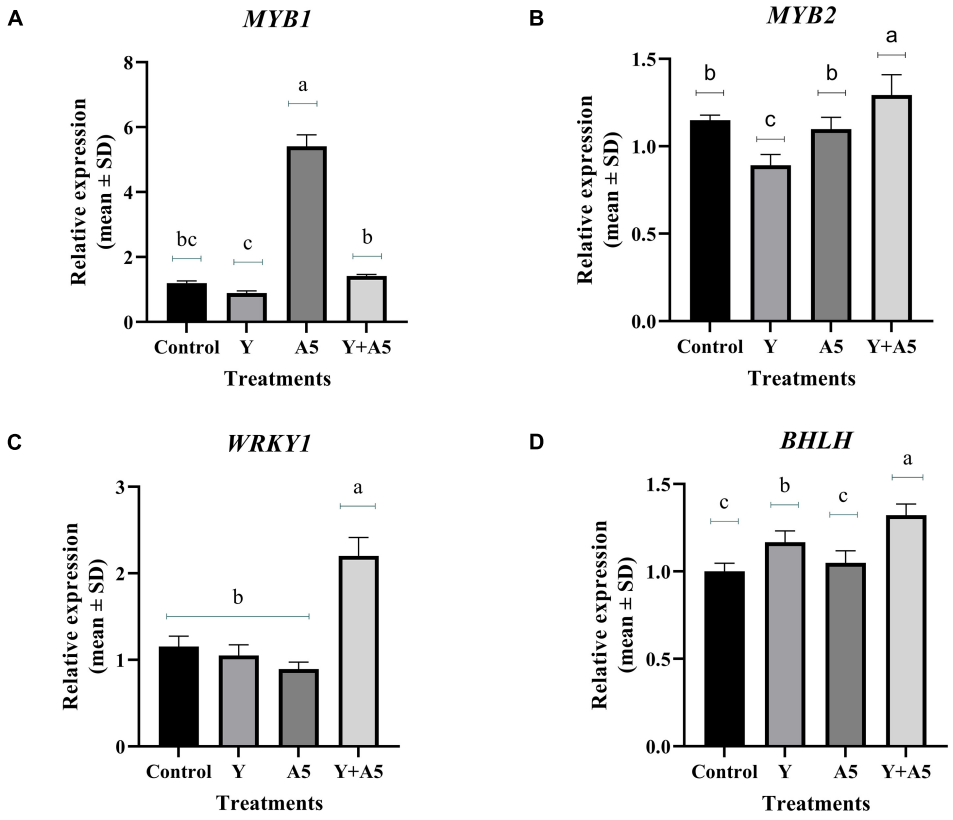

FIGURE 9
Influence of endophyte inoculation on the expression of genes involved in bacoside biosynthesis. Total RNA was isolated from the leaves of 60 days old *Bacopa monnieri* plants inoculated with endophytes A5 and Y individually and in the form of consortium (A5 + Y), reverse-transcribed and used for expression analysis by SYBR Green-based RT-qPCR leaves on untreated plants were considered as control. Expression of *MYB 1* **(A)**, *MYB 2* **(B)**, *WRKY* **(C)**, and *BHLH* **(D)** was analyzed. Actin was used as a reference transcript. Data are means ± SE (n = 3 biological replicates), and Y-axis represents relative expression which was calculated using $2^{-\Delta\Delta Ct}$ method. Different Duncan's letters denote significant difference at $P < 0.05$.

phenylpropanoid pathways along with transcription factors was studied (Figures 7–9). A very interesting observation was recorded in the study as significant increase in phenylpropanoid genes was observed in the case of plants inoculated with *B. subtilis,* while an overall increase in MEV and MVA pathway genes was observed in microbial combination set. Significant increase in cinnamoyl-CoA reductase (*CCR),* cinnamyl-alcohol dehydrogenase *(CAD),* chalcone synthase (*CHS*), and shikimate O-hydroxycinnamoyltransferase (*HCT)* genes was observed in *B. subtilis* [A5] inoculated plants in comparison with the control set plants (Figure 7). Likewise, a significant fold change in gene expression was recorded for *HMGR, PMVK, FDPS, SQS,* and β-*AS* genes in the case of plants inoculated with microbial combination. β-*AS* was one gene which showed enhanced expression in all the microbial treatments with respect to the control set plants (Figure 8). In case of transcriptional factors, *MYB* 1 was significantly enhanced in *B. subtilis* inoculated plants, while *MYB-2*, *WRKY-1,* and *BHLH* were most profoundly upregulated in treatment having both the microbes (Figure 9).

Discussion

Bacopa monnieri plant houses diverse category of microbes as the culturable bacterial and fungal diversity varied significantly with respect to different plant parts. A notable observation pointed out toward more abundance of fungal endophytes in leaf and stem tissues which could be possible because of the presence of higher content of bacosides in the aerial tissues. Our speculation is in line with a study carried out on *Lycoris radiata* where amaryllidaceae alkaloids were hypothesized to play a crucial role in fine-tuning the balance and distribution of fungal endophytes in different plant parts (Zhou et al., 2020). Since the endophytes like other beneficial microbes enhance the plant growth by producing various plant growth-promoting traits, selection of potential strains for further studies was based on their ability for producing such traits. All the isolates except three cultures were found to be positive for one or the other plant growth-promoting traits in *in vitro* assay. Therefore, out of the 47 endophytic cultures, screening of cultures for

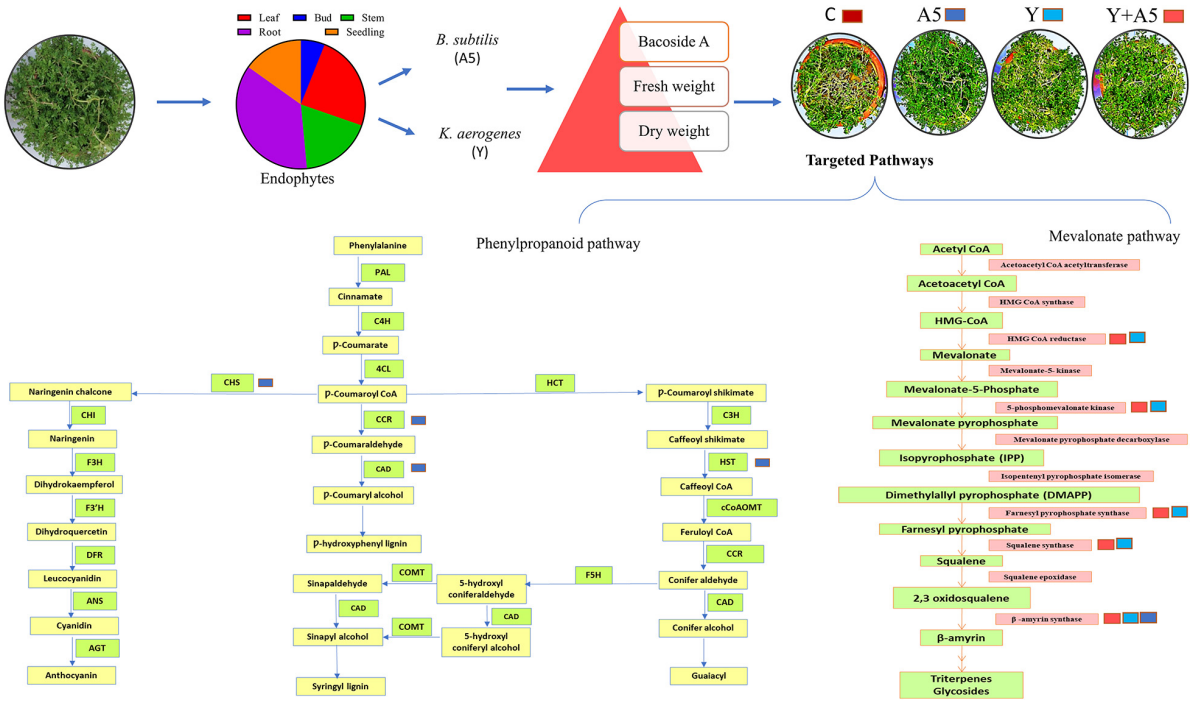

FIGURE 10
Schematic representation of the impact of endophytic cultures *B. subtilis* (A5) and K.aerogenes (Y) singly as well as in combination on plant fitness and secondary metabolite biosynthetic pathways.

in vivo plant growth promotion was based on the selection of functionally diverse group of microbes showing different plant growth-promoting traits as obtained in *in vitro* assays. The selection done was in accordance with the previous studies where endophytes with plant growth-promoting traits not only enhanced the plant productivity but also the secondary metabolites of pharmaceutical importance (Pandey et al., 2016; Ray et al., 2019; Mastan et al., 2020; Tripathi et al., 2020). In this study, better plant productivity and photosynthetic efficiency in inoculated plants could be possibly because of the growth hormones and other growth-promoting factors produced by the endophytes. Among the 17 endophytic cultures selected for *in vivo* greenhouse trial, two cultures namely *B. subtilis* (A5) and *K. aerogenes* (Y) significantly enhanced the Bacoside A content. Both *B. subtilis* and *K. aerogenes* have been previously reported to have beneficial impact on the plant fitness and health (Berg, 2009; Mukherjee et al., 2020). Keeping in mind the beneficial attributes of the application of synergistic combination of microbes in MAPs (Ray et al., 2019; Tripathi et al., 2020), the study was finally aligned toward evaluation of combinatorial effects of both the cultures with respect to single inoculation. In this investigation, nitrogen fixed by *K. aerogenes* and the synergistic combination resulted in an increase in the concentration of nitrogen in the plant while non-significant increment was observed in uptake of potassium. The increased nitrogen content which is reported to be responsible for influencing the vegetative and the reproductive status of the plants was positively correlated with the herb yield, as well the nitrogen fixation test.

The beneficial microbes induce complex metabolic changes at cellular level which fine-tune the various pathways occurring inside the plant cell for making a "win–win" situation for the plants (Singh et al., 2013a; Gupta et al., 2017b). The interplay between the reactive oxygen species, different metabolic pathways, and antioxidant status is essential for successful colonization of an endophyte which ultimately decides the fate of the plants (Waszczak et al., 2018). Previously, inoculation with beneficial group of microbes has shown promising leads in regulating the reactive oxygen species levels, thereby helping plants to perform its usual activities both in normal and adverse conditions (Hamilton et al., 2012; Zhou et al., 2016). In our study, significant enhancement was observed in SOD enzyme and total phenolic content which is in concurrence with a study carried out by Huang et al. (2007) who observed high level of production of antioxidants by 292 endophytic morphotypes isolated from 29 plant diverse plant families. One interesting observation was recorded that single inoculation of *B. subtilis* significantly enhanced the total phenolic content which correlated well with the enhanced lignification pattern in the stem of treated plants. The enhanced defensive state of plants treated with *B. subtilis* is in line with the previous observations where microbes have shown encouraging results

in terms of enhancing the plant immunity (Van Wees et al., 2008; Yu et al., 2022). Contrary to the above findings, significant upregulation in the bacoside biosynthetic pathway genes was observed in synergistic microbial combination treatment. The results suggested upregulation in phenylpropanoid pathway genes upon inoculation with *B. subtilis,* while the synergistic combination specifically upregulated the genes of bacoside biosynthetic pathway pointing toward the mutualistic effect of both the microbes.

The precursors of all kinds of isoprenoids produced by plants are synthesized by two independent pathways: The MVA pathway occurs in the cytoplasm, while the MEP pathway functions in the plastids (Vranová et al., 2013). The MVA pathway starts with acetyl-coenzyme A through which sequence of events is converted to isopentenyl diphosphate, an intermediate molecule of both MVA and MEP pathways. Our findings revealed upregulation in bacoside biosynthesis pathway genes like *HMGCR* maximally in treatment having synergistic combination of microbes. It is speculated that upregulation in the above gene might have provided pool of substrates for proper functioning of the lower MVA pathway genes like *PMVK*, *FDPS*, *SQS,* and β- *AS* genes which finally yielded higher amount of bacosides as confirmed by HPLC analysis. Our results are in accordance with a recent study carried out by Chen et al. (2021) who showed upregulation in expression level of some important genes like *DXS*, *DXR*, *HMGCR,* and *GGPPS* primarily involved in tanshinone biosynthesis pathway upon inoculation with an endophyte *Mucor circinelloids* DF20 in *Salvia miltiorrhiza*. However, contrary to the above findings, inoculation of *B. subtilis* singly significantly triggered the expression of phenylpropanoid pathway genes. The synthesis of defensive metabolites in the presence of beneficial microbes holds an important strategy adopted by plants to create its niche in its surrounding. A study in support of the above hypothesis reported augmentation in the production of phenylpropanoids and terpenoids in *Atractylodes lancea* upon inoculation with endophytic *Gilmaniella* sp. (Yuan et al., 2016).

Lignin, a constitutively present polymer of phenylpropanoid group in plants, is known to enhance plant cell wall rigidity, thereby promoting mineral transport through the vascular bundles. In addition, it also acts as an important barrier against various biotic stresses. Some reports suggest enhancement in lignification content in response to inoculation of beneficial microbes and their metabolites (Singh et al., 2013b; Singh et al., 2016; Patel et al., 2017). Upregulation in transcripts of phenylpropanoid pathway genes like *CCR, CAD, HST,* and *CHS* genes in *B. subtilis* treatment is also in line with the above studies. We hypothesize that the upregulation in the above genes could be the main reason for enhanced lignifications in the above treatment as CAD is the final enzyme responsible for converting sinapaldehyde to sinapyl alcohol which finally leads to formation of syringyl lignin. In addition to the role of primary genes involved in the biosynthetic pathways, the expression of plant secondary metabolites is under the governance of various kinds of transcription factors belonging to diverse families of *bHLH, MYB,* and *WRKY* (Yang et al., 2012; Patra et al., 2013; Gandhi et al., 2015). Enhanced expression of *WRKY 1* in combination treatment is in accordance with previous studies which state the role of *WRKY* transcription factors in regulation of plant development and production of secondary metabolites (Xu et al., 2004; Ma et al., 2009; Suttipanta et al., 2011). Likewise, higher expression of *MYB 1* in *B. subtilis* alone points out toward its role in lignin biosynthesis as reported in previous studies (Ambawat et al., 2013; Miyamoto et al., 2020).

Overall, the results of this study strongly suggest that the deployment of combined inoculation of *B. subtilis* with *K. aerogenes* enhances the transcription of bacoside biosynthetic pathway genes while single inoculation of *B. subtilis* specifically impacted the phenylpropanoid pathway (**Figure 10**). The leads obtained can be exploited to use specific microbes as per requirement, that is, *B. subtilis* inoculation alone in future can be used for managing biotic/abiotic stresses while combined inoculation of the endophytes could be suggested as a sustainable value added strategy for cultivation of *B. monnieri*. On the whole by using native endophytic microbes, *in planta* production of pharmaceutically important bacosides in *B. monnieri* can be enhanced which can provide non-chemical and cheap alternative for sustainable agriculture for future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

AS conceived and designed the experiments and wrote the manuscript. NS, DS, AT, PK, RG, SS, and KS performed the experiments. AS and RKG analyzed the data. All authors read and approved the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.896856/full#supplementary-material

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Overview of biofertilizers in crop production and stress management for sustainable agriculture

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With the increase in world population, the demography of humans is estimated to be exceeded and it has become a major challenge to provide an adequate amount of food, feed, and agricultural products majorly in developing countries. The use of chemical fertilizers causes the plant to grow efficiently and rapidly to meet the food demand. The drawbacks of using a higher quantity of chemical or synthetic fertilizers are environmental pollution, persistent changes in the soil ecology, physiochemical composition, decreasing agricultural productivity and cause several health hazards. Climatic factors are responsible for enhancing abiotic stress on crops, resulting in reduced agricultural productivity. There are various types of abiotic and biotic stress factors like soil salinity, drought, wind, improper temperature, heavy metals, waterlogging, and different weeds and phytopathogens like bacteria, viruses, fungi, and nematodes which attack plants, reducing crop productivity and quality. There is a shift toward the use of biofertilizers due to all these facts, which provide nutrition through natural processes like zinc, potassium and phosphorus solubilization, nitrogen fixation, production of hormones, siderophore, various hydrolytic enzymes and protect the plant from different plant pathogens and stress conditions. They provide the nutrition in adequate amount that is sufficient for healthy crop development to fulfill the demand of the increasing population worldwide, eco-friendly and economically convenient. This review will focus on biofertilizers and their mechanisms of action, role in crop productivity and in biotic/abiotic stress tolerance.

KEYWORDS

abiotic stress, biotic stress, biofertilizers, crop productivity, plant-root interaction

Introduction

The world population will reach 9 billion by 2050 in accordance with Food and Agricultural Organization; as a result, there should be an enhancement in crop yield to meet the food demand. Soil is an important source of food production in human lifespan. In the last decades, due to the increase in agricultural practices

such as pesticides and chemical fertilizers it has been degraded at a universal scale and causes lower fertility due to loss in biodiversity, water retention, and disturbance in biogeochemical cycles. Soil health and plant productivity are severely influenced by numerous interactions among plant, soil, and microorganisms (Harman et al., 2020). Soil microbes cooperate with one another and also with plant roots in numerous means providing a wide variety of essential acts which are valuable for sustaining the ecological balance in soil (Kumar et al., 2021c). Plant microbial interactions are positive if they improve plant survival, nutritional status, and crop productivity and they are negative if they reduce plant growth. Soil fertility is inextricably linked to the balance of microorganisms and plants (Vishwakarma et al., 2020). The application of biofertilizers can be a probable approach to improve soil microbial status that stimulates the natural soil microbiota therefore influencing nutrient accessibility and decomposition of organic matter (Chaudhary et al., 2021). It was observed that the supply of biofertilizers in apricot modifies the microbial composition and degradation process which could be efficient in nutrient cycles in soil under field conditions (Agri et al., 2021; Baldi et al., 2021). The capability of biofertilizers to form a high-level microbial diversity in soil may outcome better crop productivity for sustainable agriculture (Agri et al., 2022). Recently, many studies reported the positive impact of beneficial soil microbes on crop productivity, but the role of consortium in agriculture is not entirely unstated. Usage of consortium has positive impact on nutrient uptake efficiency by plants, protection from pathogens, and stress conditions (Aguilar-Paredes et al., 2020). This review provided information on effective approaches such as biofertilizers which help in the restoration of agricultural soil thus improving crop health for sustainable agriculture. This can permit agriculturalists to enhance farming and reach a high standard of soil quality and subsequently lead to raised plant development.

Nutrients are required by every living creature in this world. A total of 17 essential plant nutrients are mandatory for the proper development of plants (Kumar et al., 2021a). These 17 nutrients are divided into three classes based on the amount required such as major nutrients (carbon, hydrogen, oxygen, nitrogen, phosphorus, and potassium), minor nutrients such as sulfur, calcium, and magnesium, and micronutrients (nickel, zinc, molybdenum, manganese, iron, copper, chlorine, and boron). The plant takes up oxygen, hydrogen, and carbon from air and water, but the other nutrients are taken from soils in inorganic forms (Gong et al., 2020). Biofertilizer or biological fertilizer is a material that contains living or dormant microorganisms that colonize the rhizosphere or present inside the plants and directly or indirectly promotes the growth of plants by supplying nutrition (Malusa and Vassilev, 2014; Fasusi et al., 2021). Microorganisms present in soil used as biofertilizers can mobilize the nutrient from soil and convert them into a usable form from unusable form through biological processes like nitrogen fixation, phosphorus solubilization, zinc solubilization, siderophores production, and producing plant growth-promoting substances (Bhattacharjee and Dey, 2014; Mazid and Khan, 2015). Biofertilizers are applied to seed, root, soil, or by the foliar spray to enhance the microbial activity through their multiplication which then mobilizes the nutrients to target plants which remarkably improved the soil fertility and sooner increases the crop health and production (Pandey and Singh, 2012; Ismail et al., 2013).

Biotic stress is responsible to damage plants by pathogenic organisms like bacteria, fungi, viruses, parasites, and insects and by other harmful plants. They lead to declining the crop productivity by causing diseases such as vascular wilts, leaf spots, cankers, nutrient deficiency, systematic damage, chlorosis, stunting and reduce plant vigor, ultimately causing the death of the plants (Iqbal et al., 2021). Plant protects themselves to biotic stress *via* direct mechanisms like synthesis of secondary metabolite, hormones, cell-wall-degrading enzymes, and antioxidants (Kaur et al., 2022). The indirect mechanisms include the induction of acquired systematic resistance, plant pathogen molecular patterns (PAMPs) which in turn trigger the immunity and plant resistance proteins (Yu et al., 2022). Microorganisms solubilize the phosphorus and zinc, fixing the nitrogen and other macro- and micronutrients which promote the growth of the plants under biotic stress condition by providing nutrition (Singh et al., 2022a). They also enhance the stress resistance in plants by expressing the gene of phytohormones and stress-related metabolite. Some microorganisms also produce the volatile organic compounds (VOCs) such as melatonin to protect the plant from pathogens (Moustafa-Farag et al., 2019). When pathogen attacks, the plant produces various compounds within the tissues that lead to the activation of defense mechanisms inside the plants such as induced systematic resistance, peroxidases, phenylalanine ammonia-lyase, polyphenol oxidase, and hypersensitivity (Kaur et al., 2022).

Climatic change is one of the major factors for enhancing abiotic stress on crops which results in reduced crop productivity (Liu et al., 2017a). Climatic-related abiotic stresses included drought, waterlogging, excessive heat, and soil-related abiotic stresses are fertility, heavy metals, and salinity; all these are responsible for the poor yields of crops around the whole globe (Upadhyay et al., 2019). There is less water available to plants during drought conditions, and biofertilizers have the potential to produce cytokinin, gibberellins, abscisic acid, and IAA, which cause the plant to increase its growth, root length, total surface area, and the formation of root hairs and lateral roots, which increases water absorption from water-deficient soil (Kenneth et al., 2019; Raza et al., 2019). Pollutants released from industry without any further operation if released in the environment then they cause the accumulation of heavy metals such as copper, lead, nickel, zinc, etc., which have detrimental effects on the plants and animals (Popp et al., 2013). These heavy metals are

removed from the environment by micro- and macro-nutrient solubilizing and mineralizing microorganisms (Bhojiya et al., 2021). Heat stress causes cellular changes like production of reactive oxygen species, reduction in cell turgidity, reduction in water uptake, reduction in growth of plants, ultimately leading to death of plant by showing initial symptoms like leaf senescence, damages to chloroplast, wilting of plant, and chlorosis (Ahluwalia et al., 2021), whereas low temperature causes the inactivation of protein and reduces the cell membrane fluidity leading to increases in photosynthesis, imbalance of water transport (Odoh et al., 2020). All these temperature-related stresses coped up by plants after the accumulation of the hydrophilic and osmolytes protein. Huang et al. (2015) reported that due to high salt concentration there is increased toxicity to cell due to accumulations of sodium and chloride ions inside the cell which in turn disturb the photosynthetic processes, stomatal opening and closing, shrinkage of cell within pant tissue. Various studies showed that bacteria and arbuscular mycorrhizae fungi help in surviving the plants under salinity stress condition by enhancing the plant growth and development. In this review, we will discuss about the biofertilizers and its mechanism for crop production and biotic/abiotic tolerance for sustainable agriculture.

Biofertilizers

In India, biofertilizer refers to the use of microorganisms to meet nutritional needs, whereas in other countries, the term microbial bioinoculant is used (Mitter et al., 2021). Biofertilizers are bio-based organic fertilizers that either could be from plant or animal sources or from living or dormant microbial cells that have the potential to improve the bioavailability and bioaccessibility of nutrient uptake in plants (Lee et al., 2018; Abbey et al., 2019). Bhardwaj et al. (2014) reported that live microbial mass is a major ingredient of biofertilizers. So biofertilizers are properly defined as "the preparations containing live microbes that help in enhancing soil fertility by fixing atmospheric nitrogen, solubilizing phosphorus or decomposing organic wastes or by elevating plant growth through the production of growth hormones with their biological activities" (Okur, 2018). Biofertilizers are generally applied in solid or dry forms, which are prepared after packing on suitable carriers such as clay minerals, rice bran, peat, lignite, wheat bran, humus, and wood charcoal. Carriers increase the shelf life and enable the easy handling of microbial inoculants (Bhattacharjee and Dey, 2014). The benefits of biofertilizers include low cost, enhanced nutrient availability, improved soil fertility, protect plants from soil-borne pathogens, sustainable agricultural production, enhanced biotic and abiotic stress tolerance, promote phytohormone production, improve soil health, causing less environmental pollution, and its continued use improves the fertility of soil considerably (Chaudhary et al., 2021, 2022a). Based on the source and raw material, global biofertilizer is marketed under two major categories like organic residue-based biofertilizer and microorganisms-based biofertilizer. Green manure, crop residues, treated sewage sludge, and farmyard manure are generally organic-based biofertilizers. While on the contrary, microorganism-based biofertilizers contain beneficial microorganisms like bacteria, fungi, and algae. Directly or indirectly, these biofertilizers mediate the performance of plant growth (Figure 1). Direct mechanisms that act upon plants directly include nitrogen fixation, phosphate solubilization, micronutrient solubilization, and the production of phytohormones (Chaudhary et al., 2021). The indirect mechanism generally protects the plant from the deleterious effect of the pathogens by releasing lytic enzymes, antibiotics, siderophores, and cyanide production (Mahmud et al., 2021).

Types of biofertilizers and their role in crop production and soil health maintenance

Various types of biofertilizers are classified based on microorganisms such as bacteria and fungi and function of the biofertilizers as shown in Figure 1.

Nitrogen-fixing biofertilizers

Nitrogen is the vital macro-nutrient essential by plants because it improves the growth of the shoot system, helps in reproduction, is a constituent of chlorophyll responsible for the deep green color, and also increases the size of the grains (Sandhu et al., 2021). Although the nitrogen content in the atmosphere is 78% by a mass fraction, dinitrogen contains triple bonds and is an unavailable form of nitrogen present in the air for the plants. Dinitrogen should be first converted into soluble non-toxic form ammonia by the diazotrophs through the biological process of nitrogen fixations (Abbey et al., 2019). This ammonia is then converted to the nitrite and nitrate by the ammonia-oxidizing bacteria and by nitrifying bacteria, respectively (Roy et al., 2020). The unused nitrate is converted to the atmospheric nitrogen in the deeper soil horizons through the process of denitrification which will then escape to the atmosphere as dinitrogen gas. This is the typical path of the nitrogen cycle (Mahanty et al., 2017). *Azotobacter* and *Bacillus* sp. are involved in N fixation, growth promotion of maize plants, and forest crops (Etesami et al., 2014; Azeem et al., 2022). Inoculation of *Bradyrhizobium japonicum* in soybean plants improved plant biomass, nodulation, and N fixation (Htwe et al., 2019). *Azotobacter chroococcum* improved the plant height and chlorophyll content in maize plants (Jain et al., 2021). *Bradyrhizobium* sp. showed nitrogen fixation, IAA,

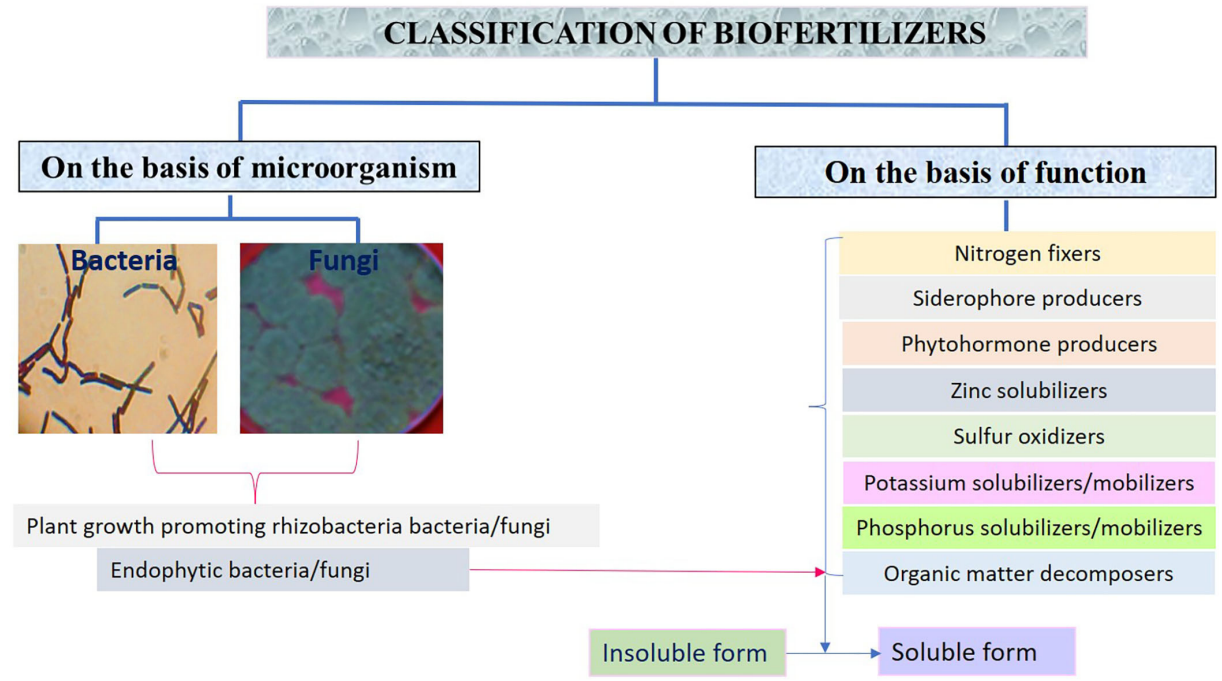

FIGURE 1
Types of biofertilizers on the basis of microorganism and functional characteristics.

and siderophores production and improved the yield of mung bean (Alkurtany et al., 2018). Nitrogen-fixing microbes are considered as symbiotic, free-living, and associative nitrogen-fixing bacteria (Aasfar et al., 2021). Jing et al. (2020) reported that the application of *Pseudomonas protegens* promoted plant growth in nitrogen-deficient conditions.

Symbiotic nitrogen-fixing microbes

In the process of symbiosis, macro-symbiont is the plant and microsymbionts are the prokaryotic bacteria**.** *Rhizobium* and legume symbiosis is one of the most studied mutualistic relationships between plant root nodules and nitrogen-fixing microorganisms. Mutualistic relationships are initiated when the plant began to secrete the flavonoids and iso-flavonoids in its rhizosphere, where it is recognized by *Rhizobium* (Hawkins and Oresnik, 2022). It started to do infection by differentiating root hairs, developing infection thread up to the root hair cell where infectious thread releases all its bacteria in the cytoplasmic region. Then, bacterial cell are terminally differentiated into the bacteroides, and the further development of bacteroides leads to the formation of symbiosome which is the site of nitrogen fixation (Cissoko et al., 2018; Jimenez-Jimenez et al., 2019; Suzaki et al., 2019). This atmospheric nitrogen fixation inside the nodule is carried out by the nitrogenase enzyme (Brahmaprakash and Sahu, 2012). Examples include *Rhizobium* associated with leguminous plants, *Frankia* (actinomycetes) associated with non-leguminous plants (*Alnus, Casuarina*), *Azolla* and the blue-green alga *Anabaena azollae,* and association of cyanobacteria with gymnosperms (Ghodhbane-Gtari et al., 2021). Fixation of N helps to improve the soil fertility and crop productivity. Mondal et al. (2020) reported that *Rhizobium meliloti* involved in N_2 fixation produced chitinase enzyme and improved the yield of peanut plants. The alfalfa-Rhizobium symbiotic system can stimulate plant N fixation, increase phytohormone production, and promote plant growth (Fang et al., 2020).

Free-living nitrogen-fixing bacteria

Mostly *Azotobacter* is studied because it is a free-living, non-symbiotic, and phototropic bacterium. *Azotobacter chroococcum* can be used as a biofertilizer because it has the potential to fix 10 mgN/g of carbon source supplied *in-vitro* (Mukherjee et al., 2022). Plant hormones such as indole acetic acids, gibberellic acids, naphthalene acetic acid, and vitamin B complex are produced by *Azotobacter.* It inhibits the root pathogens while promoting root growth, helps in mineral uptake, and improves soil fertility (Sumbul et al., 2020). Examples include *Azotobacter*, *Bacillus*, *Clostridium*, and *Azospirillum.* Application of *Bacillus*

sp. significantly enhanced the growth of *Arachis hypogea* plant, protects plants from stress, and exhibits the production of ammonia and IAA (Gohil et al., 2022). *Azospirillum brasilense* reduces N fertilization, improves plant nutrition, and increases plant biomass and wheat grain yield as reported by Galindo et al. (2022).

Associative nitrogen-fixing bacteria

The *Spirillum lipoferum* was firstly isolated by M.W. Beijerinck in 1925. *Spirillum* was found associated with the roots of the grain which were also capable of fixing nitrogen (Soumare et al., 2020). *Azospirillum* is gram-negative, non-nodulating, aerobic-associative nitrogen-fixing bacteria with plants having a C4 dicarboxylic pathway of photosynthesis, such as sugarcane, maize, sorghum, bajra, and cereals like wheat, rice, barley (Yasuda et al., 2022). They also produce cytokinin, gibberellins, and indole acetic acid, which aid in the uptake of N, P, and K and promote the growth of roots. Examples such as *Gluconobacter, Acetobacter, Herbaspirillum,* and *Azoarcus.*

Phosphorus-solubilizing biofertilizers

Phosphorus is the second macro-nutrient that is responsible for limiting the growth of plants (Bechtaoui et al., 2021). It is an important constituent of organic and nucleic acids and is responsible for the synthesis of ATP and several amino acids. P helps in the nodulation process, amino acid synthesis, and proteins in leguminous plants (Wang et al., 2020). Soluble form of phosphorus is phosphate anion (orthophosphate), and their uptake is facilitated by rhizospheric microbes which help in plant nutrition. There are different microbes which can solubilize the remaining unavailable form of P into available form *via* organic acid production by bacteria which lowers the pH of the soil, leads to the dissolution of the phosphate compounds, and makes them available for the plant's nutrition (Mahanty et al., 2017). Examples of phosphate-solubilizing bacteria and fungi (PSB and PSF) are *Bacillus, Rhizobium, Aerobacter, Burkholderia*, *Aspergillus,* and *Penicillium.* Inoculation of *Alcaligenes* sp. improved plant growth parameters *via* P solubilization and IAA production (Abdallah et al., 2016). *Rhizobium leguminosarum and Pseudomonas moraviensis* enhanced the yield and growth of wheat plants and showed IAA and solubilization (Igiehon et al., 2019; Fahsi et al., 2021). Application of Arbuscular fungi can make greater availability of P in plants and protects them from stress condition as reported by Nacoon et al. (2020). *Bacillus subtilis* is also known as PSB which improved safflower growth and protects plants from salinity stress as reported by Zhang et al. (2019). NanoPhos containing phosphate-solubilizing bacteria enhanced the maize production *via* increasing the soil enzymes and bacteria population under field conditions (Chaudhary et al., 2021).

Phosphorus-mobilizing biofertilizers

They are beneficial bacteria that effectively mobilize the soluble phosphorus and mineralization of the organic phosphorus compound, both are unavailable form of phosphorus. *Bacillus, Pseudomonas,* and *Rhizobium* are representative phosphorus-mobilizing microorganisms (PMB) (Kirui et al., 2022). Three different mechanisms have been reported for this process. First, PMB is releasing the phosphatases enzyme. Second, PMB is producing organic acids. The last one added PMB may interact symbiotically with the other fungal mycorrhiza which mobilizes the soluble phosphorus from distant places where plant roots cannot reach by absorbing soluble phosphate by hyphae (Nassal et al., 2018; Etesami et al., 2021). One of the major advantages of Arbuscular mycorrhiza is transporting both inorganic and organic forms of phosphorus to plants. Examples of arbuscular mycorrhiza fungi (AMF) include *Acaulospora* sp., *Glomus* sp., *Entrophospora,* and *Paraglomus* sp. and ectomycorrhiza include *Amanita, Laccaria,* and *Boletus* spp. Fungal endophyte (*Serendipita*) increased the K content in maize and protects plants from salinity stress (Haro and Benito, 2019).

Potassium-solubilizing biofertilizers

Subsequently, potassium (K) is the third major constituent of the macro-nutrients required by plants. It is mainly intricate in the regulation of stomatal closing and opening, nutrient uptake, protein synthesis improving the quality of products and provides resistance against stress environment (Santosh et al., 2022). K is present in different forms in soil depending upon the type of the soil composition like water-soluble, available form, and non-available form of the K (Basak et al., 2022). K is present in immobilized forms in silicate minerals like illite, orthoclase, biotite, illite, feldspar, etc. K solubilization occurs by both bacteria and fungi, and the major mechanism for solubilization of the unavailable form of K is acidification (means release of organic acids) (Varga et al., 2020). There are mechanisms also for solubilization of the K, namely, siderophores production, exchange reaction, and complexation (Sattar et al., 2019). Examples of potassium-solubilizing bacteria include *Bacillus mucilaginous, B. edaphicus, B. circulans, Acidithiobacillus ferrooxidans*, *Frateuria aurantia, Herbaspirillum spp*., and *Clostridium spp.,* and potassium-solubilizing fungi include *Aspergillus spp*. and some arbuscular mycorrhiza fungi. *Bacillus cereus* showed K solubilization and improved potato plant health parameters and yield (Ali et al., 2021). Dal et al. (2020) reported that the combination

of *Rhizophagus irregularis* and *A. vinelandii* improved soil enzyme activities and plant growth of wheat plants *via* P and K solubilization.

Potassium-mobilizing biofertilizers

The potassium-mobilizing microorganisms (PMMs) effectively release the unavailable potassium through the solubilization process (Patel et al., 2021). PMM is also recognized as potassium-dissolving bacteria or potassium-solubilizing bacteria. Ghaffari et al. (2018) observed that *Frateuria* and *B. megaterium* are efficient K-mobilizing bacteria used for crop farming purposes. *Azotobacter* showed K mobilization and solubilization in wheat plant and improved growth and soil microbial activities as reported by Game et al. (2020). *Enterococcus* and *Pseudomonas aeruginosa* also showed P and K solubilization and improved the maize height, yield, and nutrient acquisition (Kumar et al., 2021b). *Bacillus aryabhattai* showed K solubilization, protects plants from stress, and improves their growth *via* the expression of K-solubilizing genes (Chen et al., 2022).

Sulfur-solubilizing biofertilizers

Sulfur helps in chlorophyll formation, activation of a certain enzyme, amino acid formation, vitamin formation and promotes nodulation, vital for the development of all plants (Wang et al., 2019). Sulfur solubilizers are also known as sulfur-oxidizing bacteria because they are transforming the most insoluble form of sulfur that is hydrogen sulfide (H_2S) into an available form of sulfur known as sulfate ($SO4^{-2}$), and the reverse of this process is known assimilatory sulfate reduction which is mediated by sulfate-reducing bacteria (Wang et al., 2019). Sulfur transformation in the soil is primarily due to the microbial activity through the processes of mineralization, immobilization, oxidation, and reduction (Malik et al., 2021). Examples of aerobic sulfur-oxidizing bacteria include *Bacillus, Beggiatoa, Aquifer, Paracoccus, Sulfolobus, Thiobacillus, Thermithiobacillus, Xanthobacter;* phototropic anaerobic sulfur-oxidizing bacteria include *Allochromatium, Chlorobium, Rhodobacter, Rhodopseudomonas;* non-phototrophic obligate anaerobes include *Wolinella succinogenes;* and aerobic sulfur-oxidizing archaea include *Sulfolobales* members (Kusale et al., 2021). *Thiobacillus thiooxidans* and *Bradyrhizobium japonicum* are sulfur-oxidizing biofertilizers which showed better effect on cereal crops, medicinal plants, and forage crops (Zhang et al., 2018). *Halothiobacillus* bacteria tolerated the high salt concentration and improved crop production in saline soils (Boroujeni et al., 2021).

Zinc-solubilizing biofertilizers

Zinc is required during protein synthesis, DNA–protein interaction, growth hormone production, seed development, production of chlorophyll and protects plants from stress conditions (Umair Hassan et al., 2020). Insoluble forms of zinc are mostly ZnO, $Zn_3(PO_4)_2$, $ZnCO_3$, and metallic Zn. The usable form of zinc by the plant is divalent cations (Ayoub et al., 2022). Zinc-solubilizing fertilizers contain the zinc solubilization bacteria which produce the organic acids to solubilize the insoluble zinc to Zn^{+2}, thereby enhancing zinc uptake in plants (Nitu et al., 2020). Examples of zinc-solubilizing bacteria and fungi are *Bacillus subtilis, Pseudomonas striata, Serratia, Burkholderia cenocepacia*, *Aspergillus niger, A. nomius,* and *A. oryza* which improved the soil enzyme activities and availability of Zn in crop plants (Batool et al., 2021). *Leclercia adecarboxylata* solubilizes Zn and produced siderophores which enhanced the Zn uptake in the roots of cucumber plants (Kang et al., 2021). *Bacillus* spp. and *Pseudomonas taiwanensis* showed a positive impact on the growth and chlorophyll content of maize plants (Chaudhary and Sharma, 2019; Hussain et al., 2020). Inoculation of *Trichoderma longibrachiatum* and *Bacillus megaterium* improved the seed germination of soybean plants in the pot experiment (Bakhshandeh et al., 2020). The application of PSB along with fertilizers improved the growth of faba bean in sandy soils (Ding et al., 2021).

Phytohormone-producing biofertilizers

Plant hormone or phytohormone plays a substantial role in plant development, secreted by both plants and microorganisms (Usman et al., 2022). Plant hormone production is an important feature of the beneficial microbes which is producing the indole-3-acetic acids, gibberellins, cytokinin, etc. (Eichmann et al., 2021). Auxin helped in the differentiation and division of plant cells. Cytokinin prevents the premature leaf senescence of plants (Wu et al., 2021a). Abscisic acid is also identified as hormone which is produced by plants during stress conditions. Gibberellins are involved in seed germination, shoot elongation, flowering, and fruiting (Binenbaum et al., 2018). These hormones are generally secreted by microorganisms under environmental stress conditions to protect the plants by modulating the phytohormone level inside the host plants (Lopes et al., 2021). *Bacillus thuringiensis* has the genes required for IAA production which improved the growth of tomato plants (Batista et al., 2021). *B. licheniformis* is known for the production of IAA, ABA, and gibberellin which improved the growth of grapevine and protects plants from stress conditions (Salomon et al., 2014).

Siderophores producing biofertilizers

Iron (Fe) is a micronutrient that performs various functions like photosynthesis, respiration, chlorophyll, and many of the enzymatic reactions in plants (Gao et al., 2022). The unavailable form of iron in nature present under aerobic environment predominately is Fe^{+3} and is more probable form of insoluble oxyhydroxides and hydroxides complex. So, bacteria are producing the low-molecular weight iron-binding protein molecules called siderophores (Lurthy et al., 2020). Siderophores are water-soluble molecules that exist in two forms, namely, extracellular and intracellular. After capturing Fe^{+3} by siderophore inside bacteria, Fe^{+3} is reduced to the Fe^{+2} inside the cytoplasmic membrane which is then transported inside the cytoplasm by gating mechanisms (Gu et al., 2020). This available form of iron is given by the bacteria to the host plant for its development (Mahanty et al., 2017). Plant assimilates the iron with the help of siderophores by releasing the chelating agent *via* bacteria. Examples include *Pseudomonas fluorescens* C7, *Pseudomonas aeruginosa* RSP5, and *Pseudomonas aeruginosa* RSP8. Application of siderophore-producing *Bacillus* sp. improves the growth of groundnut (Sarwar et al., 2020). *Pseudomonas koreensis* inoculation in maize plants inhibited the growth of plant pathogens *via* the production of siderophore and antioxidant enzymes (Ghazy and El-Nahrawy, 2021).

Organic matter decomposer biofertilizers

Soil organic matter is a mixture of living organisms consisting of bacteria, fungi, and insects, and the non-living part which includes fresh organic residues or waste, the dead and decaying matter of living organisms is generally known as humus (Lou et al., 2022). In organic matter generally, cellulose, lignin, hemicellulose, chitin, and lipids are present which are degraded by microbes such as bacteria, actinomycetes, and fungi. The organic-matter-degrading organisms break down the SOM into simpler or inorganic from which they derive energy and carbon for their growth. Examples of bacteriainclude *Bacillus subtilis* and *Pseudomonas fluorescens* and of fungi include ectomycorrhizal fungi. *Trichoderma* spp. involved in the degradation of litter at a faster rate releases antimicrobial compounds, improves the physicochemical properties of soil, and improves microbial diversity (Baldi et al., 2021). *Bacillus subtilis* and *B. hisashii* are involved in lignocellulose biodegradation by secreting the microbial enzymes as reported by Niu and Li (2022).

Endophytic bacteria as biofertilizers

Mutualistic microorganisms that employ the whole or part of their life cycle inside the plant tissues are known as endophytes (Fadiji and Babalola, 2020). Endophytes are of interest because they improve the nutritional requirements of the non-leguminous and leguminous plants by nitrogen fixation, phosphate solubilization, or by siderophores production (Janati et al., 2021). These bacteria have the potential to suppress pathogenic effects by activating the plant defense system (Dicko et al., 2021). Examples of endophytic bacteria include *Klebsiella spp., Pseudomonas spp., Serratia spp., Bacillus spp., Burkholderia spp., Citrobacter spp.* and endophytic fungi include *Colletotrichum*, *Fusarium*, *Alternaria,* and *Aspergillus. Penicillium* and *Aspergillus* isolated from roots of *Taxus wallichiana* solubilized P and produced phosphatase and phytase enzymes (Adhikari and Pandey, 2019). Kang et al. (2014a) observed that *Bacillus megaterium* regulates the content of amino acids and carbohydrates to promote the growth of mustard plant. Endophytes isolated from rice such as *Bradyrhizobium* sp., *Paraburkholderia* sp., showed acetylene reduction properties and high sugar content contributing to high nitrogen-fixing ability. High content of sugar in different crops such as sweet potato, pineapple, and sugar has known to assist endophytic N-fixing activity among non-leguminous plants (Okamoto et al., 2021).

Plant growth-promoting rhizobacteria

PGPR is used as biofertilizers; it represents the variation of soil bacteria that live in association with the rhizosphere, rhizoplane associated to root surface, and endophytes present inside the intercellular places (Vandana et al., 2021). PGPRs are soil bacteria which increase the growth and enhance the tolerance of plants toward stress conditions (Ghosh et al., 2019). There are diverse mechanisms shown by PGPR which support the plant growth such as N_2 fixation, macro- and micronutrient mineralization, secretion of exopolysaccharides, phytohormone production, siderophore, hydrogen cyanide to prevent the growth of phytopathogens, antibiotics, etc. (Gouda et al., 2018; Numan et al., 2018). *Rhizobium lupini* increased alfalfa growth and enhanced nutrient uptake efficiency (Duan et al., 2022). Application of biofertilizers such as *Pseudomonas taiwanensis, Bacillus* spp., and *Pantoea agglomerans* improved the maize growth, yield, and soil health parameters (Khati et al., 2018; Chaudhary et al., 2022b). Application of *Bacillus* spp. improved the plant/soil health parameters and maize productivity as reported by Chaudhary et al. (2021). Kukreti et al. (2020) reported that *Pseudomonas taiwanensis* improved maize plant health and soil enzyme activities in the pot experiment.

Role of biofertilizers in biotic stress management

The outbreak of plant diseases in nature necessitates sustainable agriculture with minimum use of agrochemicals. For

a long time, the use of chemicals has posed a significant risk to the environment and the agricultural sector (Akanmu et al., 2021). Long-term use of pesticides, on the other hand, harms both plant/soil health and eventually leads to significant crop loss. Thus, effective and eco-friendly phytopathogen control strategies such as biofertilizers are required. The exploitation of potential biofertilizers as endophytes could be useful to improve crop plants from various bacterial and fungal diseases (Collinge et al., 2022). Biological control of plant diseases occurs *via* destruction of pathogens *via* beneficial microbes such as *Bacillus* spp., *Pseudomonas* spp., *Streptomyces, Pantoea* spp., and several fungal spp. (Köhl et al., 2019; Chaudhary et al., 2021). Such endosymbiont group of biocontrol agents being friendly, they not only colonize internal plant tissue but also protect host plant throughout its life cycle without causing any apparent damage (Lahlali et al., 2022). The use of *Bacillus* sp. for crop growth promotion and biocontrol has a long history (Zhu et al., 2021). *Bacillus thuringiensis* (Bt), a producer of endotoxins that can be used as biopesticide and a source of genes for the creation of transgenic plants that are resistant to insects, is currently the most effective biopesticide on the market (Sujayanand et al., 2021).

Biofertilizers in the form of potential biocontrol agents represent a safe alternative to harmful chemicals like fertilizers, herbicides, pesticides, and insecticides (Hernández-Fernández et al., 2021). Consequently, the use of biofertilizers is receiving special attention for the management of phytopathogens that are comprised of bacteria, fungi, virus, aphids, and nematodes (Table 1). Their ubiquitous nature and the ability to reside within plant tissues make them unique, showing multidimensional interactions within the host plant (Khare et al., 2018). The biodiversity of endophytes is hyperdiverse in almost every other plant species ranging from small non-vascular plants to large conifers like *Pinus radiata* (Liu et al., 2017b). Some of the known endophytes are *Burkholderia*, *Stenotrophomonas*, *Rhizobium*, *Microbacterium, and Bacillus spp.* (Kandel et al., 2017).

There are several enzymes which protect the plants from stress conditions such as antioxidant enzymes like peroxidase (POD), polyphenol oxidase, phenylalanine ammonia-lyase (PAL), lipoxygenase, and chitinase (Cataldo et al., 2022). Lipoxygenase enzymes have its place to non-heme iron comprising dioxygenases which contribute to stress response *via* lipid oxidation. Also, it is found to act as signals for communication with the plant host, with associated endophytes and pathogens (Singh et al., 2022b). In response to pathogen attack, endophytes boost plant immunity by priming induced systemic resistance (ISR) and systemic acquired resistance (SAR) *via* several phytohormones (Romera et al., 2019; Oukala et al., 2021). Pathogenesis-related proteins with antimicrobial properties are produced and accumulated by several endophytes symbiotically living with their host plants. Many endosymbionts have the capability to complement the inefficient antioxidative system of plants by different mechanisms (Shukla et al., 2022). In some strains, production of lipopeptides, surfactin, plipastatin, and mycosubtilin differentially activated the plant innate immune response (Kumar et al., 2021c). Production of surfactin may have an important role in the suppression of *Fusarium* infestation on germinating seeds (Eid et al., 2021). *Bacillus* strains inhibited the verticillium wilt caused due to *Verticillium dahliae* by the production of secondary metabolites such as surfactin, fengycin, and bacillibactin, as well as expressing defense-related genes such as SOD and PAL (Hasan et al., 2020). *Bacillus atrophaeus* inhibits *Meloidogyne incognita* growth by producing volatile dimethyl disulfide and antioxidant enzymes (Ayaz et al., 2021). According to Nie et al. (2019), *Bacillus cereus* inhibits the growth of *Pseudomonas syringae* by producing antioxidant enzymes. *Pseudomonas fluorescent* controls the iron uptake genes and protects plants from phytopathogens as reported by Desrut et al. (2020). *Acrophialophora jodhpurensis* defends tomato plants from *Rhizoctonia solani* which causes crown root disease *via* the production of peroxidase enzyme, chitinase, and phenylalanine (Daroodi and Taheri, 2021). Plants are protected from *Botrytis cinerea* by *Trichoderma atroviride via* the production of glutamate and glyoxylate aminotransferase (González-López et al., 2021).

Secondary metabolites play the foremost role in defense mechanism toward pathogens, pests, and herbivores. Many plants microbiome especially endosymbionts regulate defense mechanisms through secreting various metabolites (Divekar et al., 2022). Secondary plant metabolites belonging to the family of steroids, alkaloids, phenolics, flavonoids, and terpenoids function in innate immunity and defense response signaling (Pang et al., 2021). Volatile compounds from endophytes modulate plant microbiome and possess antimicrobial properties. A variety of fungi, including *Ascomycetes* and *Deuteromycetes,* are inhibited by a mixture of VOCs produced by the fungal endophyte *Phomopsis* sp. (Hummadi et al., 2022). Three VOCs, including caryophyllene, 2-methoxy-4-vinylphenol, and 3,4-dimethoxystyrol, produced by endophytic fungi *Sarocladium bravhiariae* HND5 have been found to have antifungal activity against *Fusarium oxysporum* (Yang et al., 2021). Alkaloid produced by *Epichloe* sp. in a variety of grass species is one of the well-known secondary metabolites produced by endophytic fungi. Hennessy et al. (2022) reported *Epichloe festucae* colonized agricultural forage grasses and offered the plant defense against herbivorous insects. *Streptomyces hydrogenans* metabolites can be used as safe biocontrol agents against *Meloidogyne incognita* and plant growth promoters for *Solanum lycopersicum* (Sharma et al., 2020). *Bacillus velezensis* is a potential pesticide due to its strong biocontrol activity and ability to strengthen host defense against *Magnaporthe oryzae* fungi, which cause rice blast disease in plants (Chen et al., 2021). An isolate of *Trichoderma asperellum* increased the resistance in tomato seedling to the disease *A. alternata* leaf spot (Yu et al., 2021). *Trichoderma asperellum* also produces mycolytic enzymes such as chitinase and 1,3, glucanase

TABLE 1 Role of biofertilizers in biotic stress tolerance.

Biofertilizers	Host plant	Pathogen	Response	References
Bacillus subtilis	*Atractylodes macrocephala*	*Ceratobasidium* sp.	Inhibit growth of pathogen and promote plant growth	You et al., 2018
Bacillus cereus	*Arabidopsis*	*Botrytis cinerea*	Regulates signaling pathway such as JA and MAPK	Nie et al., 2019
Bacillus velezensis	*Arabidopsis*	*Myzus persicae*	Protects host plant from pathogen *via* systemic resistance response	Rashid et al., 2017
Bacillus safensis	*Vaccinium*	*Botrytis cinerea*	Enhanced the chitinase, hydrolytic, protease production and protects plants from pathogen	Hassan et al., 2021
Pseudomonas aeruginosa	*Cruciferous vegetables*	*Xanthomonas campestris*	Protects plants from pathogen *via* chitinase production	Mishra and Arora, 2012
Gluconacetobacter diazotrophicus	*Arabidopsis thaliana*	*Ralstonia solanacearum*	Protects from pathogen and activates defense response in plants	Rodriguez et al., 2019
Streptomyces spp.	*Oryzae sativa*	*Xanthomonas oryzae*	Provides immunity to plants and protect from disease *via* increasing antioxidant enzymes	Hata et al., 2021
Paenibacillus polymyxa	*Brassica napus*	*Verticillium spp.*	Increased production of volatile fatty acids and antibiotics	Rybakova et al., 2017
Bacillus subtilis	*Solanum lycopersicum*	*Fusarium oxysporum*	Increased expression of auxin-related genes and improved plant growth	Samaras et al., 2021
Trichoderma koningii	*Nicotiana tabacum*	*Tobacco Mosaic Virus*	Enhanced proline content and pathogen-related enzymes and inhibit the growth of pathogens	Taha et al., 2021
Aureobasidium pullulans	Olive trees	*Colletotrichum acutatum*	Increased production of volatile fatty acids and improves seed germination	Sdiri et al., 2022
Trichoderma harzianum	*Zea mays*	*Curvularia lunata*	Provides protection to plants from pathogen *via* JA signaling and platelet-activating factor	Yu et al., 2015
Bacillus amyloliquefaciens	*Solanum lycopersicum*	Viruses	Induced SA and JA signaling and protects plants from disease	Beris et al., 2018
Bacillus endophyticus, Pseudomonas aeruginosa	*Solanum lycopersicum*	*Spodoptera litura*	Increased secondary metabolite, phytohormone production and improved plant growth	Kousar et al., 2020
Bacillus subtilis	*Solanum lycopersicum*	*Fusarium oxysporum*	Increased plant growth and suppress the growth of pathogens	Sundaramoorthy and Balabaskar, 2013
Bacillus sp.	*Common bean*	*Rhizoctonia solani*	Inhibit growth of pathogens *via* production of cyanogens and lytic enzymes	Kumar et al., 2012
Pseudomonas spp.	*Gossypium*	*Fusarium spp.*	Inhibit pathogens *via* production of HCN and enzymes	Zain et al., 2019
Bacillus halotolerans, Agrobacterium fabrum	*Common bean*	*Alternaria sp.*	Improved plant growth and increased chitinase, siderophore, and IAA production	Sendi et al., 2020
P.putida	*Solanum tuberosum*	*Phytophthora infestans*	Increased production of HCN against pathogens	Anand et al., 2020
Aureobasidium pullulans	*Crops*	*Botrytis cinerea, Alternaria alternata*	Increased production of volatile organic acids and inhibit pathogen growth	Don et al., 2020

which may be capable of destroying phytopathogens cell walls (Win et al., 2021). *Trichoderma* spp. also have biocontrol potential against *V. dahliae*, which causes olive tree wilting, and inhibit the pathogenic fungus mycelial growth (Reghmit et al., 2021). *Trichoderma* sp. has been shown successfully to suppress *Sclerospora graminicola*, the cause of pearl millet downy mildew disease, and develop systemic resistance (Nandini et al., 2021).

Some endophytes can also regulate stress management through SAR mediated by salicylic acid. SAR offers long-lasting stress management and broad-spectrum effectiveness against a variety of pathogens (Xia et al., 2022). It frequently involves the accumulation of chitinase and pathogenesis-related proteins (PR). In a study by Samain et al. (2019), *Paenibacillus* strain (PB2) used to control *Mycosphaerella graminicola* induced pathogenesis-related proteins (PR1) which is considered as marker of SAR. Application of *Bacillus aryabhattai* activated a durable defense response against pathogens facilitated through salicylic acid/ethylene pathways (Portieles et al., 2021). *Trichoderma harzianum* helps to improve plant immediate resistance against *Nezara viridula* feeding invasion *via* enhancing JA marker gene transcript levels (Alınç et al., 2021). *Bacillus subtilis* and *Pseudomonas fluorescens* mediated systemic alleviated the biotic stress in *Solanum lycopersicum* against *Sclerotium rolfsii*. Heat-killed endophytic strain *B. aryabhattai* (HKEB) induced defense-related genes protein (PR1) and phytoalexin-deficient 3 in *A. thaliana*. PR1 gene expression was found to be 20-fold higher in treated plants than control, and other genes found in the study were associated with jasmonic and salicylic acid pathways (Portieles et al., 2021). Endophytes exhibit different gene upregulation and a distinct signaling pathway in response to distinct colonization strategies (Morelli et al., 2020). *Trichoderma* spp. demonstrated antagonistic activity against phytopathogens such as B. *cinerea, Fusarium solani,* and *Rhizoctonia solani* used as biocontrol agent in greenhouse experiment (Sánchez-Montesinos et al., 2021).

Role of biofertilizers in abiotic stress management

Climate change is one of the major reasons for the increasing abiotic stresses on the crops, which results in reducing the world's agriculture productivity. Abiotic stresses like drought, salinity, waterlogging, and excessive heat are responsible for the poor yield of crops (He et al., 2018). In recent years, the abiotic stress has increased so fast, because of the fluctuation of climates or climate change, and it has caused an unusual rise in the weather conditions and incidents, which is responsible for the substantial losses of crops around the globe. These abiotic stresses induce several physiological, biochemical, and morphological changes in plant that finally affect the economic yield of crop plants, and it was reported that the yield loss from abiotic stress is about 51–82%, which if continues will affect the goal of sustainable food production (dos Santos et al., 2022).

The use of beneficial microbes such as endophytes capable of producing growth hormones, like IAA, ACC deaminase, augmented the K uptake in plant tissues but decreased the ethylene level which helps in tolerance of stress in diverse plants (Figure 2). Biofertilizers as endophytes are found to have diverse associations with its host plant such as symbiotic, parasitic, and mutualistic and colonize plant tissues without causing any disease, thus benefiting for plants (Chaudhary et al., 2022c). Endophytes may benefit from mutualistic associations as they obtain nutrients from the hosts, and they spread by host seed transmission. They are also able to enhance the nutrients uptake like nitrogen, magnesium, zinc, and phosphorus from soil and provide to the host plant for better growth and survival (Bamisile et al., 2018). It is well-identified that plant biofertilizers play a significant role in supporting the growth of crops under different abiotic stresses (Table 2). Actinobacteria are well-known for plant growth *via* metabolite production and antibiotics under stress conditions (Yadav et al., 2018). Abd El-Daim et al. (2014) observed that the application of *Pseudomonas* sp. improved plant growth under heat stress *via* HSPs and ROS reduction. *Paenibacillus* sp. improved *Phaseolus vulgaris* growth *via* facilitating the siderophore, IAA and HCN production under salinity stress conditions (Gupta and Pandey, 2019). *Trichoderma harzianum* inoculation in rice plants improved root growth and protects from drought stress as reported by Shukla et al. (2012). Mukhtar et al. (2020a) reported that *Bacillus cereus* enhanced the production of ACC deaminase and exopolysaccharide which protects *Solanum lycopersicum* plants from heat stress.

Drought stress

Drought stress is one of the main abiotic stresses which causes water scarcity to meet the plant necessity and causes economic fatalities in agriculture production. The normal progress of plants is hindered due to decrease in water shortage in their cells. Drought stress decreased the rate of photosynthesis, germination in plants, and loss in crop productivity (Lata et al., 2018). Inoculation of beneficial biofertilizers (rhizospheric and endophytic microbes) improved plant growth and development *via* different direct/indirect mechanisms under stress situations. Stress can be overcome *via* using biofertilizers which produced growth hormones such as IAA and cytokinins and improved plant development (Fasusi et al., 2021). Inoculation of *Pseudomonas putida* boosted the flavonoids, salicylic, and abscisic acid production which protects soybean plants from drought stress (Kang et al., 2014b). Inoculation of *Pseudomonas* spp. protects maize plants and improved biomass and sugar content in treated plants from drought stress *via* upregulation of dehydrin proteins and

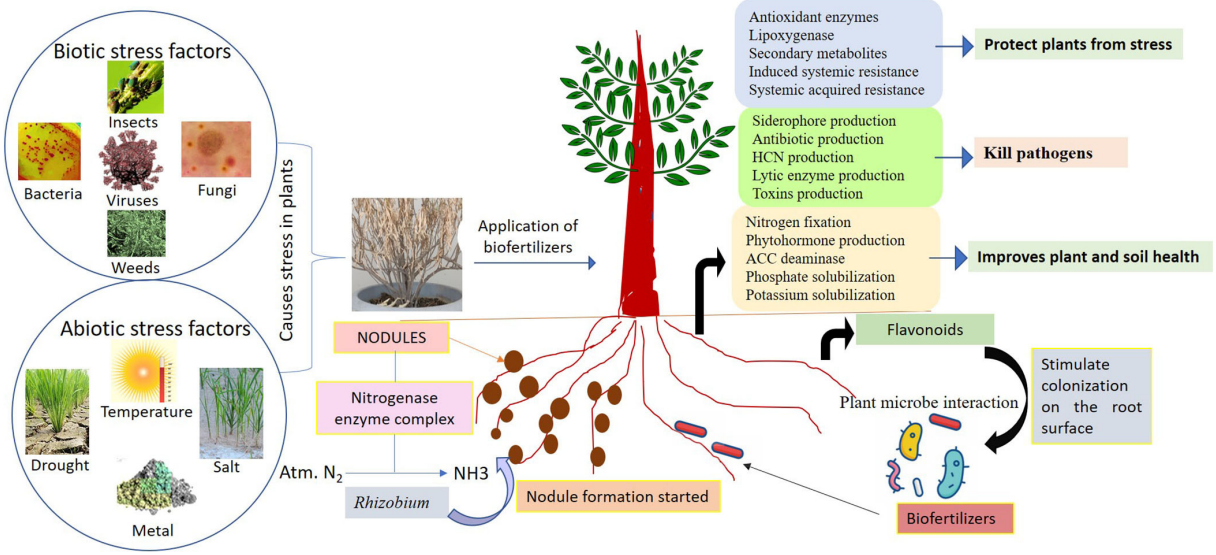

FIGURE 2
Role of biofertilizers for maintenance of crop productivity and soil health.

proline content (Sandhya et al., 2010). Khan et al. (2018) found that *Bacillus thuringiensis* improved chickpea growth under drought conditions *via* production of volatile organic compounds. Application of *Microbacterium* sp. improved maize plant growth, root length, photosynthetic rate, and yield under drought stress (Romera et al., 2019). Usage of *Phoma* improved the drought tolerance in *Pinus tabulaeformis* plants and increased seedling growth by improving the mechanism of water uptake, proline, and SOD (Zhou et al., 2021). Sheteiwy et al. (2021) reported that *Bradyrhizobium japonicum* and AMF improved the yield of soybean bacterial count and enzyme activities of soil *via* improving the nutrient accessibility in soil under drought stress. AMF and *Rhizobium* inoculation improved the *Glycyrrhiza* plant growth and phosphorus content in roots in drought stress (Hao et al., 2019). Combined inoculation of arbuscular fungi and bioinoculants improved plant biomass and chlorophyll content in date palm (*Phoenix dactylifera*) under water-deficit conditions *via* enhanced antioxidant enzyme activities, soluble sugars, and proteins (Anli et al., 2020). Inoculation of *Glomus mosseae* and *Bacillus amyloliquefaciens* in *Phaseolus vulgaris* significantly improved the photosynthetic rate and yield under water stress conditions (Salem and Al-Amri, 2021).

Salinity stress

Accumulation of salt in agricultural soil will have a negative impact on plants including its physiological, morphological, and molecular aspects. This affects plants *via* creating osmotic stress, ion toxicity and reducing the photosynthesis, CO_2 fixation, and transpiration rate in plants. Availability of nutrients and microbial diversity are also affected due to the salinity stress (Luo et al., 2021). Usage of bioinoculants is enormously supportive in countering the lethal properties of soil salinity *via* improving the soil physicochemical properties and thus improved crop production (Jiménez-Mejía et al., 2022). Interaction between microbes and plants can overcome stress problem. Gond et al. (2015) reported that inoculation of *Pantoea agglomerans* in tropical corn under salt stress (0–100 mM) improves tolerance and growth of plants due to the upregulation of aquaporins. *Bacillus megaterium* also regulates the aquaporin genes during salt stress in maize plants and improved root growth and leaf water content (Marulanda et al., 2010). Waqas et al. (2012) reported that *Penicillium* and *Phoma glomerata* improved the rice plant growth under salinity stress *via* increased production of CAT, POD, and IAA. Checchio et al. (2021) observed that *Azospirillum brasilense* improved resistance in corn plants *via* enhancing the production of antioxidant enzymes and glycine betaine. Application of *Pseudomonas* sp. improves *Arabidopsis thaliana* germination and growth *via* upregulation of lipoxygenase genes which are involved in tolerance mechanism *via* jasmonic pathway (Chu et al., 2019). The *Arthrobacter nitroguajacolicus* improved wheat growth under salt stress *via* upregulation of IAA, ACC, flavonoid, stilbenoid, terpenoids, and cytochrome P450 genes (Safdarian et al., 2019). Inoculation of *Planococcus rifietoensis* protects Cicer arietinum plants from salt stress (200 mM) *via* EPS and biofilm production (Qurashi and Sabri, 2012). Gupta and Pandey (2019) observed that inoculation of *Paenibacillus* sp.

TABLE 2 Role of biofertilizers in abiotic stress tolerance.

Biofertilizers	Host plant	Stress	Response	References
Bacillus aryabhattai	*Oryza sativa*	Salinity, heavy metals	Improved salt tolerance ability *via* exopolysaccharide production	Sultana et al., 2020
Bacillus amyloliquefaciens	*Arabidopsis*	Salt	Improved plant growth *via* regulation of JA pathway and antioxidant enzymes	Liu et al., 2020
Bacillus licheniformis	*Chrysanthemum*	Salt	Improved salt tolerance ability in stressed plants *via* production of antioxidant enzymes and Fe attainment	Zhou et al., 2017
Bacillus HL3RS14	*Zea mays*	Salt	Increased weight of roots and shoots *via* production of IAA, proline, and glycine betaine	Mukhtar et al., 2020b
Bacillus subtilis, Pseudomonas sp.	*Solanum melongena*	Salt	Increase chlorophyll content and protects plants from stress	Mokabel et al., 2022
Bacillus sp.	*Pisum sativum*	Salt	Improved plant growth and photosynthesis *via* antioxidant enzyme and AAC and siderophore production	Gupta et al., 2021
Gluconacetobacter diazotrophicus	*Zea mays*	Drought and nitrogen	Increase plant biomass and chlorophyll content	Tufail et al., 2021
Pseudomonas pseudoalcaligenes	*Glycine max*	Salt	Improved plant health parameters *via* production antioxidant enzyme, proline contents in shoots and roots	Yasmin et al., 2020
Alternaria alternata	*Triticum aestivum*	Drought	Improved photosynthesis *via* increasing antioxidant enzymes	Qiang et al., 2019
Aspergillus flavus	*Glycine max*	Salt	Increased antioxidant enzyme activity and chlorophyll content	Asaf et al., 2018
Aspergillus violaceofucus	*Helianthus annuus*	Heat	Improved plant height, biomass, and chlorophyll content	Ismail et al., 2020
Funneliformis mosseae	*Trifoliate orange*	Drought	Improved phenols, terpenoids and soil protein and enzyme activities	Cheng et al., 2021
Glomus lomus	*Date palm*	Salt	Improved shoot weight and growth	Meddich et al., 2018
Piriformospora indica	*Triticum aestivum*	Nutrient	Improved Zn uptake and root and shoot biomass	Abadi et al., 2021
Piriformospora indica	*Arabidopsis*	Cold	Increased proline content and cold stress tolerance genes	Jiang et al., 2020
Trichoderma atroviridae	*Arabidopsis*	Cold	Improved auxin production and cold-related gene expression	González-Pérez et al., 2018
Rhizophagus intraradices	C3 plants	Salt	Improved chlorophyll content in plants	Chandrasekaran et al., 2019
AMF and *Bradyrhizobium japonicum*	*Glycine max*	Drought	Increased yield and protects from stress *via* upregulation of CAT and POD activity	Sheteiwy et al., 2021
AMF and *Rhizobium* spp.	*Glycine max*	Drought	Improved plant health and microbial diversity in soil Triggered CAT, proline, and IAA production	Igiehon et al., 2021
Serratia marcescens	*Lactuca sativa*	Salinity		Fortt et al., 2022

protects and improved *Phaseolus vulgaris* plant growth under salinity stress *via* the production of IAA and ACC deaminase. Meena et al. (2020) reported that *Nocardioides* sp. improved seedling growth of *Triticum aestivum* under salt stress (0–100 mM) *via* increasing the CAT and POD genes. Inoculation of *Penicillium* and *Ampelomyces* spp. improved drought and

salinity stress tolerance in tomato plants *via* the production of osmolytes, stress-responsive genes, and antioxidant enzymes (Morsy et al., 2020). Inoculation of *Piriformospora indica* highly enhanced plant development and attenuated NaCl-induced lipid peroxidation which helps to build tolerance during salinity stress (Ghaffari et al., 2018). Studies show that the inoculation of *Trichoderma longibrachiatum* T6 in wheat increased the levels of antioxidant enzymes (SOD, POD, and CAT) which helped to improve the stress tolerance in plants during salt stress (Zhang et al., 2016). *Agrobacterium* and *Raoultella* showed production of IAA, HCN, and ACC under salt stress and improved growth of *Tetragonia tetragonioides* plants (Egamberdieva et al., 2022). Fortt et al. (2022) reported that the application of PGPR improved the growth of lettuce under salt stress *via* the production of IAA and antioxidant enzymes which provide protection to plants.

Temperature stress

Global warming is a serious risk to all living creatures and is becoming a worldwide concern. Temperature stress such as heat and cold greatly limits the growth and development of plants (Yadav et al., 2018). Heat stress causes modification in homeostasis, degradation of proteins, which have lethal effects on physiology of plants as it delays the seed germination, damages to seeds and affects agricultural production (Imran et al., 2021). Cold stress causes dehydration due to ice formation which is responsible for protein denaturation. It also causes plant leaves lesions, yellowing of leaves, and rotting. It also affects the seed germination and yield of crops (Wu et al., 2021b). The application of several microbes alleviated the damaging effects of heat stress in various plants such as wheat, tomato, and sorghum *via* producing phytohormones, biofilm formation, and enhancing heat shock proteins (Issa et al., 2018; Sarkar et al., 2018). *Bacillus cereus* inoculation in tomato plants increased the production of HSPs, IAA, essential amino acids, and organic acids and protects plants from stress conditions (Khan et al., 2020). Inoculation of *Azospirillum* and *B. amyloliquefaciens* improved the heat tolerance *via* reducing oxidative damage in wheat seedling (Abd El-Daim et al., 2014). Duc et al. (2018) reported that *Glomus* sp. tolerates heat stress and protects tomato plants *via* scavenging ROS generation. *Bacillus velezensis* improved wheat plant survival under cold stress *via* increase in cold stress-related proteins as reported by Abd El-Daim et al. (2019). Zulfikar et al. (2011) reported that *Pseudomonas putida* also improved the growth of wheat plants under heat stress *via* enhanced production of proline, sugars, and antioxidant enzymes. *R. irregularis* and *F. mosseae* increased plant height, transpiration rate in maize, and nutrient composition in roots of *triticum aestivum* during heat stress (Cabral et al., 2016). *Paraburkholderia phytofirmans* having ACC deaminase-producing efficiency helps in normal growth of tomato plants under heat stress as reported by Esmaeel et al. (2018). Bacterial inoculants such as *Rhodococcus* and *Burkholderia* protect the medicinal plant *Atractylodes lancea* from heat stress and improved their growth *via* enriched root-associated microbes (Wang et al., 2022a).

Heavy metal stress

Extreme usage of inorganic chemical fertilizers in agriculture system causes the accumulation of toxic metals such as nickel, manganese, cadmium, iron, and zinc in soil (Ghori et al., 2019). These metals are beneficial for plants at low level, but if their concentration increases cause stress *via* decrease in plant growth due to the decrease in photosynthesis, deprived nutrients, membrane integrity, and enzyme activities. It causes oxidative stress *via* ROS and H_2O_2 generation and reduces plant growth and crop productivity (Ahmad et al., 2019; Gong et al., 2020). ROS generation occurs both under favorable and unfavorable circumstances, and it has a negative impact on vital macromolecules (Köhl et al., 2019). *Rhizobium* inoculation at nickel-contaminated site improves the chlorophyll content and increased lentil plant growth (Wani and Khan, 2013). *Bradyrhizobium* increased IAA production and siderophore production and improved the shoot weight of *Lolium multiflorum* at cadmium-contaminated site (Guo and Chi, 2014). *Candida parapsilosis* and *B. cereus* protect *Trifolium repens* plants from heavy metal stress conditions as reported by Azcón et al. (2010). Toxicity of arsenic in *Brassica juncea* is reduced by *Staphylococcus arlettae via* enhanced production of dehydrogenase and phosphatase enzyme in soil (Srivastava et al., 2013). Inoculation of *Talaromyces pinophilus in Triticum aestivum* plants stimulates plant growth *via* the production of gibberellic acid under heavy metal stress (El-Shahir et al., 2021). Paredes-Páliz et al. (2018) reported that inoculation of metal-resistant bacteria such as *B. aryabhattai* and *Pantoea agglomerans* brings production of phenylalanine ammonia-lyase enzyme and SOD which protects plants from metal stress. The addition of bioinoculants like *P. aeruginosa* and *Burkholderia gladioli* reduced Cd toxicity in *Solanum lycopersicum* by producing phenols, organic acids, and osmoprotectants (Khanna et al., 2019). Application of *Serratia marcescens* and *E. bugandensis* improved spinach (*Ipomoea aquatica*) growth *via* the production of polyamine under Pb and Cd toxicity (Wang et al., 2022b). *Citrobacter* and *Enterobacter cloacae* mitigate the Cd and Pb toxicity, improve the wheat plant health parameters, and protect from stress *via* the generation of antioxidant enzymes (Ajmal et al., 2022). Oubohssaine et al. (2022) reported that *Pseudarthrobacter oxydans* improved *Sulla spinosissima* growth and can be used as biofertilizer at heavy metal-contaminated sites. Cadmium tolerance bacteria such as *Curtobacterium ocenosedimentum* having P-solubilizing,

IAA, and siderophore-producing possessions improved chili growth and increased shoot/root length (Patel et al., 2022). Inoculation of *Pseudoarthrobacter* and *Vibrio neocaledonicus* improved the *Salicornia ramosissima* growth at As- and Cu-polluted sites (Mesa-Marín et al., 2020). *Rhizobium* inoculation can promote soil nutrient cycling by increasing enzyme activity in metal-contaminated soil, thereby providing more N and P for microbial activity and growth of plants (Ma et al., 2021; Duan et al., 2022). Heavy metal toxicity is a growing problem in the world; therefore, finding appropriate microbes proficient to depollution of the metals can benefit to improve the crop efficiency. Application of biofertilizers for sustainable food crop production and boosting various stress tolerance of plants are gaining popularity. Still, further studies are crucial to unravel the potential role of biofertilizers in responding to the impact of different stresses at molecular level.

Conclusion

Agriculture systems have to face the task of food production, stress management, and dependency on agrochemicals. The presence of pest and pathogen in crops causes decrease in crop yield and heavy crop losses every year. The occurrence of abiotic stresses due to the change in climatic conditions leads to difficult challenge to crop production worldwide. Different effective approaches should be employed to reduce crop output loss and control diseases. Hence, the necessity to implement the eco-friendly approaches such as biofertilizers is of great importance for sustainable agriculture. The application of biofertilizers not only improves plant heath parameters but also enhances the crop productivity, soil health and protects from stress environment. More research has been focused on physiological and molecular aspects under different conditions with different crops using biofertilizers under field conditions.

Author contributions

PC: conceptualization and wrote the manuscript. SS, AC, AS, and GK: editing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Effects of single- and mixed-bacterial inoculation on the colonization and assembly of endophytic communities in plant roots

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The introduction and inoculation of beneficial bacteria in plants have consistently been considered as one of the most important ways to improve plant health and production. However, the effects of bacterial inoculation on the community assembly and composition of the root endophytic microbiome remain largely unknown. In this study, 55 strains were randomly isolated from tomato roots and then inoculated into wheat seeds singly or in combination. Most of the isolated bacterial strains showed an ability to produce lignocellulose-decomposing enzymes and promote plant growth. The results demonstrated that bacterial inoculation had a significant effect on the wheat root endophytic microbiome. The wheat root samples inoculated with single-bacterial species were significantly separated into two groups (A and B) that had different community structures and compositions. Among these, root endophytic communities for most wheat samples inoculated with a single-bacterial strain (Group A) were predominated by one or several bacterial species, mainly belonging to *Enterobacterales*. In contrast, only a few of the root samples inoculated with a single-bacterial strain (Group B) harbored a rich bacterial flora with relatively high bacterial diversity. However, wheat roots inoculated with a mixed bacterial complex were colonized by a more diverse and abundant bacterial flora, which was mainly composed of *Enterobacterales*, *Actinomycetales, Bacillales, Pseudomonadales,* and *Rhizobiales*. The results demonstrated that inoculation with bacterial complexes could help plants establish more balanced and beneficial endophytic communities. In most cases, bacterial inoculation does not result in successful colonization by the target bacterium in wheat roots. However, bacterial inoculation consistently had a significant effect on the root microbiome in plants. CAP analysis demonstrated that the variation in wheat root endophytic communities was

significantly related to the taxonomic status and lignocellulose decomposition ability of the inoculated bacterial strain ($p < 0.05$). To reveal the role of lignocellulose degradation in shaping the root endophytic microbiome in wheat, four bacterial strains with different colonization abilities were selected for further transcriptome sequencing analysis. The results showed that, compared with that in the dominant bacterial species Ent_181 and Ent_189 of Group A, the expression of lignocellulose-decomposing enzymes was significantly downregulated in Bac_133 and Bac_71 ($p < 0.05$). In addition, we found that the dominant bacterial species of the tomato endophytic microbiome were more likely to become dominant populations in the wheat root microbiome. In general, our results demonstrated that lignocellulose-decomposing enzymes played a vital role in the formation of endophytes and their successful colonization of root tissues. This finding establishes a theoretical foundation for the development of broad-spectrum probiotics.

KEYWORDS

plant endophytic microbiome, probiotics, bacterial inoculation, bacterial colonization, lignocellulase

Introduction

Plants harbor a set of taxonomically and functionally diverse microbial communities on their surfaces and within their tissues. Similar to the human microbiome, the plant microbiome contains an enormous number of genes, and the genetic content far exceeds that of the host plant itself (Yun, 2020). These microorganisms establish an intimate relationship with their hosts, ranging from beneficial or neutral interactions to harmful interactions (Bulgarelli et al., 2013; Turner et al., 2013; Nair and Padmavathy, 2014; Santoyo et al., 2016). In recent years, the plant microbiome has attracted much attention for its role in protecting the host against abiotic and biotic stresses, promoting plant nutrient uptake and utilization, protecting plants from pathogens, and improving plant health and production (Ma et al., 2011; Gaiero et al., 2013; Truyens et al., 2014; Hardoim et al., 2015; Blain et al., 2017; Iqbal et al., 2017). Plant microorganisms can directly antagonize pathogenic microorganisms through parasitism, secretion of antibiotics, or spatial nutrition competition and can indirectly inhibit microbial pathogenicity by inducing plant resistance or biological control agent (BCA) activity. The microbial communities associated with plants are regarded as one of the important functional driving forces for these eukaryotic hosts and can extend the host genome and metabolic capacity, providing or promoting a range of basic life-sustaining functions, including nutrient acquisition, immune regulation, and biological stress resistance (Cordovez et al., 2019). However, the application of beneficial bacteria in the field cannot achieve the expected results in practice. One reason for this is that not all beneficial bacteria have the ability to successfully colonize the plant rhizosphere or root tissue and play a role in biocontrol (Zhou et al., 2018). This phenomenon may be due to insufficient settlement in the rhizosphere or plant tissue, as the introduced microorganisms are usually washed away and do not survive at a meaningful functional density in plants or soil ecosystems. In this context, it is essential to understand the common pathways associated with the mechanisms of the assembly, activity, and persistence of the plant-associated microbiota and the interactions among them (Weller, 1988; Gibbons et al., 2016; Maldonado-Gomez et al., 2016; Sessitsch et al., 2018; Cordovez et al., 2019).

Studies showed that the assembly of the plant microbiome is a continuous and multistep process that is jointly determined by active diffusion, species interactions, the environment, and the host. Early colonizing microorganisms can be transmitted vertically through parental seed transmission routes. Once seeds germinate, the assembly of microorganisms is likely to be driven by horizontal transfer. Seed-borne microorganisms preferentially combine with aboveground plant tissues, while soil-derived microorganisms mainly combine with the rhizosphere and roots (Trivedi et al., 2020). The root microbiome is likely to be dynamically recruited and assembled during the life cycle of its plant host (Trivedi et al., 2020). A two-step selection model for root microbial flora differentiation has been previously reported. In this model, during the first step of differentiation, the characteristics of the root sediment and host cell wall promote the growth of bacteria that use organic nutrients, which lead to the migration of soil biological communities and the formation of rhizosphere communities.

Taking strains of *Pseudomonas* as an example, when these strains come in contact with the plant root surface, they first form a colony and then form biofilms. In this way, *Pseudomonas* strains can colonize host plants as well as the fungi in the rhizosphere (Yang et al., 2010). During the second step of differentiation, the community structure of the rhizosphere and root is finely tuned. A comparison of the bacterial and fungal root microflora of mature poplar in two natural sites showed that the endophytic community composition was significantly different compared with that of the surrounding rhizosphere (Gottel et al., 2011), potentially because not all rhizospheric bacteria can become root endophytes (Bulgarelli et al., 2012; Tian et al., 2015). Due to host genetic factors and the selective colonization resistance of the host's inherent microflora, only a small number of microorganisms are successful in colonization. Through verification of *Pseudomonas aeruginosa* (*P. chlororaphis*) strain PCL1391, Yang et al. (2010) found that mutant strains lacking colonization ability completely lost the ability to control damping off in tomatoes compared with the wild-type strains. The relationship between the colonization level of biocontrol strains of *P. fluorescens* in different parts of wheat roots and the number of spots of wheat take-all disease was studied, and the results confirmed that the colonization level of the biocontrol strains was inversely proportional to the number of disease spots on the host plants. The higher the level of colonization was, the lower the number of disease spots. When the root colonization level reached 10^7–10^8 CFU/cm, almost no disease spots were produced (Yang et al., 2010).

Colonization is a key step for limiting the biocontrol effect of beneficial microorganisms in plants (Yang et al., 2010). In general, the colonization ability of bacteria in host plant roots is related mainly to the genetic characteristics of the bacterial strains. First, microorganisms can attach themselves to plant surfaces for successful colonization, which is achieved through flagella and fimbriae (de Weert et al., 2002; Anna et al., 2017). The second step involves bacterial chemotaxis. de Weert et al. (2002) demonstrated the role of chemotaxis in the colonization ability of *Pseudomonas fluorescens* by constructing mutants of cheA, a key gene involved in the process of chemotaxis. Compared with that of the wild type, the colonization ability of the four mutants was weakened in all parts of the root, and this weakening increased from the root base to the root tip, indicating that chemotaxis played a very important role in the process of competitive site colonization. Third, a study showed that polymer-degrading enzymes, such as endoglucanase and polygalacturonidase, play an important role in helping bacteria penetrate into the endosphere of the root (Compant et al., 2005). Bulgarelli et al. (2012) and Xl et al. (2020) proposed that the lignocellulosic properties of plant hosts play a more important role than the internal environment of plants in determining whether rhizospheric bacteria become endophytes. There is evidence that colonization by endophytes in the internal tissues of plants involves the production of cellulases and pectinases, such as endoglucanase, pectinate lyase, and polygalacturonase (Alden et al., 2001). Thus, cell wall–degrading enzymes are most likely the key determinants of the initial entry and colonization by bacteria in plant hosts, and natural endophytic bacteria have the ability to secrete a variety of enzymes that help them penetrate the polysaccharide barrier, enabling them to survive in plants. After successful colonization by plant bacteria, the amount of bacteria colonizing the rhizosphere soil or plant roots plays a vital role in the biocontrol or beneficial effect of the rhizobacteria or endophytes.

In this study, 55 bacterial strains with plant growth-promoting effects were screened from isolated tomato endophytes and then used to inoculate wheat seeds. High-throughput sequencing and transcriptome sequencing were used to explore the following aspects: (1) examine the effect of bacterial inoculation with a single bacterium or a mixed bacterial complex on the community assembly and composition of the root endophytic microbiome of plants; (2) identify the factors that determine whether a microorganism becomes a root endophyte or successfully colonizes the inner tissue of plant roots; and (3) identify the reasons for the differences in colonization ability, which is why some strains exhibit colonization or even become excellent endophytes. A better understanding of the colonization ability of bacterial endophytes will contribute to the study of plant–endophyte interactions in agroecosystems and natural ecosystems.

Materials and methods

Isolation and identification of endophytic bacterial strains from tomato roots

The tomato cultivar Xinzhongshu No. 4, which was grown in a greenhouse under natural light conditions, was used for the isolation of endophytic bacteria. After 55 days of growth with nutrients, the tomato plants were pulled from the soil, and their roots were shaken to remove large soil particles. The harvested roots were carefully rinsed with tap water to remove the tightly attached soil, separately placed in 75% ethanol solution for 1 min and 5% NaClO solution for 3 min for sterilization, and then rinsed again with sterile water three times. The sterilized tomato roots were placed on LB agar plates at 37°C overnight to assess sterility to ensure that the isolated bacteria were from endophytic bacteria within the roots. The surface-sterilized root tissues were ground, serially diluted to 10^{-7}, inoculated on different media, and incubated in an incubator at 28° or 37°C. The experimental medium included Luria-Bertani agar (LB), nutrient agar (NA), tryptic soy agar (TSA), Gauze No. 1 medium, and Hoagland nitrogen-free medium. The bacterial colonies on the plate were selected according to size, color, and shape. The isolated endophytic bacteria were tested for growth

on LB at 28° or 37°C and transferred to a new plate two times for purification. Then, the purified strains were divided into two portions for seed preservation and stored at 20% glycerol in a -20°C freezer for further study.

The genomes of the isolated bacterial strains were extracted according to instructions for the Ezup Column Bacteria Genomic deoxyribonucleic acid (DNA) Purification Kit. A pair of universal primers, 27F and 1492R, was used for PCR amplification of bacterial 16S rDNA, using the extracted DNA as a template. The basic conditions for amplification were as follows: denaturation at 94°C for 30 s, followed by 30 cycles of annealing at 60°C for 30 s, and extension at 72°C for 90 s. The reaction volume was 50 μL, and 2 μL of DNA template was added for each reaction. The concentration of the PCR products was determined by 1% agarose electrophoresis. The purified PCR products of the 16S rRNA gene for each strain were sent for sequencing and molecular identification. After removing the repetitive strains, 55 isolated endophytic strains were selected for the subsequent bacterial colonization experiments, and the results of the molecular identification are displayed in **Table 1**.

Screening of the cellulase and xylanase activities of the isolated bacterial endophytes

In this experiment, the cellulase and xylanase activities of 55 isolated bacterial strains were detected. To screen for cellulase activity, the overnight cultured bacterial solutions were spotted on CMC agar plates (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethyl cellulose (CMC), 0.02% peptone, and 1.7% agar in 1,000 mL of H_2O) with a sterile tip. The CMC agar plates were incubated at 28°C for 48 h and stained with Gram's iodine solution (2.0 g of KI and 1.0 g of iodine in 300 ml of distilled water) for 3–5 min. The cellulase activity was determined by observing the presence or absence of hydrolytic circles. Standard cellulose solution was used as a positive control, and sterile water was used as a negative control. To screen for xylanase activity, similar agar plates were prepared using xylan as a substrate rather than CMC, and then, the plates were incubated at 28°C for 48 h and stained with Gram's iodine solution for 3–5 min.

Bacterial inoculation and colonization experiment with wheat plants

For the single-bacterial inoculation experiment, the 55 endophytic strains were separately inoculated in liquid LB medium and cultured at 28°C (120 r · min^{-1}) for 8 h (some strains were shaken for 10–24 h). The concentrations of the bacterial cultures were determined at an OD_{600} of 0.5, and 100 μL of bacterial suspension was collected for each strain. Wheat seeds were surface-sterilized with NaClO (5%) for 10 min and thoroughly rinsed with sterile water three times. The sterilized seeds were sown in sterile plastic chambers with 2–3 layers of filter paper and incubated overnight at 28°C under dark conditions. Ten healthy wheat seeds were then placed in sterile chambers with 2–3 layers of filter paper and were separately treated with 50 μL of bacterial culture suspension. All the treated samples were transferred into a light incubator at 28°C for 3–5 days and then incubated under natural light at room temperature for 10–12 days. Sterile water was added promptly to keep the filter paper moist. The control plants were treated with a sterile medium solution. The experiment for each bacterial strain was carried out in triplicate.

After the single-bacterial inoculation experiment for the 55 strains, we selected 4 bacterial strains with the different ability to colonize wheat roots, Ent_181 (absolute dominance), Ent_189 (absolute dominance), Bac_71 (relative dominance), and Bac_133 (non-dominance), according to their abundance in the inoculated root samples. Furthermore, we constructed 3 mixed bacterial complexes using the 4 selected bacterial strains and their taxonomic relatives (**Table 2**). Based on these 4 strains and the 3 mixed bacterial complexes, we compared the effects of single- and mixed-bacterial inoculation on the root endophytic microbiome in wheat. The experiment with 4 single-bacterial and 3 mixed-bacterial inoculations was performed using the same protocol as that described above.

Genomic deoxyribonucleic acid extraction for the wheat root samples with single- and mixed-bacterial inoculations and Illumina high-throughput sequencing

The healthy wheat plants were separately collected for each treatment, and roots were removed with sterile scissors. Wheat roots were disinfected in 5% NaClO solution for 30 s with sterilized forceps and then rinsed with sterile water three times. Subsequently, they were disinfected with 75% ethanol for 5–10 s and rinsed with sterile water three times again. The sterilized roots were used for the extraction of total genomic DNA by using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States) according to the instructions. The extracted total genomic DNA was stored at –80°C until being subjected to high-throughput sequencing.

DNA fragments of the V5–V7 region of the bacterial 16S rRNA gene were amplified using the primer pair 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) and fused with Illumina MiSeq adapters and a 6-bp barcode sequence unique to each sample. PCR amplifications were carried out in triplicate in a 50-μL reaction system using the extracted genomic DNA as templates. The PCR amplification products were subsequently

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TABLE 1 Taxonomic and lignocellulose identification of 55 endophytic strains isolated from tomato root microbiota.

Strains	Molecular identification	Sequence Identity (%)	Gene IDs	Xylanase activity	Cellulase activity
Ach_45	*Achromobacter insuavis*	100	ON242109	+ ++	+++
Aci_112	*Acidovorax monticola*	98.5	ON242156	+	+
Bac_1	*Bacillus velezensis*	100	ON242110	++	++
Bac_14	*Bacillus amyloliquefaciens*	99.93	ON242115	−	−
Bac_27	*Bacillus nitratireducens*	99.72	ON242122	+	+
Bac_36	*Bacillus subtilis*	99.79	ON242123	+	+
Bac_43	*Bacillus subtilis*	99.93	ON242124	−	+
Bac_44	*Bacillus cereus*	99.86	ON242125	−	+
Bac_51	*Bacillus cereus*	99.39	ON242126	+ ++	+++
Bac_64	*Bacillus aryabhattai*	100	ON242127	+ +	++
Bac_67	*Bacillus megaterium*	99.86	ON242128	+ +	++
Bac_68	*Bacillus megaterium*	99.57	ON242129	−	−
Bac_71	*Bacillus cereus*	99.79	ON242130	+ ++	+++
Bac_79	*Bacillus cereus*	100	ON242131	+ ++	+++
Bac_92	*Bacillus aryabhattai*	99.56	ON242132	+ +	++
Bac_98	*Bacillus subtilis*	99.78	ON242133	+ +	++
Bac_101	*Bacillus velezensis*	99.16	ON242111	+ +	++
Bac_133	*Bacillus subtilis*	99.86	ON242112	+ +	++
Bac_138	*Bacillus cereus*	99.79	ON242113	+ ++	+++
Bac_139	*Bacillus cereus*	96.68	ON242114	+ ++	+++
Bac_152	*Bacillus anthracis*	99.65	ON242116	+ ++	+++
Bac_165	*Bacillus velezensis*	99.29	ON242117	+ +	++
Bac_183	*Bacillus altitudinis*	100	ON242118	+ +	++
Bac_186	*Bacillus paranthracis*	99.7	ON242119	+ ++	+++
Bac_204	*Bacillus subtilis*	100	ON242120	+ +	++
Bur_95	*Burkholderia ambifaria*	99.84	ON242134	−	−
Ent_2	*Enterobacter ludwigii*	99.43	ON242141	+	+
Ent_4	*Enterobacter ludwigii*	99.58	ON242147	++	++
Ent_15	*Enterobacter mori*	99.26	ON242136	−	+
Ent_17	*Enterobacter cloacae*	99.03	ON242137	+	+
Ent_22	*Kosakonia oryzendophytica*	99.93	ON242142	−	−
Ent_29	*Enterobacter cloacae*	98.78	ON242143	−	−
Ent_33	*Enterobacter ludwigii*	99.56	ON242144	+	+
Chr_38	*Chryseobacterium sediminis*	99.55	ON242145	−	+
Ent_39	*Enterobacter* sp.	99.79	ON242146	−	+
Ent_113	*Enterobacter cloacae*	100	ON242135	+	+
Ent_117	*Enterobacter quasiroggenkampii*	96.74	ON242152	++	++
Ent_181	*Enterobacter cloacae*	99.71	ON242138	+	+
Ent_188	*Enterobacter cloacae*	100	ON242139	+++	+++
Ent_189	*Enterobacter cloacae*	100	ON242140	+	+
Lel_129	*Lelliottia amnigena*	99.46	ON242148	−	−
Lys_159	*Lysinibacillus xylanilyticus*	99.44	ON242149	+	−
Pan_120	*Pantoea*_sp.	99.86	ON242150	−	−
Pse_104	*Pseudomonas frederiksbergensis*	99.48	ON242151	+ ++	+++
Pse_61	*Pseudomonas plecoglossicida*	99.72	ON242153	+ +	++
Pse_77	*Pseudomonas plecoglossicida*	99.78	ON242154	+ ++	+++
Pse_97	*Pseudomonas extremaustralis*	98.67	ON242155	+	+
Pse_208	*Pseudomonas nicosulfuronedens*	99.43	ON242121	++	++
Rhi_114	*Rhizobium radiobacter*	99.02	ON242157	+	+
Rhi_130	*Rhizobium larrymoorei*	99.84	ON242158	+	+
Rhi_34	*Rhizobium radiobacter*	99.34	ON242159	+	+
Ser_99	*Serratia GRIMESII dsm*	99.78	ON242160	−	−
Sta_52	*Staphylococcus pasteuri*	99.93	ON242161	+ +	++
Sta_72	*Staphylococcus epidermidis*	99.93	ON242162	+ +	++
Ste_26	*Stenotrophomonas maltophilia*	99.41	ON242163	+	+

+ means enzymatic activity, −means no enzymatic activity.
+ (Within 0.5 cm of hydrolysis circle), + + (within 0.5 1.9 cm), + + + (above 2 cm).

TABLE 2 Bacterial strains of the inoculated bacterial complex in the wheat root.

Groups	Bacterial strain	Taxonomy	Abundance status
Single	Bac_71	*Bacillus*	Dominant bacteria
	Bac_133	*Bacillus*	Non-dominant bacteria
	Ent_181	*Enterobacter*	Dominant bacteria
	Ent_189	*Enterobacter*	Dominant bacteria
BacM	Bac_71	*Bacillus*	Dominant bacteria
	Bac_133	*Bacillus*	Non-dominant bacteria
	Bac_64	*Bacillus*	Dominant bacteria
	Bac_79	*Bacillus*	Dominant bacteria
	Bac_68	*Bacillus*	Dominant bacteria
	Bac_27	*Bacillus*	Non-dominant bacteria
	Bac_138	*Bacillus*	Non-dominant bacteria
	Bac_139	*Bacillus*	Non-dominant bacteria
	Bac_186	*Bacillus*	Non-dominant bacteria
EntM	Ent_181	*Enterobacter*	Dominant bacteria
	Ent_189	*Enterobacter*	Dominant bacteria
	Ent_113	*Enterobacter*	Dominant bacteria
	Ent_29	*Enterobacter*	Dominant bacteria
	Ent_17	*Enterobacter*	Dominant bacteria
Mix	Bac_71	*Bacillus*	Dominant bacteria
	Bac_133	*Bacillus*	Non-dominant bacteria
	Bac_64	*Bacillus*	Dominant bacteria
	Bac_79	*Bacillus*	Dominant bacteria
	Bac_68	*Bacillus*	Dominant bacteria
	Ent_181	*Enterobacter*	Dominant bacteria
	Ent_189	*Enterobacter*	Dominant bacteria
	Ent_113	*Enterobacter*	Dominant bacteria
	Ser_99	*Serratia grimesii*	Dominant bacteria
	Bur_95	*Burkholderia*	Dominant bacteria

purified, combined in equimolar ratios, and subjected to high-throughput sequencing with the Illumina MiSeq sequencing platform, which produced 250 paired-end nucleotide reads, at Sangon Biotech (Shanghai, China). Two rounds of sequencing were conducted to ensure that an adequate amount of bacterial DNA (no less than 4,000 reads for each sample) was available for subsequent analysis after the removal of all the chloroplast sequences.

Data processing and statistical analysis of the endophytic microbiome of wheat roots inoculated with bacteria

The raw sequences were separated according to barcode tags, and pairs of short-read sequences (reads) were spliced with FLASH software (version 1.2.3). Barcodes and primer sequences were removed using Cutadapt (version 1.9.1). Low-quality sequences with ambiguous bases, average quality scores < 25, or sequences shorter than 200 bp were removed to control sequence quality. Then, chimeric sequences were identified and removed with a *de novo* method using USEARCH (version 8.1.1861) (Edgar, 2010). After sequence control, the obtained effective sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using Usearch (version 8.1.1861). The sequence with the highest abundance in each OTU was selected as the representative sequence. The obtained representative sequences were compared and annotated with the SILVA database using the QIIME package RDP Classifier to obtain the taxonomic information corresponding to each OTU (Haas et al., 2011). According to the obtained classification information of the OTUs, the OTUs classified as plant chloroplast and mitochondrial were deleted from the representative sequence. New OTU clustering and classification annotation were carried out to generate OTU tables for subsequent data analysis. To ensure the consistency of the results, a fixed number of sequence reads from each sample were separately rarefied on the bassi of the sample with the smallest number of reads. OTU clustering and classification analysis were then performed to generate OTU tables for subsequent data analysis.

The taxonomic units and their relative abundances in each sample were visualized by drawing bar charts and heatmaps based on the number of reads using the R package gplots (version 2.17.0). The diversity indexes (Shannon and Simpson index) and species richness estimators (Chao1 diversity and ACE index) for each sample with respect to a sequence depth of 3% were calculated using QIIME (version 1.8.0). Rarefaction and rank abundance curves were generated at a 97% OTU similarity level. Principal co-ordinates analysis (PCoA) and hierarchical cluster analysis in QIIME were used to evaluate the beta diversity of samples and the similarities and differences in community composition of different samples. Statistical analysis was performed based on the unweighted UniFrac distances and Bray–Curtis matrix to determine the significant differences between samples. The differences in bacterial taxa among the groups of samples were identified by LEfSe (LDA effect size) analysis (Edgar, 2013). First, the species with significant differences in relative abundance between different treatments were calculated using the R package edgeR with a p-value less than 0.05 (and FDR to control the false-positive rate at less than 5%). Through statistical analysis of the degree of influence of species on the samples, the value of the influence of each significantly different species on the sample was obtained, and significantly different microorganisms with a value of influence greater than two could be visualized.

Metatranscriptomic analysis for wheat root samples inoculated with bacterial strains

To reveal the potential functional traits corresponding to the bacterial localization in the wheat root microbiome, four endophytic strains, Ent_181, Ent_189, Bac_71, and Bac_133, with colonization ability were selected for inoculation into

wheat roots in sterile Petri dishes. The inoculated plants were grown, and the roots were harvested as described above. After surface disinfection, the sterilized plant roots were cutoff and placed into sterile bowls for grinding. Then, Phosphate Buffer Saline (PBS) was added to dilute the grinding solution to prepare the bacterial suspension. The bacterial suspension was obtained by serially filtering through 100, 20, and 11 μm membranes to remove plant tissues. Finally, the obtained endophytic bacterial precipitates for each sample were collected and sent to a commercial service for RNA extraction and transcriptional sequencing by using Illumina HiSeq^{TM}.

The obtained raw data for each sample were first evaluated by FastQC, and then, reads containing adapters and low-quality bases (Q-score $\leq$ 5) were removed to obtain clean data. The clean data were assembled into transcripts *de novo* (Tjaden, 2015) and then mapped to the gene-coding sequences by using Bowtie2. The read counts were normalized to the gene lengths, and the total number of non-rRNA metatranscriptomic reads was converted to reads per kilobase of transcript per million reads mapped (RPKM) values for subsequent statistical analysis (Langmead and Salzberg, 2012; Wang et al., 2012). The collected assembled transcripts were annotated using blastx against the NCBI nr database. The top hits assigned to amino acid sequences with taxonomic information were then used for downstream analysis.

According to the annotation results for the transcripts, the annotated GeneOntology (GO) function information was obtained, and the top GO functions were used for the analysis of GO enrichment to draw the network diagram. KAAS was used to obtain the annotated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway information of the transcripts, and clusterProfiler was used for the analysis of the KEGG pathways and COG enrichment (Kanehisa and Goto, 2000; Tatusov et al., 2000; Yuki et al., 2007). Salmon was used to calculate the expression levels of gene, and WGCNA was used for the analysis of gene coexpression levels. Analysis of the expression of differential gene was performed using DESeq2 (Tatusov et al., 2000).

Results

Sequencing data statistics for the root endophytic microbiome of wheat infected with a single-bacterial strain or mixed bacteria

The total genomic DNA of wheat root samples infected with 55 single-bacterial strains, parallel control samples, and mixed bacteria was amplified and sequenced in the V5–V7 region of bacterial 16S rDNA using Illumina MiSeq with 2 × 250 bp reads. The obtained raw sequence reads were separately merged and filtered for quality control (removal of primer and vector sequences, sequences with low-quality scores, chimeric sequences, etc.). Finally, a total of 23,36,079 high-quality sequences for 58 root samples were obtained. On average, 41,713 high-quality sequences were obtained per sample (min = 27,871, max = 67,899). For the inoculation experiment with the mixed bacterial complex, 806,643 pairs of raw sequences (reads) were obtained for 21 root samples from 7 groups after high-throughput sequencing. These sequences were also filtered for quality control, and 690,046 high-quality sequences were obtained. On average, 32,859 high-quality sequences were obtained per sample (min = 32,206, max = 33,291).

Identification of wheat root endophytes and the effects of single-bacterial inoculation on the community structure and composition of the wheat root microbiome

The 55 identified plant growth-promoting bacterial strains belonged to 13 bacterial taxa, including *Achromobacter*, Bacillaceae, *Chryseobacterium*, *Lysinibacillus*, *Pantoea*, Pseudomonadaceae, *Acidovorax*, Rhizobiaceae, *Serratia*, *Staphylococcus*, *Stenotrophomonas*, and *Burkholderia*, covering a broad range of bacterial phyla. The wheat root samples with single-bacterial inoculation were divided into Group A, Group B, and the control group for further bioinformatic analysis according to the Shannon index and community diversity of the endophytic microbiome. Compared with those of the control wheat group, the community structure and the composition of the root endophytic root microbiome of the inoculated groups changed greatly, suggesting that single-bacterial inoculation greatly affected the structure and proportion of the flora in the wheat roots (**Figure 1A**). In the control samples, Bacillales (74.9%) was the predominant bacterial group of the wheat root microbiome, followed by Actinomycetales (14.3%), Rhodospirillales (4.12%), and Enterobacteriales (2.49%). In contrast, Bacillales was not the dominant bacterial group in most wheat root samples with single-bacterial inoculation (**Figure 1A**). Moreover, the OTU richness and bacterial diversity of the root microbiome were significantly different between the control samples and the root samples with bacterial inoculation ($p < 0.05$), suggesting that single-bacterial inoculation had a significant effect on the bacterial diversity (**Figures 1B,C**). The grouping results showed that the diversity and abundance of the bacterial flora of Group A, Group B, and the control group were significantly different due to inoculation. Regardless of whether the corresponding bacterial strains were inoculated, Group A, which included 36 root samples, was mostly colonized by a certain type of flora, mainly the pathogenic bacterium of the genus *Enterobacter*. Compared with those of Group A, the root samples of Group

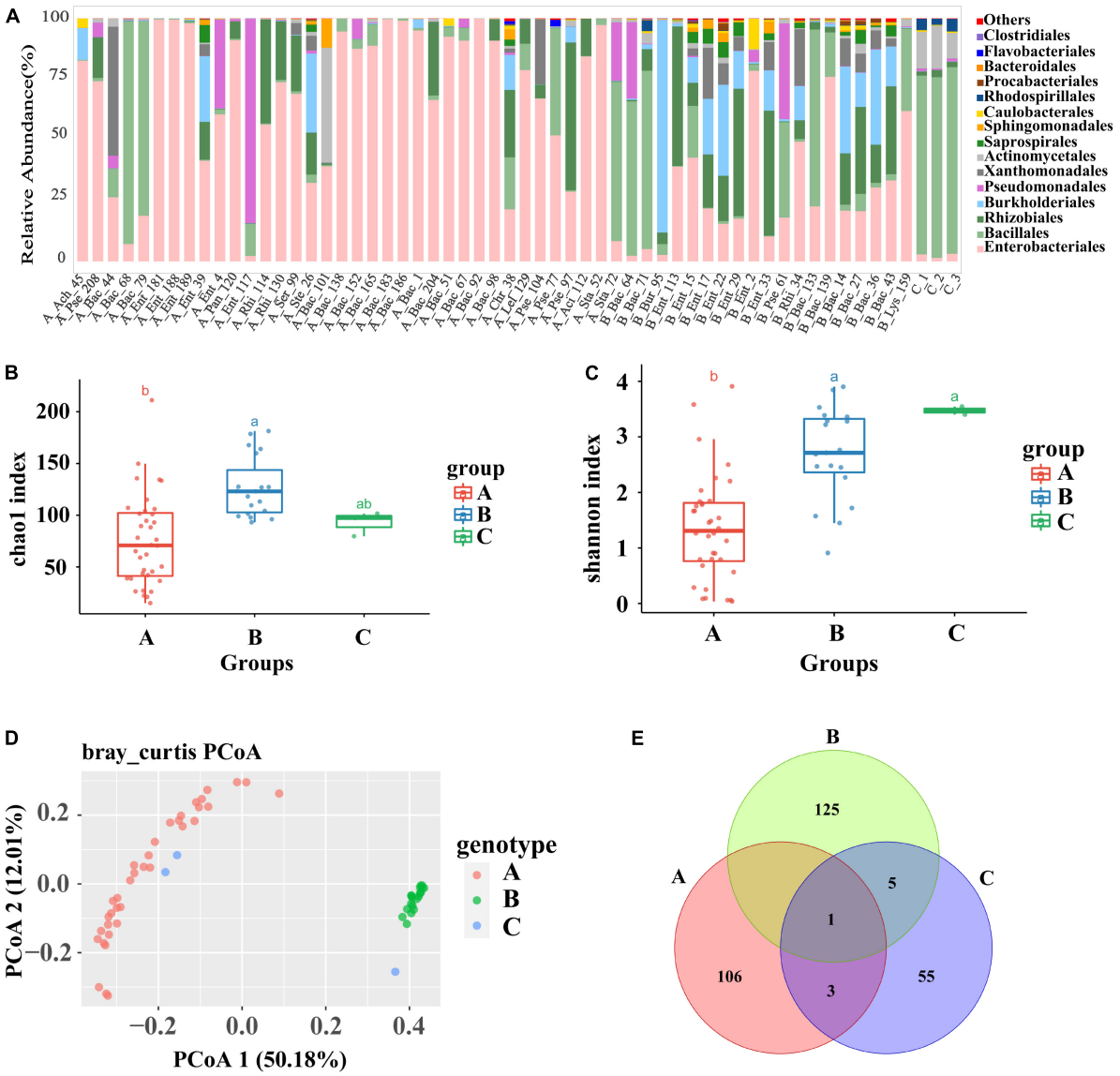

FIGURE 1
Analysis of the composition and diversity of the flora of the roots of tomato infested with 55 single bacteria and the control group. **(A)** Diagram of the species structure of 55 single bacteria and the control group (at the order level, selecting the top 15 flora in terms of abundance). **(B,C)** The Chao1 boxplot and Shannon boxplot of 55 single bacteria and control group (the letters above represent the differences between groups). **(D)** PCoA plots of 55 single bacteria and the control group, principal coordinated analysis (PCoA) derived from dissimilarity matrix of Unweighted UniFrac distance. **(E)** A Venn plot of 55 single bacteria and the control group. Among them, 55 single bacteria were divided into group A and group B due to the difference in the diversity of root flora.

B were colonized by more abundant and diverse bacterial communities. The Chao1 and Shannon plots showed significant differences in OTU richness and bacterial diversity between Group A and Group B. The OTU richness and bacterial diversity of the flora within Group B were significantly higher than those of the flora within Group A, which was consistent with the results of the histogram analysis for single bacteria ($p < 0.01$) (Figures 1B,C). Simultaneously, the PCoA diagram showed that the root samples were significantly separated into two different groups, i.e., Group A and Group B, and both of them were significantly different from the control (Figure 1D; ANOSIM: $R = 0.302$, $p = 0.001$). The result was further verified by a Venn diagram, in which only a few OTUs were shared among the three groups or between pairs of samples (Figure 1E). In summary, single-bacterial inoculation significantly affected the community assembly and composition of the endophytic microbiome in wheat. In most cases, single-bacterial inoculation resulted in a lower abundance

and diversity of endophytic communities in plant roots and a higher proportion of pathogenic bacteria.

Identification of endophytic strains that colonized wheat roots

Before identifying the endophytic strains that colonized wheat roots, bacterial strains with more than 99% sequence identity were first merged and regarded as one type of representative strain by pairwise alignment and the construction of a phylogenetic tree using the full-length 16S rDNA sequences of the 55 bacterial strains. Finally, 22 representative bacterial species with unique sequences were obtained (Figure 2A). To identify the endophytic strains with the ability to colonize wheat roots, high-throughput sequencing reads for 55 wheat root samples were separately mapped on the 22 representative sequences at a sequence identity threshold of 97%. The mapped

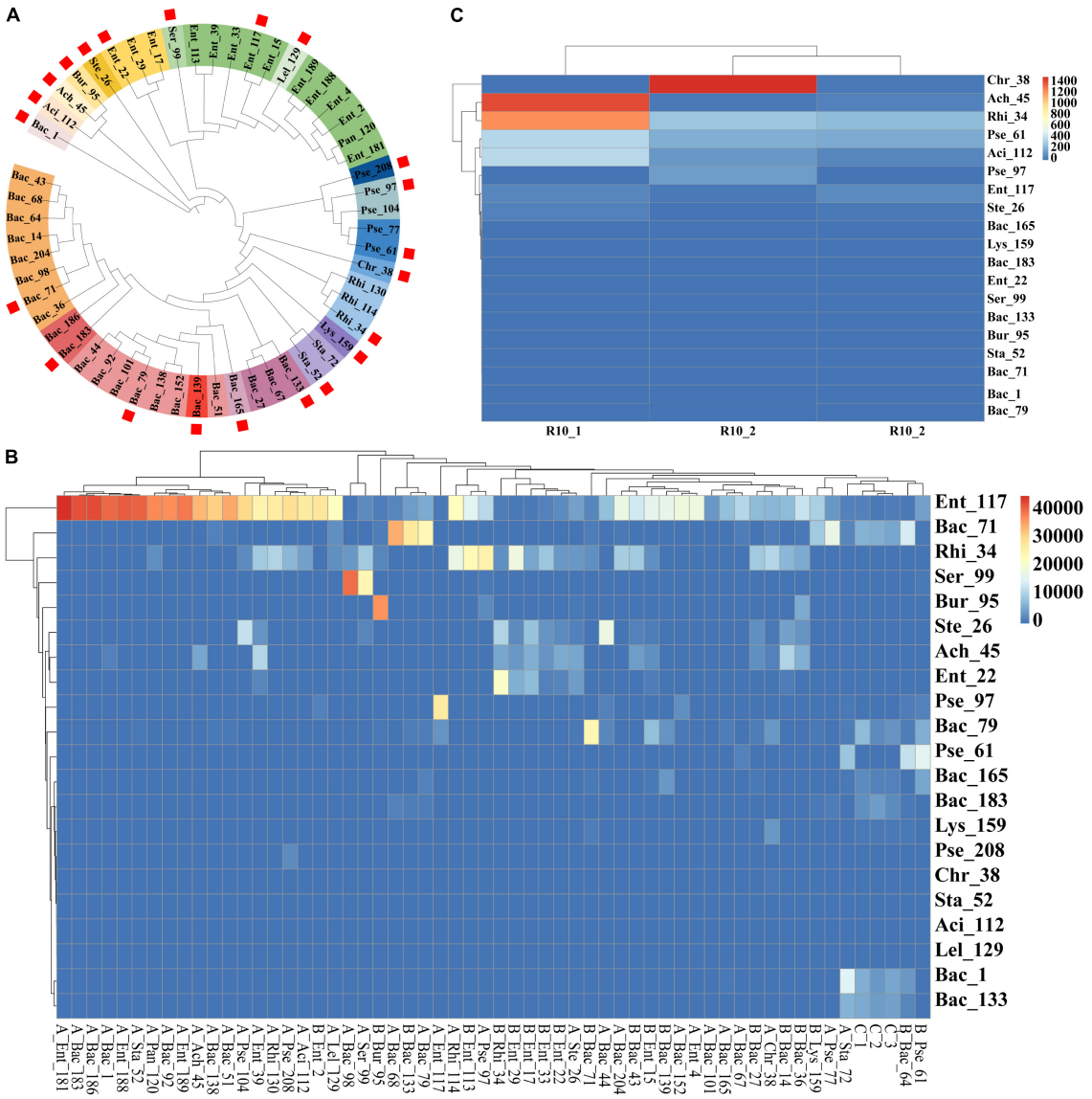

FIGURE 2
High-throughput sequencing of wheat and healthy tomato and heatmap of 55 single bacteria. High-throughput sequencing data of 55 infected wheat roots and the data of healthy tomato roots were mapped with a similarity of 97% with the representative sequences of 55 monocultures, respectively. **(A)** Screening dendrogram of representative sequences of 55 single bacteria. The representative sequences of the 55 single strains were screened, and each sample was blasted with the other samples. The similarity between the representative samples and the samples within the group was more than 99%, and the similarity between the samples within the group was more than 98%. A kind of color represents a set of representative sequences, and samples marked with squares are representative strains. **(B)** Heatmap of high-throughput data and representative sequences with 55 infected wheat roots. **(C)** High-throughput data within healthy tomato roots and heatmap of representative sequences.

reads were collected and visualized in a heatmap (**Figure 2B**). In the mapping results, the Bac_139 sample did not map to the corresponding strain, so there were 21 strains in the heatmap. The strains with more than 1,000 mapped reads were designated dominant bacterial species of the wheat root microbiome. Finally, 28 of the 55 bacterial strains were found to constitute the dominant bacterial group in the wheat root microbiome (**Figure 2B**). However, in most cases, bacterial inoculation did not always result in successful colonization or in the strain becoming the dominant population in the inoculated wheat roots. It is likely that the bacterial species belonged to the dominant bacterial taxa in the wheat root microbiome. To further compare the differences in the dominant bacterial populations between the tomato and wheat root microbiomes, we also downloaded the raw sequencing reads of the tomato root microbiome (wherein we isolated the 55 bacterial strains from the tomato plants) and mapped them onto the 22 representative sequences of type bacterial strains (Tian et al., 2015). Finally, a total of 8 bacterial strains were identified as the dominant population in the tomato root microbiome (**Figure 2C**). Among these, only 5 bacterial strains of the 55 strains, including Rhi_34, Rhi_114, Rhi_130, Ach_45, and Pse_61, were found to be dominant bacteria in both wheat and tomato roots. The results showed that a small portion of the dominant bacterial species coexisted in different plant species or that the dominant bacterial species were not always dominant in another plant. However, at a higher taxonomic level, most of the dominant bacterial orders in the tomato root microbiome, including Actinomycetales, Pseudomonadales, Rhizobiales, Burkholderiales, and Enterobacteriales, were also the dominant bacterial communities in the wheat root microbiome. This was also true at the bacterial phylum level (Tian et al., 2015).

Identifying the main factors that affect bacterial colonization and community assembly in wheat roots

To identify the main factors that affect bacterial colonization and the community differences in the wheat root microbiome between the groups, a constrained analysis of principal components (CAP) with the environmental variables was performed (**Supplementary Table 1**). The results showed that lignocellulose-decomposing enzyme activities and the taxonomy of the inoculated bacterial strains were the main factors that affected the community assembly of and differences between Group A and Group B (ANOVA: $p = 0.005$). Among these, cellulase activity was significantly related to the root samples that harbored the dominant bacterial population in the endophytic microbiome (ANOSIM: $R = 0.1358$, $p = 0.025$), indicating that lignocellulose-decomposing enzymes play an important role in the mechanism of bacterial infection in plants. Among the 55 endophytic bacterial strains used in this study, most strains showed clear cellulase and xylanase activities (**Table 1**), indicating that they originated from the inner tissue of plant roots. A comparison of the enzyme activities of the bacterial strains from Group A and Group B showed that most of the strains from Group A had higher cellulase and xylanase activities than those of Group B, which might explain why wheat root samples from Group A always harbored the predominant bacterial population in the endophytic microbiome. In summary, lignocellulose-decomposing enzyme activity has a significant effect on the wheat root microbiome, which further verified the role of enzymes during the process of flora establishment. The lignocellulose decomposition ability was a key factor affecting inoculation with beneficial bacteria, for both bacterial localization in root tissue and assembly of the root endophytic microbiome.

Effect of inoculation with a mixed bacterial complex on the community composition and bacterial diversity of the endophytic microbiome in wheat roots

To further verify the effect of bacterial inoculation and inoculated bacteria on the community assembly and composition, we selected 4 bacterial strains from the 55 endophytic strains, Ent_181 (absolute dominance), Ent_189 (absolute dominance), Bac_71 (relative dominance), and Bac_133 (non-dominance), according to their abundance in the inoculated root samples. Furthermore, we constructed 3 mixed bacterial complexes using the selected 4 bacterial strains and their taxonomic relatives (**Table 2**). Based on these 4 strains and the 3 mixed bacterial complexes, we compared the effects of single- and mixed-bacterial inoculation on the root endophytic microbiome in wheat. Consistent with the previous inoculation experiment, inoculation with a single strain or single-bacterial species in wheat roots, including with Ent_181, Ent_189, Bac_71, Bac_133, mixed *Bacillus* strains (BacM), and mixed *Enterobacter* strains (EntM), resulted in an Enterobacteriales-dominant microbiome, with a proportion of 45–50% (**Figure 3A** and **Table 2**). In contrast, inoculation with the bacterial complex Mix, which included 10 different species from *Enterobacter*, Bacillus, *Serratia,* and *Burkholderia*, resulted in a lower abundance of Enterobacteriales, with Actinomycetales accounting for the highest proportion (**Figure 3A** and **Table 2**). Compared with single-bacterial inoculation, mixed-bacterial inoculation led to a more balanced diversity of flora composition, and the probability of the emergence of a single bacterium with absolute dominance was lower, especially for pathogenic bacteria (**Figures 3B,C**). However, among the four single-bacterial strains, the Bac strains were more diverse than the Ent strains, precisely as a result of the high abundance

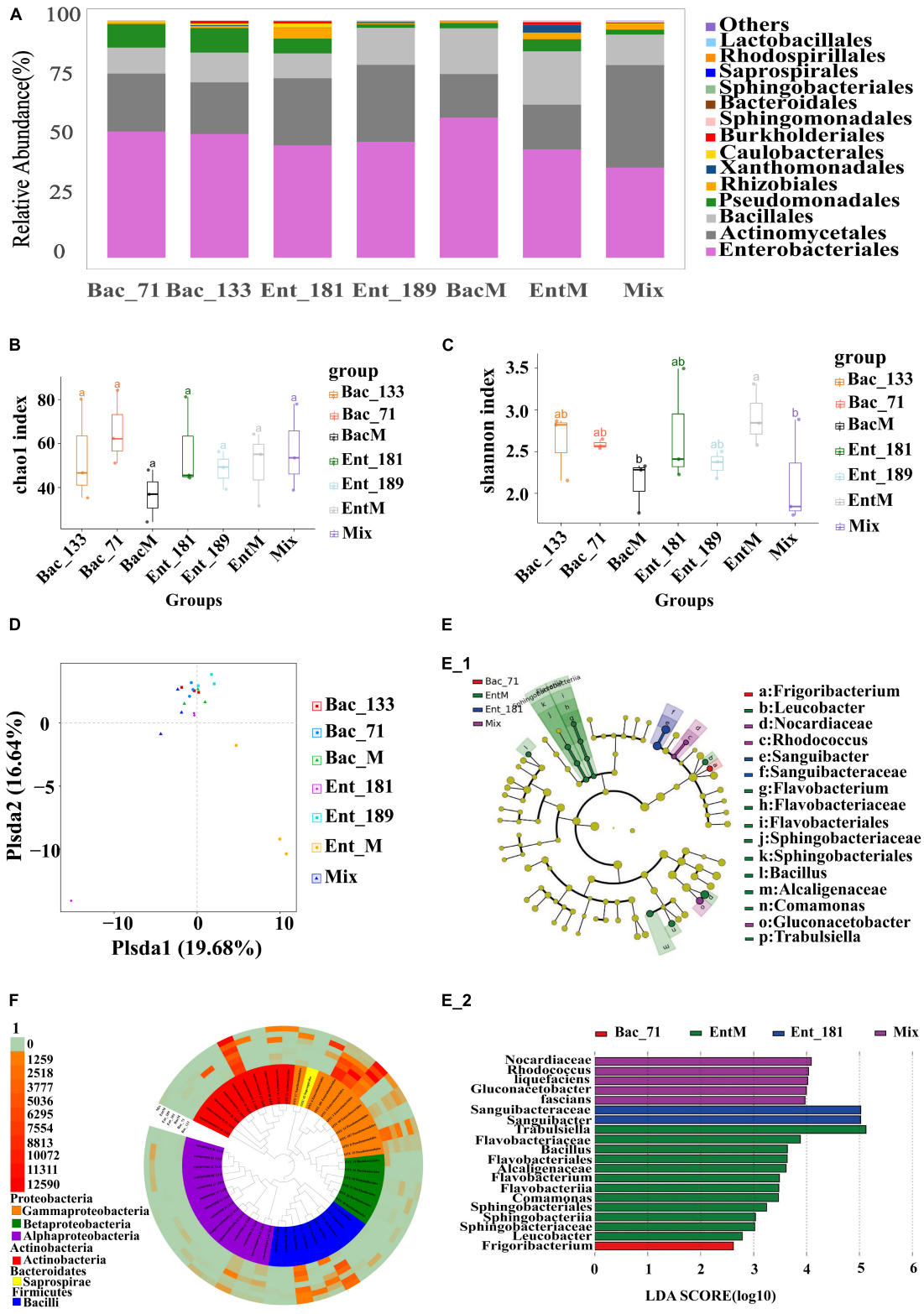

FIGURE 3
Secondary analysis of the composition and diversity of the flora of the roots of tomato after the infestation of four single and mixed bacteria. **(A)** Diagram of the species structure of 4 single bacteria and the mixed bacteria (at the order level, selecting the top 15 flora in terms of abundance). **(B,C)** The Chao1 boxplot and Shannon boxplot of 4 single bacteria and the mixed bacteria (the letters above represent the differences between groups). **(D)** PCoA analysis of single bacteria and the mixed bacteria, principal coordinated analysis (PCoA) derived from

(Continued)

FIGURE 3 (Continued)
dissimilarity matrix of Unweighted UniFrac distance. **(E)** LEfSe difference analysis diagram of single bacteria and mixed bacteria (different circles represent different hierarchical classification, from inside to outside, followed by phylum, class, order, family, and genus. Each node represents a species, and yellow means that the species is not significantly different within several groups. Other colors indicate that there are differences in the corresponding samples, and the species with specific differences are marked on the right side). **(F)** Tree diagram with OTU high value of single bacteria and mixed bacteria (the top 50 OTUs in terms of abundance were selected to draw the tree diagram, and different colors represent different classes. The outer layer of the tree diagram is the abundance of OTUs, and different colors represent different abundances).

of *Enterobacter* colonization. There was also variability among the mixed bacteria, with differences in bacterial diversity observed between BacM and EntM and between EntM and Mix, probably due to the influence of the *Enterobacter* species. The *Enterobacter* strain corresponding to OTU3 was highly abundant in the Ent_189 and Ent_M samples. The PCoA results demonstrated that the root samples Bac_71, Bac_133, BacM, and Mix shared a higher community similarity, while the *Enterobacter*-inoculated samples had significant differences among themselves and were also different from the *Bacillus* and Mix samples (**Figure 3D**).

The results demonstrated that the inoculated *Enterobacter* strains could easily infect and colonize root systems *via* complex interactions. The *Enterobacter* strain corresponding to OTU101 had a relatively high abundance in Ent_181. Although the inoculated *Bacillus* strains were also found to be localized in wheat roots, such as the *Bacillus* strain corresponding to OTU5 in Bac_71 and that, corresponding to OTU4 in BacM, the abundance of the corresponding *Bacillus* OTUs was relatively low, and the infection was less effective than that with the *Enterobacter* strains (**Figure 3A**). We further identified the differences in the communities between the root samples by using LEfSe (**Figures 3E,F**). Most of the identified differentially abundant bacterial taxa were from Actinomycetales and Enterobacteriales, among which the difference in Enterobacteriales was the largest. The results seemed to be consistent with the analysis of community structure and composition, and *Enterobacter* strains were among the dominant colonizers.

Identification of functional traits and genes corresponding to bacterial infection and localization in wheat roots using transcriptome analysis

To reveal the potential functional traits corresponding to bacterial localization and the main factor that affected community assembly in the wheat root microbiome, we performed transcriptomic sequencing for the samples inoculated with bacterial strains Ent_181, Ent_189, Bac_71, and Bac_133. The Venn diagram results showed that the number of specifically expressed genes in the *Enterobacter* samples Ent_181 and Ent_189 was higher than that in *Bacillus* samples Bac_71 and Bac_133, and the number of specifically expressed genes in the *Enterobacter* sample Ent_181 was 931 (**Figure 4A**). In addition, the results for the difference in the expression of gene between samples were also consistent with the results of the correlation analysis. The heatmap of the correlation analysis between samples showed that the correlation between Ent_181 and Ent_189 was relatively high, with a value of 0.73, while the correlations between Ent_181 and Bac_71 and between Ent_181 and Bac_133 were both 0.43, with good repeatability for each group of samples (**Figure 4B**). In the KEGG pathway analysis, we analyzed and compared the genes that were upregulated or downregulated during bacterial infection and their localization in the different samples (**Table 3**). The results showed that the levels of four pathways related to bacterial colonization, mainly starch and sucrose metabolism, bacterial chemotaxis, tryptophan metabolism, and flagellar assembly, were significantly different between samples. Studies showed that lignocellulose-decomposing enzymes might be involved in root colonization by bacterial flora, and there is evidence that colonization by endophytes in internal plant tissues involves the production of cellulase and pectinase (Reinhold-Hurek et al., 2006; Rosenblueth and Martínez-Romero, 2006). To gain a deeper understanding of the role of these enzymes, the expression levels of genes related to lignocellulose degradation were further compared. The results showed that α-amylase, beta-glucosidase, maltase-glucoamylase, beta-fructofuranosidase, alpha-trehalase, glucan 1,3-beta-glucosidase, 6-phospho-beta-glucosidase, beta-galactosidase, pectinesterase, and other genes encoding cell wall–degrading enzymes, acting mainly on cellulose and pectin, were identified (**Table 4**). Compared with the *Bacillus* sample, most of the identified lignocellulose genes were upregulated in *Enterobacter* samples Ent_181 and Ent_189 (**Table 4**). The transcriptomic analysis results verified that lignocellulose-decomposing enzymes might play an important role in bacterial colonization of plant roots, and the *Enterobacter* sample had higher expression levels of enzymes related to lignocellulose degradation, especially pectinases, which was consistent with the pathogenic trait of the *Enterobacter* strains (Rafique et al., 2021). In addition to the role of enzymes, the colonization by the Bac samples in plants might be related to bacterial chemotaxis and flagellar assembly, which illustrated the complexity of the colonization process.

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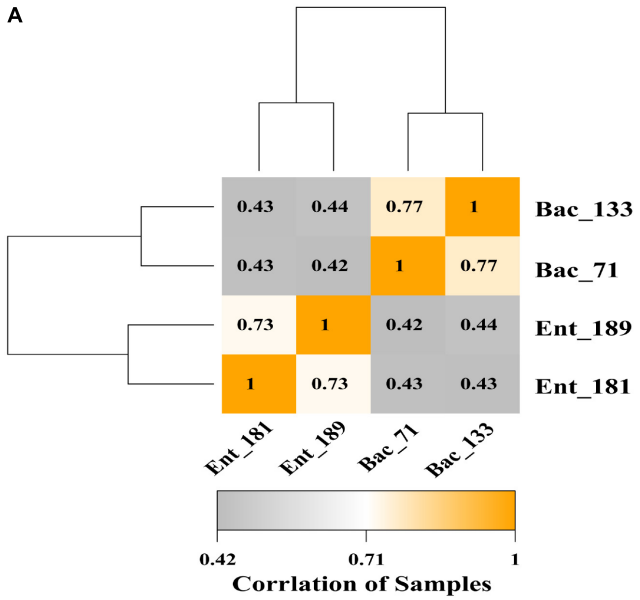

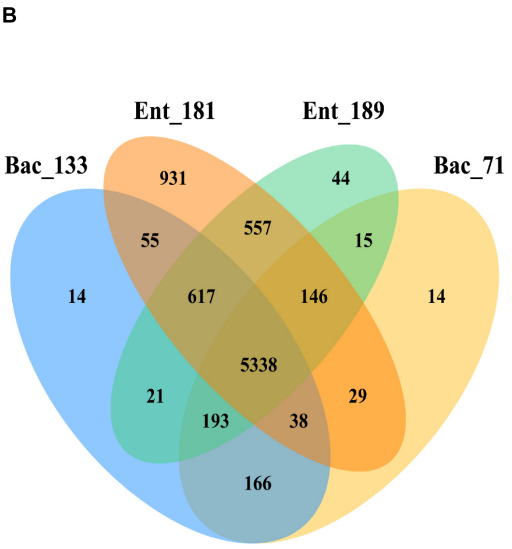

FIGURE 4
Transcriptome analysis of 4 significant colonizing bacteria. **(A)** Pearson correlation coefficient heat map of transcriptome expression of different samples. **(B)** A Venn map of gene expression between different samples.

TABLE 3 The identified differential expression level for the KEGG pathway using transcriptomic analysis.

Sample pair (*P*-value)	Starch and sucrose metabolism	Bacterial chemotaxis	Flagellar assembly	Tryptophan metabolism
Bac_71_vs_Ent_181.down	0.038***	0.59	0.99	0.07**
Bac_71_vs_Ent_181.up	0.89	0.07**	5.19E-08***	0.96
Bac_71_vs_Ent_189.down	0.000357315***	6.19E-05***	0.62172586	0.48
Bac_71_vs_Ent_189.up	0.56	0.98	0.19*	0.8
Bac_133_vs_Ent_181.down	0.08**	0.82	0.99	0.02***
Bac_133_vs_Ent_181.up	0.94	0.16*	7.54E-09***	0.87
Bac_133_vs_Ent_189.down	0.02***	0.000007 ***	0.102	0.23
Bac_133_vs_Ent_189.up	0.7	0.9	0.53	0.8
Ent_181_vs_Ent_189.down	0.97	0.006***	1E-11***	0.65
Ent_181_vs_Ent_189.up	0.93	0.99	no	0.07**
Bac_71_vs_Bac_133.down	0.4*	no	no	0.68
Bac_71_vs_Bac_133.up	0.72	0.92	0.8	0.26

Differential up- or downregulation of gene expression is reflected by the p-value.
*0.1 < p<0.5; **0.05 < p<0.1; ***p < 0.05.

Analysis of the assembly process of the endophytic microbiome in wheat roots

We studied the role of deterministic and stochastic assembly processes in the wheat root endophytic microbiome by calculating the relationship between βNTI and infection with different bacterial inoculation. The βNTI values of the 55 strains used for single-bacterial inoculation were divided into three groups: A, B, and C (Figure 5A). The results of pairwise comparisons showed that the differences in microbial populations among Group A, Group B, and the control group were mainly dominated by stochasticity (|βNTI| < 2). However, the differences between the two inoculation groups were significantly different from those between each of them and the control group; that is, bacterial inoculation had a significant effect on the community assembly and composition of the wheat root endophytic microbiome. Similar results were obtained for wheat roots inoculated with mixed bacterial complexes, which also showed that the community composition of the root endophytic microbiome was mainly dominated by stochasticity (Figure 5B). This result was consistent with those of studies showing that the community assembly process of soil- and

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TABLE 4 The identified differential expression level for the lignocellulose genes using the transcriptomic analysis.

EC	CAZymes	Bac_133_ vs_Ent_181	Bac_133_ vs_Ent_189	Bac_71_ vs_Ent_181	Bac_71_ vs_Ent_189	Bac_71_ vs_Bac_133	Ent_181_ vs_Ent_189
3.2.1.1	alpha-amylase	2_down	2_down	2_down	2_down	2_down	2_balance
3.2.1.20	maltase-glucoamylase	2_down	2_balance	2_down	2_down	2_balance	2_balance
3.2.1.21	beta-glucosidase	3_balance	3_balance	3_up	3_up	3_balance	3_balance
3.2.1.26	beta-fructofuranosidase	2__down	2_down	2_down	2_down	2_balance	2_up
3.2.1.28	alpha-trehalase	1_down	1_down	1_down	1_down	1_balance	1_up
3.2.1.58	glucan 1,3-beta-glucosidase	1_down	1_balance	1_down	1_balance	1_balance	1_up
3.2.1.86	6-phospho-beta-glucosidase	1_down	1_balance	1_down	1_balance	1_balance	1_balance

up, gene upregulation; down, gene downregulation; The numbers represent the number of occurrences of this enzyme gene.

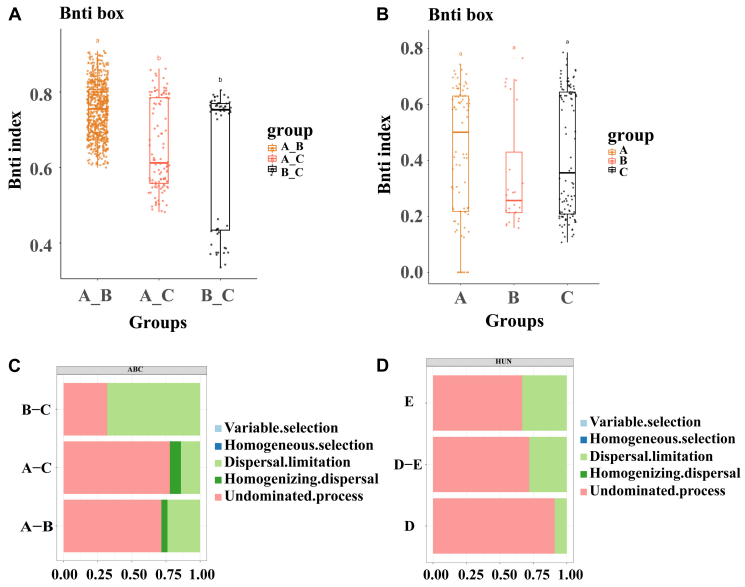

FIGURE 5
Analysis of microbiota assembly process. **(A)** Microbial assembly analysis of 58 single strains, divided into three groups of A, B, and C, which are viewed as control groups. **(B)** Microbial community assembly analysis of 4 single bacteria and mixed bacteria, where D represents the combination of four single bacteria and E represents the combination of three groups of mixed bacteria. **(C)** Analysis of the causes of community aggregation and composition of 55 single bacteria and the control group (divided into three groups of A, B, and C, which are viewed as control groups). **(D)** Analysis of the reasons for the aggregation and composition of single bacteria and mixed bacteria in the mixed bacteria test, where D represents the combination of four single bacteria and E represents the combination of three groups of mixed bacteria. The letters above the bars represent the differences between groups.

root-associated microbiomes was initially controlled by random processes (Dini-Andreote et al., 2015).

In addition, we quantitatively estimated this stochastic assembly process. A comparison of bacterial inoculation between Group A and the control group showed that the undominant process was the most prevalent (Group A, 77%), and the same result was observed when comparing Group A with Group B (Group A, 71%), which indicated that the assembly process was mainly influenced by external factors; that is, inoculation had an effect on the assembly of the bacterial flora in wheat roots (Figure 5C). Among the mixed-bacterial inoculations, the non-dominant process of single bacteria of Group D (90%) was significantly higher than that of Group E (66%), which was related to the larger proportion of single bacteria in the single-bacterial inoculation group, and the strain composition of Group E was relatively balanced (Figure 5D). This result was also consistent with the results for the 55 strains of single bacteria, so it could be speculated that the establishment of the flora in the process of inoculation was mainly related to the non-dominant process.

Discussion

Plant roots can release a large number of chemicals or organic compounds, such as amino acids, proteins, and organic acids, into soils to affect the microbial community of the rhizosphere and further affect the recruitment of bacterial

endophytes in plant roots (Bulgarelli et al., 2012; Kawasaki et al., 2016; Pétriacq et al., 2017; Shyam et al., 2017). However, bacterial entry and localization in the root inner tissue are determined by many factors, such as bacterial chemotaxis, lipopolysaccharides, and flagella (Böhm et al., 2007). In addition, it is believed that endophytes can penetrate the root endodermis by secreting cell wall–degrading enzymes (CWDEs), including cellulase, xylanases, pectinases, and polygalactosidase, which enables them to continue to colonize and move in the endoderm (Lodewyckx et al., 2002; Compant et al., 2005; Sabbadin et al., 2021). In agriculture, the introduction of bacteria that are beneficial to plants is an important practice for improving plant productivity and stress resistance without the use of pesticides and inorganic fertilizers and for promoting the phytoremediation of heavy metals and hydrocarbons (Anna et al., 2017). Therefore, understanding the mechanism of bacterial colonization and the effect of bacterial inoculation on the community assembly and composition of the root endophytic microbiome will provide a basis for developing more successful applications for broad-spectrum probiotics.

With regard to microbial ecology, it has been hypothesized that microbial assembly is mainly dominated by deterministic and stochastic processes (Zhou et al., 2013). The stochastic process was mainly proposed based on neutral theory, which assumes that all species or individuals are ecologically absolutely equal, so community composition and distribution patterns are completely determined by stochastic processes. Many studies have demonstrated that stochastic processes drive the community assembly and composition of the rhizospheric microbiome in plants (Luan et al., 2020; Qiu et al., 2021). Few studies have been performed to reveal the community assembly process of the endophytic microbiome in plant roots. In this study, through βNTI analysis of the community differentiation of the root microbiome upon inoculation with single or mixed bacterial strains, we found that the community differences and composition of the root endophytic microbiome were also mainly dominated by stochastic processes ($|\beta NTI| < 2$). Compared with the control group, root samples with single-bacterial inoculation showed significantly different community assembly and composition, wherein the root samples of Groups A–B showed mainly non-dominant processes. Dispersal limitation (67%) was dominant in Groups B–C, which was related to the high diversity of Group B and the absence of single bacteria, further indicating that the flora differed during the process of inoculation. The results for the mixed bacteria and the single bacteria were basically consistent. The significant difference in the main factors in the assembly process of Group A and Group B of single bacteria was consistent with that in the diversity analysis of Group A and Group B. CAP analysis was performed on Group A and Group B, and the *p*-value in the combined model with cellulase activity was less than 0.05.

In the single-bacterial inoculation experiment for 55 strains, we found that infection by single bacteria was clearly dominated by 1–3 bacterial species with high abundance, and the dominant bacteria were most likely to be pathogenic bacteria, although these bacteria were also found to be the earliest colonizers among root endophytes in previous studies. In contrast, the community composition of root samples inoculated with the mixed bacterial strains was highly diverse and varied, with a relatively high abundance of Actinomycetales. These results were consistent with the ecological process analysis for the root endophytic microbiome; that is, bacterial inoculation significantly affected the community assembly of the root microbiome in plants. In addition, CAP analysis demonstrated that the lignocellulose-decomposing enzyme activities of the inoculated bacterial strains were among the main factors that affected the community assembly and composition. The further transcriptomic analysis also showed the significant differences in the expression of functional pathways and genes involved in lignocellulose degradation. It was then speculated that the formation of the flora did not simply follow the neutral theory but that there was a certain selective deterministic process (screened by the lignocellulose barrier) within the neutral theory. Hurek and Reinhold-Hurek (2003) found that mutation of the nitrogen-fixing bacterium BH72 by knocking out the genes encoding endo-β-1,4-glucanase (cellulase) led to the failure to invade the roots of rice, indicating that the production of some lignocelluloses may be necessary for bacterial strains to become endophytes. Further studies to validate the role of lignocellulose in bacterial localization in plant roots and to determine whether they could establish themselves in the plant environment after application as biological fertilizers or biocontrol agents in the field are needed.

Data availability statement

The data presented in the study are deposited in the NCBI Short Read Archive, accession numbers SRX14891103–SRX14891181 and SRX14938537–SRX14938540 for community and transcriptomic analysis of the wheat root microbiome and the GenBank database, accession numbers ON242109–ON242163 for the full-length 16S rDNA of the isolated bacterial strains.

Author contributions

BT and YZ designated and supervised the project. TZ, JX, and RT performed the experiments. TZ performed all the bioinformatic analyses and prepared figures and tables. BT, TZ, KL, and XX contributed to the manuscript's writing and revision. YL, QZ, KL, XX, and LL suggested protocols, data analyses, and interpretation of results. All authors have read and approved the submitted version of the manuscript.

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Conflict of interest

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

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Supplementary material

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Can multi-cropping affect soil microbial stoichiometry and functional diversity, decreasing potential soil-borne pathogens? A study on European organic vegetable cropping systems

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Crop diversification in spatial and temporal patterns can optimize the synchronization of nutrients plant demand and availability in soils, as plant diversity and soil microbial communities are the main drivers of biogeochemical C and nutrient cycling. The introduction of multi-cropping in organic vegetable production can represent a key strategy to ensure efficient complementation mediated by soil microbiota, including beneficial mycorrhizal fungi. This study shows the effect of the introduction of multi-cropping in five European organic vegetable systems (South-West: Italy; North-West: Denmark and Belgium; North-East: Finland and Latvia) on: (i) soil physicochemical parameters; (ii) soil microbial biomass stoichiometry; (iii) crop root mycorrhization; (iv) bacterial and fungal diversity and composition in crop rhizosphere; (v) relative abundance of selected fungal pathogens species. In each site, three cropping systems were considered: (1) crop 1—monocropping; (2) crop 2—monocropping; (3) crop 1—crop 2—intercropping or strip cropping. Results showed that, just before harvest, multi-cropping can increase soil microbial biomass amount and shape microbial community toward a predominance of some bacteria or fungi phyla, in the function of soil nutrient availability. We mainly observed a selection effect of crop type on rhizosphere microbiota. Particularly, *Bacteroidetes* and *Mortierellomycota* relative abundances in rhizosphere soil resulted in suitable ecological indicators of the positive effect of plant diversity in field, the first ones attesting an improved C and P cycles in soil and the second ones a reduced

soil pathogens' pressure. Plant diversity also increased the root mycorrhizal colonization between the intercropped crops that, when properly selected, can also reduce the relative abundance of potential soil-borne pathogens, with a positive effect on crop productivity in long term.

KEYWORDS

intercropping, rhizosphere microbial community, root mycorrhization, nutrients, organic vegetables

Introduction

The concept of soil health is unavoidably connected to its multifunctionality and is strongly dependent on soil biodiversity. The recently changed and still evolving environmental conditions, call for management practices able to increase biodiversity and the functional redundancy of soil biological communities to ensure adequate ecosystem resilience (Griffiths et al., 2000).

The introduction of agroecological practices based on crop diversification represents one of the key strategies to ensure efficient complementation and exploitation of energy and nutrients by the soil biota, so to increase the ecosystem resilience (Tsiafouli et al., 2015). Among the alternative strategies to increase crop diversification, intercropping (IC) and strip cropping (SC), where multiple crops are contemporarily grown, can supply several ecosystem services, including nutrient cycles, crop production, control of pests, and diseases (Theunissen, 1997; Li et al., 2007, 2014; Brooker et al., 2015; Ciaccia et al., 2015; Campanelli et al., 2019; Fan et al., 2020). Particularly, crop diversification in spatial and temporal patterns can optimize the synchronization of plant nutrient demand and availability and contribute to increasing the diversity of plant and soil microbial communities, which are among the main drivers of biogeochemical C and nutrient cycle (Crews and Peoples, 2005; Prommer et al., 2019). Understanding the composition, traits, and functions of soil organisms, as well as their ecological interactions, is imperative to understand which agricultural practices can maintain plant biodiversity, soil health, and productivity (van der Heijden et al., 1998; Francaviglia et al., 2004; Zhang J. et al., 2020). Recently, it was found that crop diversification and soil disturbance strongly impact on microbiome functional diversity, as plant residues play a substantial role in defining the assortment of microbial species (Figueiredo Santos and Olivares, 2021; Orrú et al., 2021). The fungi:bacteria ratio in soil microbial community depends on environmental changes and its impact on soil functioning is a key element in the assessment of microbiome functional diversity (Strickland and Rousk, 2010). Suitable parameters and methods to study the functional diversity of soil communities include the fungi:bacteria ratio, the assessment of soil microbial C:N:P stoichiometry, the geno- and phenotype profiling of soil microbial communities, and functional, metagenomic approaches.

Elemental composition and stoichiometry of microorganisms usually stem from indirect analysis of the whole community, although in grassland ecosystems C:P and C:N ratios were found significantly higher in fungi, such as *Ascomycota* and *Basidiomycota*, than in bacteria, such as *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Mouginot et al., 2014). However, the observed mean stoichiometric ratios fell within the overall distributions reported in Cleveland and Liptzin (2007) and overlap with some recorded ratios for the microbial biomass of the whole communities. The microbial stoichiometry supplies information about organic matter decomposition, patterns of nutrient limitation, and links between fluxes of C, N, and P (Philippot et al., 2013; Mouginot et al., 2014). Anyway, soil nutrient stoichiometry was recognized as the main predictor of bacterial and fungal diversity and composition even at a regional scale, being mainly driven by variation in soil C, N, and P resources, as affected by system management (Delgado-Baquerizo et al., 2017; Bragazza et al., 2021).

When evaluating the effect of agricultural practices on soil microbial stoichiometry, the total soil organic carbon (TOC) content and nutrient availability should be considered. The quantification of mineral N (Keeney and Bremner, 1966) and the estimation of soluble organic C (HWC) and easily available P (HWP) in hot water represent simple methods to determine the C and available P (P_{av}) in soils. Hot water extraction leads to the decomposition of organic compounds due to the high temperature (Vanden Nest et al., 2014). HWC and HWP pools are often used as proxies for microbial C (Sparling et al., 1998; Ghani et al., 2003), due to the ability of soil microbiome to stabilize soil organic matter (SOM) within the soil C and nutrient dynamics.

In a review by Strickland and Rousk (2010) about the bacterial or fungal dominance in soils in response to environmental changes, the distribution of C:N ratio of bacteria, saprotrophic fungi, and mycorrhizal fungi was evaluated. The authors found that the dominance of bacteria communities corresponds to a microbial C/N ratio two times lower (between 5 and 6) than under saprotrophic and mycorrhizal fungi dominance (between 9 and 17). Recent results on

sugarcane–soybean IC showed that microbial C (C_{mic}) and microbial N (N_{mic}) increased under IC when compared to monoculture (MC), with a significant increase in microbial C:N ratio especially in intercropped soybean (Lian et al., 2019).

Coupling soil microbiome stoichiometry data with comprehension of soil microbial diversity and functioning give the chance to understand how soil microbiome may be driven by both the introduced crop diversity and soil nutrient availability. In recent years, the massive use of Next Generation Sequencing (NGS) technologies, a high-throughput method to investigate sequences of nucleotides within DNA/RNA molecules (Metzker, 2010; Klindworth et al., 2013), became a common tool in agroecological studies. This method allows us to focus on multiple species (i.e., bacterial and fungi communities) and their related functionality in soil (Moscatelli et al., 2018), for example, nutrient availability, stress resistance, or plant diseases (Sirangelo and Calabrò, 2020). The use of NGS to investigate agricultural soils, where crop diversification and no tillage, was applied for 10 years showed that some fungal species worked as indicators of soil disturbance in intensively tilled soils, while others seemed to be mostly associated with chemical characteristics of plant residues accumulated on soil surface (Ashworth et al., 2017; Orrú et al., 2021).

In general terms, a high microbial diversity is expected in highly diversified cropping systems. In fact, a positive effect of legume–cereal crop rotations on beneficial fungi (e.g., mycorrhizae) was found under Mediterranean conditions, being strongly correlated to SOC, and to occurrence and β-diversity of arbuscular mycorrhiza fungi genera (Pellegrino et al., 2020). In addition, crop diversification (e.g., IC, living mulch, and cover crops) seems to promote the beneficial plant–fungi symbiotic association (Trinchera et al., 2019, 2021).

In organic vegetable farming systems, the farmers often introduce diversification strategies as a mean to control the development of soil-borne plant pathogens. Although some papers have focused on the effects of crop diversification on soil microbiome (e.g., Li and Wu, 2018; Orrú et al., 2021), very few studies investigated how multi-cropping systems or crop rotation affect fungal communities, particularly on those with potential pathogenic activity in soils. Several pathogens were found to be reduced in a cereal–legume multi-cropping system, where wheat and maize were intercropped with faba bean (Wang et al., 2021). Similarly, in greenhouse vegetable production, a tomato–celery rotation was able to trigger a shift of fungal diversity toward less pathogenic populations (Lyu et al., 2020).

The SureVeg project "*Strip-cropping and recycling of waste for biodiverse and resoURce-Efficient intensive VEGetable production*" (CoreOrganic Cofund 2016-2021) aimed at developing and implementing new diversified, resource-efficient, and intensive organic vegetable systems in Europe. In this framework, we focused on the effect of multi-cropping on a set of biochemical and microbial indicators in different European pedoclimatic conditions, to disclose how crop diversification affects diversity and functionality of the soil microbial community and related ecosystem services. We hypothesized that increased plant diversity in multi-cropping can modify the soil microbial stoichiometry, shape the composition and diversity of bacteria and fungi communities, thus improving soil C-N-P cycles mediated by soil microbiota, promoting beneficial plant–fungal symbioses, and containing fungal diseases which afflicted horticultural crop production in those systems (e.g., *Fusarium*, *Olpidum Brassicae*).

In each experimental site, two crops, representative of the local farming systems, were intercropped row-by-row (IC) or bed-by-bed (SC), compared to MC. This study reports the results obtained on: (i) soil physicochemical parameters; (ii) soil microbial biomass stoichiometry, (iii) crop root mycorrhization; (iv) bacterial and fungal diversity in crop rhizosphere; and (v) relative abundance of selected potential pathogenic fungal species.

Materials and methods

Site and experimental design

The SureVeg field trials were established in 2018 in five experimental sites in Europe, representative of different pedoclimatic conditions:

- South-West EU: CREA (Italy, Mediterranean North region)
- North-West EU: AU (Denmark, Atlantic Continental region), ILVO (Belgium, Atlantic region)
- North-East EU: Luke (Finland, Boreal region), LatHort (Latvia, Nemoral region)

All experimental fields were under organic vegetable rotation for more than 5 years. In 2018–2019, different vegetable species rotated in a field in each site: reported data are referred to in the second year of the experiment (2019, after 2 years of multi-cropping introduction) (Figure 1).

In each experimental site, the two crops were cultivated under two cropping systems (CS): MC and multi-cropping (namely, IC or SC):

- CREA: faba bean–tomato (SC, three blocks, not fully randomized).
- AU: beetroot–white cabbage (IC, four blocks, fully randomized)
- ILVO: celeriac–leek IC, (four blocks, randomized)
- LatHort: faba bean–white cabbage (SC, three blocks, not fully randomized).
- Luke: onion–white cabbage (IC, four blocks, fully randomized)

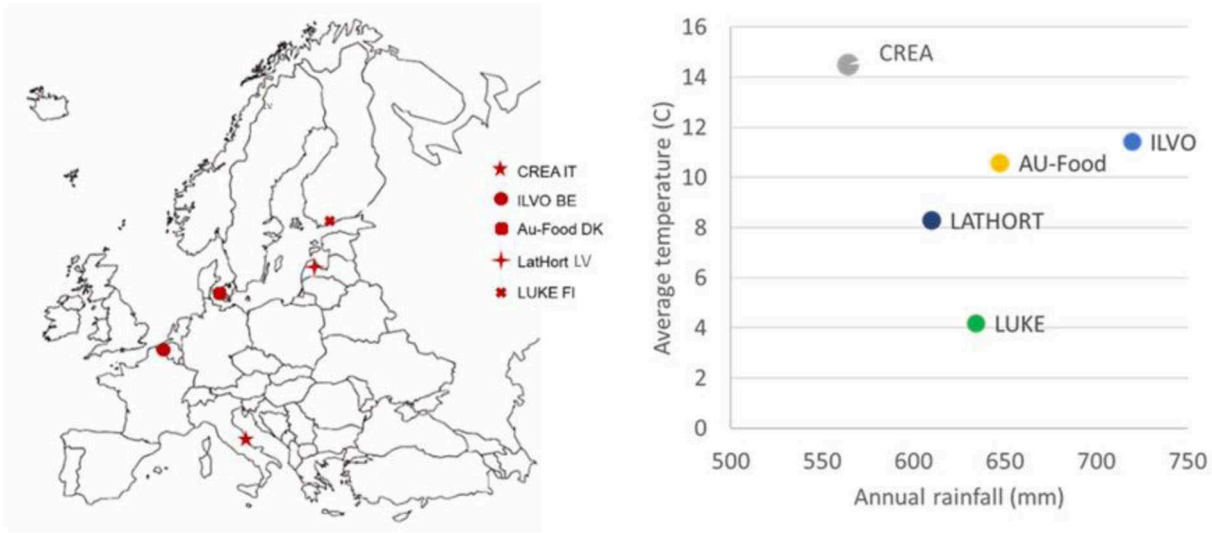

FIGURE 1
SureVeg experimental sites and related annual rainfalls (mm) and average annual temperatures (°C, 2019).

In each experimental site, the CS was tested considering three/four blocks, based on field extension and tested CS (IC or SC). A visual representation of the experimental designs applied in each site is reported in Supplementary material 1.

Irrigation was supplied according to plant needs, and fertilization was applied *via* plant-based compost to all the crops. Only at CREA site, MC and SC faba bean was not fertilized to study the local practice of tomato transplanting on flattened legume residues. Details about the pedoclimatic characteristics of EU sites, crop diversification (SC or IC), companion crops, fertilization, irrigation, weed management, sampling time, and other relevant information are reported in Table 1.

Soil sampling and physicochemical parameters

In 2019, at crop harvest, the bulk soil was sampled at 0–30 cm layer in all field trials. In AU, ILVO, and Luke sites, IC soil sampling was carried out in-between the changing rows, while at CREA and LatHort sites, SC soil sampling was performed in-between the beds' external rows. Only at CREA site, SC soil samples were collected in FB and T SC beds' external rows, being nonfertilized the soil under faba bean and fertilized that under tomato. In MC and IC/SC, after collecting four subsamples in each block, one composite soil sample/block was analyzed. The pH, N_{tot}, P_{av}, HWC, HWP, C_{mic}/TOC, C_{mic}, N_{mic}, and P_{mic} were determined at CREA, AU, and ILVO sites, while soil HWC and HWP were assessed at all the sites.

Soil moisture content was determined as the weight loss at 105°C. The pH was determined in 1M KCl (1/5 v/v), while the bulk density (in g cm^{-3}) by collecting undisturbed soil cores (100 cm^3) using an auger at depth of 0–30 cm layer (ISO 11272). Total organic C (TOC, in %) was obtained by dry combustion method at 1,050°C using LECO TOC Analyzer (mod. RC-612; LECO Corporation, 1987), after subtracting inorganic C (in %), while total N (in g kg^{-1}) by dry combustion method. To calculate P_{av} (in mg kg^{-1}), dried soil samples at a ratio 1:10 (v/v) in $CaCl_2$ solution (NEN 5704, at ILVO site) or Melich 3/Olsen solution (Mehlich, 1984; Ziadi et al., 1993, at AU and CREA sites) were extracted for 2 h and then the extracts were analyzed by simultaneous plasma emission spectrophotometer (ICP-OES Iris; Thermo Optek) or by Skalar SAN++ CFA. In LatHort plant available, phosphorus was detected by using calcium lactate according to the Egner–Riehm method (Egner et al., 1960). Soil C (HWC) and soil P (HWP) soluble in hot water (in mg kg^{-1}) were extracted using Haynes and Francis method (Haynes and Francis, 1993), by extraction of 5 g of dried soil samples in 25 ml of demineralized water for 16 h in a hot water bath at 70°C. After centrifugation and filtration on Machery–Nagel mn640d filter, the total C was determined by dry combustion by LECO TOC Analyzer or Skalar Primacs SLC TOC-analyzer. HWP was measured on water extracts by ICP-OES (ICP-OES Iris or Thermo Optek VISTA-PRO, Varian, Palo Alto, CA) or by Skalar SAN++ CFA.

Soil microbial biomass stoichiometry

Soil microbial biomass stoichiometry was assessed at CREA, AU, and ILVO sites by determination of microbial biomass C (C_{mic}), N (N_{mic}), and P (P_{mic}) content in the bulk soil collected

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TABLE 1 Experimental sites description and management practices implemented.

	CREA	AU	ILVO/Inagro	LUKE	LatHort
Experimental site	Monsampolo VEgetables organic Long-Term Experiment (MOVE LTE), Monsampolo del tronto (AP, IT)	Aarslev Research Center. Organic since 2013 (Aarslev, DK)	Inagro - Organic experimental farm (Gent, BE)	Luke Mikkeli long-term organic field (Mikkeli, FI)	Pure Research Center of LatHort (Pure, Latvia)
GPS	42° 53′ N, 13° 48′ E	55° 18′ N, 10° 27′ E	50°54,312' N, 3°7,646' E	61°41'18.85"N, 27°16'20.17"E	57°02′ N 22°54′ E
Climate	Thermo-Mediterranean (UNESCO/FAO, 1963)	Temperate	Temperate maritime	Continental subarctic (Köppen climate classification)	Temperate - Eastern-continental climate
Total annual precipitation (mm)	564	647	719.6	634	609.8
Average temperatures	Annual 14.5°C	Annual 10.6	Annual 11.43	Annual 4.2	Annual 14.3°C
Soil classification (United States Department of Agriculture, 1996)	Fine-loamy, mixed thermic (Typic Calcixerepts)	Sandy loam (Typic Agrudalf)	Eutric Retisol (Loamic)	Dystric Cambisol	Leached sod-calcareoulus soil
Experimental design	Strip-plot	Randomized split plot	Randomized split plot	Randomized split plot	Nonrandomized split plot
Replications	3	4	4	4	3
Factors	Crop: i) Faba bean (*Vicia faba* L.), FB ii) Tomato (*Solanum Lycopersicum* L.), T Cropping system: i) Monoculture (FB-MC; T-MC) ii) Bed-by-bed strip cropping (FB-SC; T-SC)* *Both crops sampled, being FB-MC and FB-SC not fertilized	Crop: i) Beetroot (*Beta vulgaris* L.), B ii) White cabbage (*Brassica oleraceae,* var. capitata f. alba), C Cropping system: i) Monoculture (B-MC; C-MC) iii) Row-by-row Intercropping (IC)	Crop: i) Celeriac (*Apium graveolens*, L.), CL ii) Leek (*Allium ampeloprasum*, L.), L Cropping system: i) Monoculture (CL-MC; L-MC) iii) Row-by-row Intercropping (IC)	Crop: i) White cabbage (*Brassica oleraceae* var. capitata f. alba), C ii) Onion (*Allium cepa* L.), O Cropping system: i) Monoculture (C-MC; O-MC) iii) Row-by-row Intercropping (IC)	Crop: i) Faba bean (*Vicia faba* L.), FB ii) White cabbage (*Brassica oleraceae* var. capitata f. alba), C Cropping system: i) Monoculture (FB-MC; C-MC) iii) Bed-by bed strip cropping (SC)
Row distance (m)	0.7	0.5	0.7	0.5 (Cabbage), 0.5 (onion)	0.7
Plant distance in row (cm)	20 (faba bean), 50 (tomato)	40 (beetroot), 35 (cabbage)	10 (leek), 40 (celeriac)	50 (cabbage), 7 (onion)	50 cabbage, 14 - bean
Plot size (m^2)	2 × 3.7 (faba bean), 2.8 × 3.7 (tomato)	10 × 4.8	6 × 8	3 × 5	3.5 × 8
Soil tillage practice	Plowing (20-25 cm) and harrowing (faba bean), no tillage (tomato). Faba bean for SC plots was flattened using in line roller crimper	Plowing (20-23 cm), cultivator 8-12 cm depth	Non inversion tillage	Harrow and rototilling (before planting and sowing)	Plowing (22-25 cm depth)
Transplanting	May 13 (tomato transplanting); January 8 (faba sowing)	June 25 (cabbage) June 6 (beetroot sowing)	May 14 (leek) May 15 (celeriac)	May 16 (onion), May 27 (cabbage)	May 31 (cabbage and faba bean)

(Continued)

TABLE 1 (Continued)

	CREA	AU	ILVO/Inagro	LUKE	LatHort
Crop irrigation	Drip irrigation: 300 L/m^2 in 25 events	Sprinkler irrigation, 125 mm in 6 events	Spray irrigation: 90 L/m^2 in three events	Sprinkler irrigation: 15 mm on event	Manual irrigation: 47 L/m^2 in five events
Fertilization	Faba bean MC and SC: not fertilized. Tomato MC and SC: on May 11, 2019, flattened faba bean residues; on May 16, 2019, Compost at 11.5 Mg ha^{-1}	Cabbage and beetroot MC and IC fertilized: May 29: fresh clover; Cabbage MC: 24 Mg ha^{-1}, beetroot MC: 26 Mg ha^{-1}; SC: 24 Mg ha^{-1}, October 3, 2019 Compost: 10 Mg ha^1 in all cropping system	Celeriac and leek MC and IC fertilized: April 2: Haspargit Potassium fertilizer 667 kg ha^{-1} April 20, 2019: organic granular fertilization (11-0-5), 500 kg/ha OPF April 25, 2019: green compost, 12 ton/ha	Cabbage and onion MC and IC fertilized: 1 October Wood based soil improver; Cabbage MC; 59 Mg ha^{-1}; onion MC; 11 Mg ha^{-1}; SC: 30 Mg ha^{-1}, May and July 2019: Biokali cabbage MC, 10 Mg ha^{-1}; onion MC: 1.9 Mg ha^{-1}; SC: 9 Mg ha^{-1}	Faba bean and cabbage MC and SC fertilized: May 3 2019: before trial establishment with green compost at 50 t ha^{-1}
Weeding	Weed cutting with mowing blade before sowing; manual weeding during cropping cycle	Interrow weeding with weed brush machine (Rath Maschinen, Germany) - late June to early Aug (5 times) Manual weeding	Mechanical weeding from late May to late Sept. Precision arrow (5 times), ridging (3 times on leek), hoeing (5 times on celeriac)	Week 23 and 24, 17.-18.6., 2.-5.7. (three times), hand weeding and harrowing	Late June to early Aug, manual weeding during cropping cycle (3 times)
Sampling time	Bulk soil: Late July (faba bean dry grains harvest); at half August (tomato harvest)	Bulk soil: Late October (after cabbage and beetroot harvest)	Bulk soil: Late October	Bulk soil: 2018 in late September	Bulk soil: Early June
	Rhizosphere soil: at late July (faba bean dry grains harvest); at half August (tomato harvest)	Rhizosphere soil (only in MC systems): late August 29 (close to harvest)	Rhizosphere soil: mid-October	None	None

in MC and IC plots (Zhang and Elser, 2017), respectively. On saturated soil samples sieved at 2 mm, soil water holding capacity (WHC) at −33 kPa (pF = 2.5) was determined using a pressure cell apparatus. Preincubation until reaching 60% of WHC for 10 days at 30°C was performed (Vance et al., 1987). Incubated soil samples were then divided into two subsamples, and one of them was fumigated with ethanol-free chloroform ($CHCl_3$) under vacuum overnight. To measure C_{mic} and N_{mic}, both fumigated and unfumigated soils were extracted with 0.5 M K_2SO_4 solution (soil/extraction solution: 1:4) at room temperature for 30 min (Brookes et al., 1985; Voroney et al., 2008). C_{mic} and N_{mic} were measured on filtered extracts using Shimadzu TOC-V-CSN analyzer (Fornasier et al., 2014). P_{mic} was determined after extraction of fumigated and not fumigated soils with 0.5 M $NaHCO_3$ solution, using ammonium molybdate–stannous chloride colorimetric method (Sparling et al., 1985), and then analyzed by a continuous-flow colorimeter Autoanalyser Technicon II or Flowsys Analyzer SYSTEA S.p.A. (absorbance of the extracted solution at $\lambda = 882$ nm). Microbial coefficient (C_{mic}/TOC) was calculated and used as ecological indicator of environmental changes induced by different CS or soil managements (Anderson, 2003; Moscatelli et al., 2005). C_{mic}/N_{mic} and N_{mic}/P_{mic} ratios were then determined to evaluate the potential shift of the soil microbiome toward bacteria or fungi community (Strickland and Rousk, 2010; Mouginot et al., 2014).

Crop root mycorrhizatin

At CREA, AU, and LatHort sites, crop roots were sampled in the field, considering four plant per treatment per block. Roots were separated from the soil by washing the sampled material under fresh water in a sieve of 0.5 mm mesh, then divided into first, second, and third orders' lateral roots. Collected third-order lateral roots (diameter < 2 mm) were stained by immerging them in a stain solution of 0.05% w/v methyl blue in lactoglycerol (1:1:1 lactic acid/glycerol/distilled water) for 1 min, and then distained in bi-distilled water for 1 min more (Grace and Stribley, 1991). A total of 10 × 1 cm root segment

(third-order lateral fine roots) per plant were selected at random from the stained root segments, by cutting them with a razor blade from 5 to 15 mm from the root tip. The total number of observed root segments per each treatment was: 10 × 4 plants × 3/4 blocks = 120/160 segments. The evaluation of mycorrhizal colonization intensity (M%) of each segment was assessed under a light microscope (Nikon E100 at 10× and 40×). The mycorrhizal infection score (Trouvelot et al., 1986) was calculated by attributing to each root fragment increasing scores from 0 to 5, applying the following formula: $M_i\ \% = (95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1)$/total number of observed segments, where n5 is the number of segments rated 5, n_4 is the number of segments rated 4, and so on.

Rhizosphere bacterial and fungal community

To evaluate bacteria and fungi diversity as affected by multi-cropping at CREA and ILVO sites, four rhizosphere soil samples per each MC and IC/SC plot were collected. In AU system, bacteria and fungi diversity were evaluated by NGS only in MC, collecting five rhizosphere soil samples per each plot. Rhizosphere soil sampling was performed using the method proposed by Lundberg et al. (2013), considering rhizosphere soil as extending up to 1 mm from the root surface. Collected roots from the soil sampled using a stainless cylinder were placed in a sterile 50 ml tube containing 25 ml phosphate buffer, vortexed at maximum speed for 15 s, to release most of the rhizosphere soil from the roots. The turbid suspension was then filtered through a 100 nm nylon mesh cell strainer into a new 50 ml tube to remove plant parts and large sediment particles, and then further centrifuged for 15 min at 3,200 rpm to form a loose pellet containing fine sediment and microorganisms. Phosphate buffer was removed, and the resulting rhizosphere pellets (250 mg) were used for DNA extraction with the PowerSoil DNA isolation kit (Qiagen) according to the manufacturer's instructions. DNA was stored at −20°C. The extracted DNA was used for identifying bacterial (V3–V4 16S rRNA gene) and fungal rhizosphere populations (ITS2) through amplicon sequencing using Illumina technology (Illumina, San Diego, CA, USA) by Admera, United States.

Libraries were constructed following Illumina 16S Metagenomic Sequencing Library Preparation protocol in two amplification steps: an initial PCR amplification using locus-specific PCR primers and a subsequent amplification that integrates relevant flow cell-binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002; Illumina Inc., San Diego, CA, USA).

For the first PCR step, 16S rRNA gene S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primers were used (Klindworth et al., 2013; Debode et al., 2016) and for ITS fungal rDNA-ITS2 region fITS7b (5′-GTGAATCATCRAATYTTTG-3′) from Ihrmark et al. (2012) and the ITS4NGSr (5′-TTCCTSCGCTTATTGATATGC-3′) primer (Tedersoo et al., 2014) (Supplementary material 2). The amplicons were sequenced on Illumina MiSeq 2× 300 bp paired-end platform (Illumina Inc., San Diego, CA, USA).

Bioinformatic elaboration and statistical analysis

Soil physicochemical (pH, bulk density, TOC, N_{tot}, P_{av}, HWC, and HWP) and biochemical parameters (C_{mic}, N_{mic}, P_{mic}, C_{mic}/TOC, C_{mic}/N_{mic}, and N_{mic}/P_{mic}) and root mycorrhizal colonization intensity (M) were analyzed by one-way ANOVA, as affected by CS, after verification by Shapiro–Wilk normality test. In CREA system, since both the crops were sampled separately under SC management, CS and crop effect were considered, together with their interaction. Shapiro–Wilk test was performed to check the normality of the data. *Post hoc* Tukey's HSD test was carried out to compare means using SPSS (IBM Corp., Armonk, NY, United States).

The principal component analysis (PCA) on soil physicochemical and biochemical indicators at AU, CREA, and ILVO experimental sites was produced using the R package factoextra.

Raw paired-end reads were processed using the R package DADA2 version 1.16.0. The Divisive Amplicon Denoising Algorithm (DADA) is based on the identification of single nucleotide sequence variants producing an amplicon sequence variant (ASV) table with a higher resolution of the traditional OTU table. The confidence level for an assignment was set at 3%, as a standard procedure with the DADA2 pipeline (97% identity level to define taxonomic units).

The Silva v132 database and the UNITE v020219 database were used to assign taxa for bacteria and fungi, respectively.

At AU, CREA, and ILVO experimental sites, the α-diversity of bacterial and fungal communities was determined by calculating richness, evenness, Shannon index, and Simpson indexes. Species richness is a measure for the total number of species in the community. Evenness refers to how close in numbers each species is in an environment.

The results of the soil microbiome were shown only with at least 0.1% relative abundance in the whole dataset (Wassermann et al., 2019). In AU site, only the crop effect was studied.

Within each condition, the normal distribution of data was verified using the Shapiro–Wilk test in R (V3.4.4), then applying ANOVA test and Tukey's *post hoc* test to compare data as affected by considered factors.

We also explored differences in the relative abundance of some fungal families that also include pathogenic fungi,

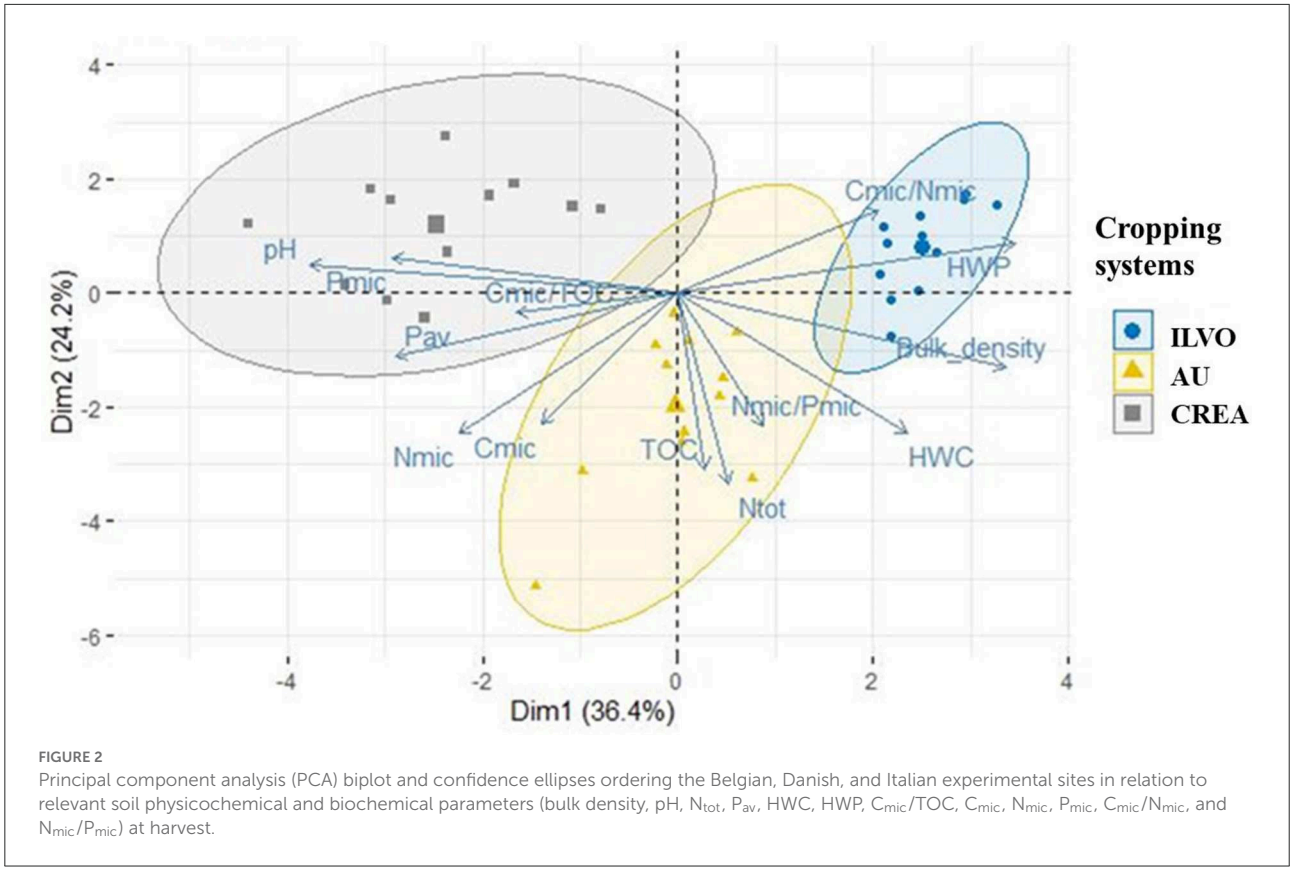

FIGURE 2
Principal component analysis (PCA) biplot and confidence ellipses ordering the Belgian, Danish, and Italian experimental sites in relation to relevant soil physicochemical and biochemical parameters (bulk density, pH, N_{tot}, P_{av}, HWC, HWP, C_{mic}/TOC, C_{mic}, N_{mic}, P_{mic}, C_{mic}/N_{mic}, and N_{mic}/P_{mic}) at harvest.

particularly *Scleroniaceae*, *Olpidiaceae*, and *Nectriaceae*. We used permutational multivariate analysis of variance (PERMANOVA) on the experimental sites. Nonmetric multidimensional scaling (NMDS) was performed on CREA and ILVO datasets with the Bray–Curtis dissimilarity index, using two dimensions ($k = 2$). These analyses were carried out using the *vegan* package.

Finally, we tested the effect of crop, block, and CS on the relative abundance of *Fusarium* spp. at ILVO and CREA, while we tested only the effects of crop and block on *Olpidium brassicae* at AU. Beta-regression models were used (*betareg* package). Shapiro–Wilk test was used to test the normality of residuals. Differences between treatment means were compared using a *post-hoc* Tukey test ($\alpha = 0.05$) (*emmeans* package). Those statistical analyses were performed in R (version 4.0.3, 2020).

Results

Soil physicochemical parameters and microbial biomass stoichiometry

The principal component analysis, applied to soil physicochemical (bulk density, pH, N_{tot}, P_{av}, HWC, HWP) and biochemical indicators (C_{mic}/TOC, C_{mic}, N_{mic}, P_{mic}, C_{mic}/N_{mic}, N_{mic}/P_{mic}) in AU, CREA, and ILVO, did not discriminate across CS. Conversely, Figure 2 shows that "site" was a discriminant factor regardless of the CS applied.

Indeed, principal components 1 and 2 explained 60.6% of variability and strongly discriminated across the CREA, ILVO, and AU systems. Along with the positive value of component 1, ILVO site was associated with bulk density, HWP, and C_{mic}/N_{mic} indicators, while CREA site to pH, P_{av} and P_{mic} content, and C_{mic}/TOC. The component 2 (Y-axis) affected mainly the AU beetroot site in terms of TOC and total N (N_{tot}), while both the components explained the variability of C_{mic}, N_{mic}, N_{mic}/P_{mic} ratio, and HWC. PCA analysis evidenced that the three sites, characterized by different soil parameters, must be evaluated separately to highlight the effect of multi-cropping management on considered microbial indicators.

In Table 2, the same soil physicochemical and microbial parameters recorded at crop harvesting are reported, as affected by CS. At CREA only, the crop effect was also evaluated, with related interaction.

At CREA, we found a CS effect on pH, which was significantly higher under faba bean SC (7.90) compared to faba bean MC (7.80, $p = 0.05023$). Soil bulk density and N_{tot} were affected by the crop × CS interaction. Similarly, P_{av} varied across crops and CS, being the lowest one in tomato MC (16.3 mg kg^{-1}) and the highest in faba bean MC and SC

TABLE 2 Soil chemical, physical and stoichiometric parameters at CREA, AU, and ILVO sites in 2019, namely: pH, bulk density (g cm^{-3}), total organic C (TOC, %), total N (g kg^{-1}), available P (mg kg^{-1}), C_{mic}, N_{mic}, P_{mic} content, plant mycorrhizal colonization intensity (M%), microbial coefficient (C_{mic}/TOC), C_{mic}/N_{mic} and N_{mic}/P_{mic} ratios at crop harvesting.

	pH	Bulk density	TOC	N_{tot}	Pav	Cmic	Nmic	Pmic	Cmic/TOC	Cmic/Nmic	Nmic/Pmic
Units		*g cm^{-3}*	*%*	*g kg^{-1}*	*mg kg^{-1}*	*mg kg^{-1}*	*mg kg^{-1}*	*mg kg^{-1}*			
Faba bean–tomato (CREA, IT)											
FB-MC	7.80 ± 0.05b	1.24 ± 0.01b	1.12 ± 0.02	1.03 ± 0.02a	34.0 ± 6.1a	51 ± 32b	10.6 ± 7.5 c	3.2 ± 0.6b	0.46 ± 0.29b	12.9 ± 11.6	3.8 ± 0.6b
T-MC	7.82 ± 0.04b	1.35 ± 0.03a	1.09 ± 0.07	0.73 ± 0.04b	16.3 ± 6.4b	220 ± 61a	37.5 ± 3.2a	3.3 ± 0.5b	2.03 ± 0.25a	5.9 ± 6.3	11.6 ± 1.5a
FB-SC	7.90 ± 0.03a	1.30 ± 0.04a	1.05 ± 0.05	1.20 ± 0.03a	44.4 ± 1.6a	87 ± 24b	6.2 ± 2.9c	4.8 ± 1.1 ab	0.82 ± 0.59b	12.5 ± 5.9	9.6 ± 1.4ab
T-SC	7.79 ± 0.04b	1.15 ± 0.06b	1.08 ± 0.10	0.73 ± 0.02 ab	22.4 ± 4.0ab	195 ± 57a	28.5 ± 9.5b	4.5 ± 0.4a	1.79 ± 0.37a	7.0 ± 0.4	6.6 ± 2.7b
C-effect	n.s.	n.s.	n.s.	n.s.	*	***	***	n.s.	***	n.s.	n.s.
CS-effect	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
C × CS	n.s.	*	n.s.	**	**	n.s.	**	***	n.s.	n.s.	**
Cabbage–beetroot (AU, DK)											
C-MC	6.70 ± 0.10	1.47 ± 0.03	1.75 ± 0.04	1.67 ± 0.07	29.7 ± 0.4	121 ± 32b	16.8 ± 4.4	4.0 ± 1.8	0.69 ± 0.14b	7.8 ± 3.5	5.51 ± 3.8
B-MC	6.70 ± 0.09	1.46 ± 0.01	1.75 ± 0.02	1.58 ± 0.04	31 ± 0.2	181.9 ± 45a	24.1 ± 11.4	3.5 ± 1.4	1.05 ± 0.27a	8.1 ± 1.9	7.16 ± 1.9
IC	6.60 ± 0.11	1.47 ± 0.02	1.75 ± 0.06	1.58 ± 0.05	32.5 ± 0.2	177.6 ± 98a	27.6 ± 27	3.0 ± 2.0	1.04 ± 0.59a	8.55 ± 3.5	15.77 ± 14.0
CS-effect	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.	n.s.
Leek–celeriac (ILVO, BE)											
L-MC	5.60 ± 0.04	1.55 ± 0.04	1.16 ± 0.02	1.07 ± 0.05	3.6 ± 0.3	120 ± 44	11.71 ± 6.4	0.68 ± 0.2b	1.02 ± 0.2	10.2 ± 0.7.2	18.70 ± 1.10 a
CL-MC	5.70 ± 0.01	1.54 ± 0.02	1.14 ± 0.05	1.04 ± 0.04	4.0 ± 0.5	105 ± 27	8.32 ± 9.5	1.33 ± 0.9 ab	0.9 ± 0.4	30.5 ± 12.9	5.25 ± 0.24b
IC	5.70 ± 0.06	1.53 ± 0.02	1.15 ± 0.05	1.07 ± 0.03	3.9 ± 0.5	119 ± 44	4.8 ± 2.5	2.33 ± 0.6a	1.03 ± 0.4	27.5 ± 11.3	2.40 ± 0.21c
CS-effect	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	*

Levels of statistical significance (p value) are: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, n.s., not significant (ANOVA).
Different letters represent significant differences (Tukey's HSD test for means comparison).

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(34.0 and 44.4 mg kg^{-1}, respectively), due to the crop × CS interaction. C_{mic} was affected only by crop type, while N_{mic} was affected by the interaction between crop type and CS. We also found a significant CS effect and crop × CS interaction on P_{mic}. SC showed a four-fold increase under faba bean SC (P_{mic} = 12.2 mg kg^{-1}) compared to MC (P_{mic} = 3.3 mg kg^{-1}). C_{mic}/TOC was instead significantly affected by the crop type. Anyway, an interaction effect of crop × CS was found on soil bulk density, N_{tot} and P_{av}, N_{mic}, P_{mic} and consequently, on N_{mic}/P_{mic}. This last ratio was lower in both the faba bean and tomato SC, and higher under tomato. N_{mic}/P_{mic} was interestingly changed by CS, increasing in faba bean (from 3.8 to 9.6 mg kg^{-1}) and decreasing in tomato, going from MC to SC (from 11.6 to 6.6. mg kg^{-1}).

In the AU site, CS affected significantly C_{mic} and C_{mic}/TOC only, with the lowest found under MC cabbage (120.9 and 6.92 mg kg^{-1}, respectively).

At ILVO, a significant effect of CS was found on P_{mic} and N_{mic}/P_{mic}. Here, the lowest P_{mic} content and the highest N_{mic}/P_{mic} content were found under MC leek (0.68 and 18.7 mg/kg, respectively).

In Figure 3, HWC and HWP, measured in bulk soil of CREA, AU, ILVO, Luke, and LatHort experimental sites by comparing the MC vs. IC/SC systems, are reported.

In CREA system, the lowest HWC was recorded in tomato SC (173.5 mg kg^{-1}), while in faba bean and tomato MC (189.7 and 181.5 mg kg^{-1}), and in faba bean SC (190.0 mg kg^{-1}), they were the highest ones. In AU experiment, no differences in soil HWC were observed in beetroot and cabbage MC compared to IC system. On the opposite, at Luke, cabbage and onion MC gave significantly lower HWC values (323.5 and 317.0 mg kg^{-1}) compared to cabbage–onion IC (345.1 mg kg^{-1}). Both at ILVO and LatHort system, no significant difference was observed between MC and IC/SC.

HWP was strongly affected by the experimental sites, being highest values recorded in ILVO and LatHort systems compared to AU, Luke, and CREA systems. In considered experimental trials, no significant effect of IC on HWP was found, except at CREA faba bean–tomato system, where soil HWP was significantly higher under tomato SC (4.90 mg kg^{-1}) compared to faba bean SC (3.72 mg kg^{-1}); intermediate values were recorded under faba bean and tomato MC (4.03 and 4.26 mg kg^{-1}, respectively).

A correlation (R = 0.6188) was found between C_{mic}/TOC and HWC, as a result of different pedoclimatic location of considered case studies.

Crop root mycorrhization

In Figure 4, the mycorrhizal colonization intensity (M%) recorded at CREA, AU, and LatHort systems is reported, as affected by SC and IC, compared to MC.

At CREA, a significant increase in crop root mycorrhization was observed in tomato SC, with an average value three times higher than that recorded in tomato MC, going from 12.5% to 37.2%. No CS effect was observed between faba bean MC and SC. In AU, even if both cabbage and beetroot are recognized as nonmycorrhizal plant species, we found a highest M% in IC beetroot (13.8%), although not significantly different from MC beetroot (6.3%; p =0.06037).

Rhizosphere bacterial and fungal community

Rhizosphere bacterial and fungal community diversity of each crop was studied by calculating richness, evenness, Shannon index, and Simpson index at CREA and ILVO sites, as affected by either IC or SC, while at AU only on MC. In CREA system, significant differences were observed only in fungal community composition (Figure 5).

The crop × CS interaction was significant at CREA. Shannon index gave the lowest value in tomato SC and the highest under faba bean MC. Similarly, Simpson index was the lowest under tomato SC and the highest under faba MC. Fungi richness was the highest under faba bean MC, and the lowest under faba bean SC.

In Figure 6, the relative abundance of bacteria and fungi phyla as affected by crop and CS at CREA, ILVO sites and at AU (here, on MC only) are reported (see also Supplementary material 3).

At CREA site, bacteria community obtained after bacterial rDNA 16S sequencing of rhizosphere soil samples revealed 10,025 different ASV. The taxonomic assignment revealed 9 unidentified ASV, while the others were assigned to 30 different bacteria Phyla (SM1). After the taxonomic assignment at CREA site 264 family and 585 genera were identified. The most represented ASV were *Proteobacteria* (31.0%), followed by *Bacteroitedetes* (13.2%), *Actinobacteria* (10.0%), *Acidobacteria*, (10.0%), *Planctomycetes* (8.9%), *Chloroflexi* (7.3%), *Verrucomicrobia* (6.2%), *Patescibacteria* (3.7%), *Firmicutes* (3.2%), *Gemmatimonadetes* (2.6%), *Cyanobacteria* (1.1%), *Armatimonadetes* (1.0%), and unindentified (1.6%) (Figure 6A).

The highest relative abundance associated with faba bean MC and SC was observed for *Proteobacteria* (35.4% and 55.5% in MC and SC, respectively; p = 0.022), *Bacteroidetes* (19.1and 21.8%), *Acidobacteria* (10.5% and 4.0%, p = 0.0524), and *Actinobacteria* (7.6% and 7.8%), being a relative abundance of other bacteria Phyla lower than 7.0% (Figure 6A). Under tomato, again *Proteobacteria* were the most abundant phyla (26.8and 29.6% in MC and SC, respectively), followed by *Actinobacteria*

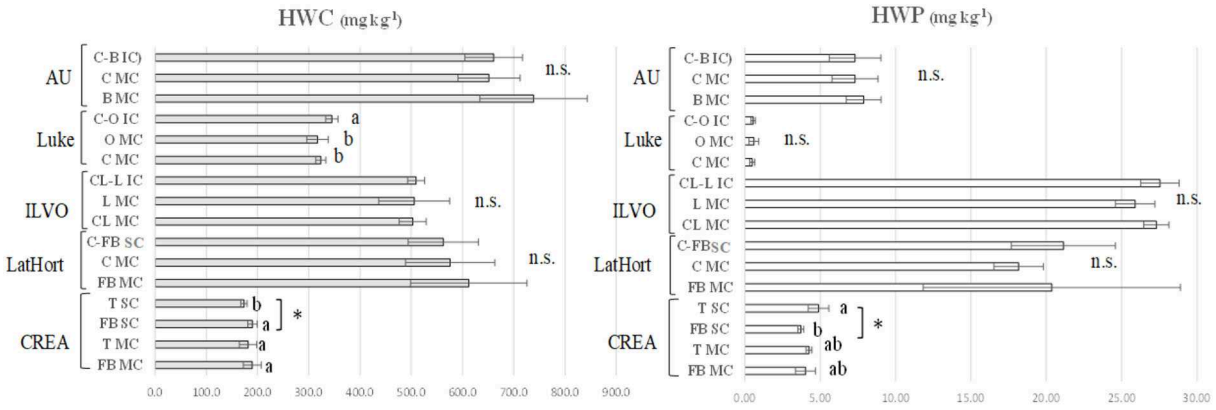


FIGURE 3
Soil HWC and HWP (mg kg^{-1}) in: monocropped faba bean (FB MC) and tomato (T MC), strip cropped faba bean (FB SC) and tomato (T SC) at CREA; monocropped faba bean (FB MC) and cabbage (C MC), strip cropped faba bean and cabbage (FB-C IC) at LatHort; monocropped celeriac (CL MC) and leek (L MC), intercropped celeriac and leek (CL-L IC) at ILVO; monocropped cabbage (C MC) and onion (O MC), intercropped cabbage and onion (C-O IC) at Luke; monocropped beetroot (B MC) and cabbage (C MC), and intercropped beetroot and cabbage (B-C IC) at AU. Levels of statistical significance (*p* value) are: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, *ns* = not significant (ANOVA). Different letters represent significant differences (Tukey's HSD test for means comparison).

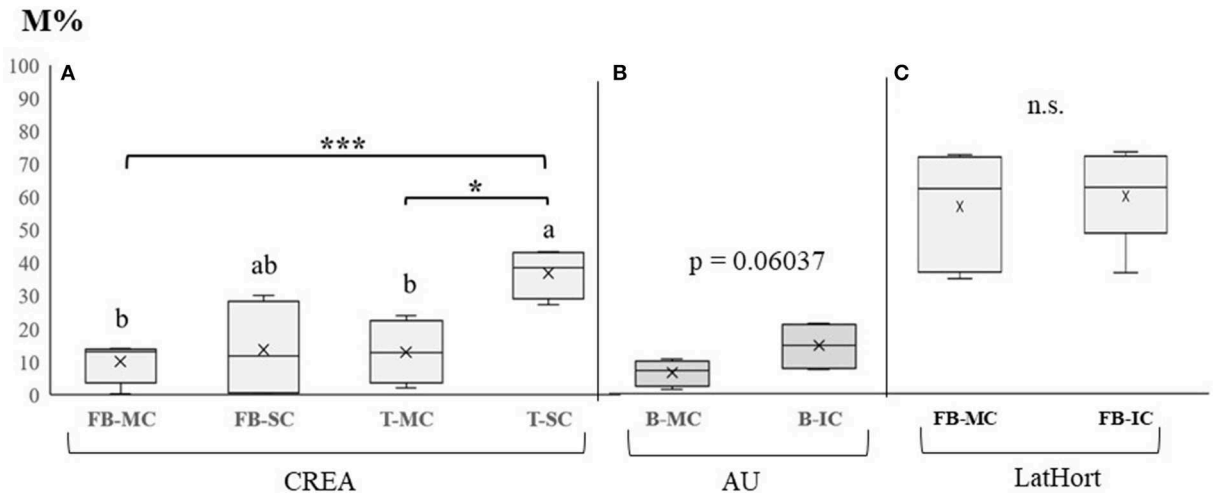


FIGURE 4
Mycorrhizal colonization intensity (M%) recorded in **(A)** monocropped and strip cropped faba bean (FB-MC, FB-SC) and monocropped and strip cropped tomato (T-MC) and strip cropped, T-SC) (CREA, IT); **(B)** beetroot monocropping (B-MC) and intercropping (B-IC) systems (AU, DK); **(C)** faba bean in monocropping (FB-MC) and intercropping (FB-IC) systems (LatHort, LV). Levels of statistical significance are: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, *ns,* not significant (ANOVA). Different letters represent significant differences (Tukey's HSD test for means comparison).

(29.5% in MC and 23.5% in SC, respectively; $p = 0.0491$) and *Bacteroitedes* (13.5% in MC and 18.7% in SC) (Figure 6A).

The overall fungal community obtained after rDNA ITS fungal sequencing of IT rhizosphere soil samples revealed 1,606 different ASV. The taxonomic assignment revealed 20% of unidentified ASV, while the others were assigned to 14 different Phyla (Supplementary material 3), particularly *Ascomycota* (44%), *Basidiomycota* (15%), *Chytridiomycota* (8%), *Glomeromycota* (4%), *Mortierellomycota* (3.2%), *Mucoromycota* (1%), and *Kickxellomycota* (0.4%), 156 Family and 245 Genera.

Under faba bean, *Ascomycota* were overrepresented in both MC and SC (70.7% and 72.8%, respectively), followed by *Basidiomycota* (12.9% and 12.9%), *Mortierellomycota* (11.5% and 12.1%), and *Chytridiomycota* (4.2%, and 1.0%) (Figure 6B). Under tomato, *Ascomycota* were the most represented Phyla (51.4% and 52.5%, in MC and SC, respectively), followed by *Basidiomycota* (40.6% and 45.7%). A reduction in relative abundance of *Mortierellomycota* was recorded under SC (7.0% in MC and 1.4% in SC, respectively; $p = 0.0646$). Most of the ASV identified

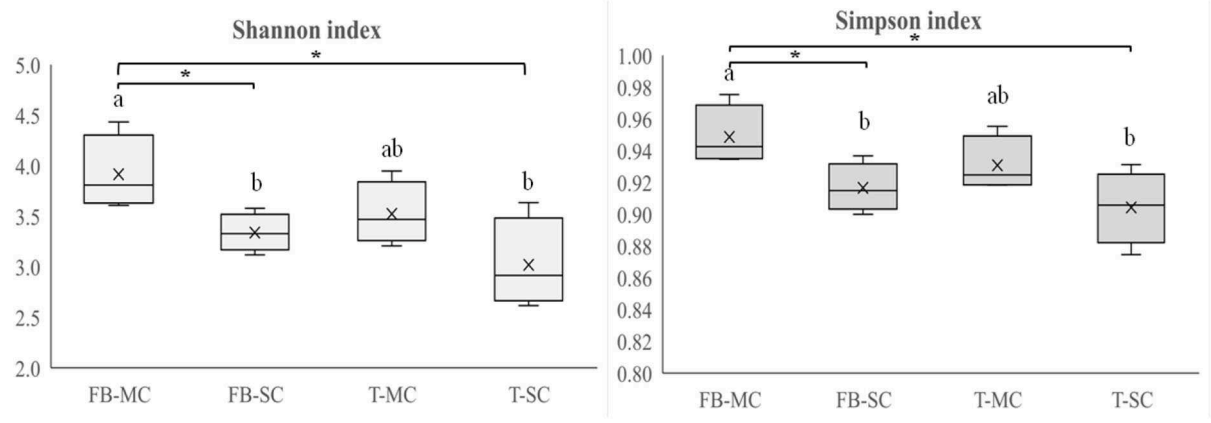


FIGURE 5
Fungal community diversity indices (Shannon index, Simpson index) recorded in faba bean (FB) and tomato (T) monocropping (MC) and strip cropping (SC) at CREA site. Levels of statistical significance (p value) are: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, *ns,* not significant (ANOVA). Different letters represent significant differences (Tukey's HSD test for means comparison).

in *Mortierellomycota* Phylum at CREA site belongs to *Mortierella* genus.

Regarding ILVO site, the bacterial rDNA 16S sequencing of rhizosphere soil samples revealed 15,334 different ASV describing the overall bacteria community. The taxonomic assignment revealed 2.5% of unidentified ASV, while the others were assigned to 31 different bacteria Phyla, 264 Family, and 585 Genera (Supplementary material 3). The celeriac–leek system was dominated by *Proteobacteria* (28.6%), followed by *Planctomycetes* (14.0%), *Bacteroitedes* (11.9%), *Acidobacteria* (9.7%), *Verrucomicrobia* (7.9) *Actinobacteria* (7.6%), *Chloroflexi* (4.8%), *Firmicutes* (4.6%), *Patescibacteria* (3.15%), *Gemmatimonadetes* (2.55%), *Chlamydiae* (1.47%), *Cyanobacteria* (1.31%), and other Phyla being lower than 1.0%.

Under celeriac, *Proteobacteria* showed the highest relative abundance in both MC and SC (33.1% and 37.6%, respectively), followed by *Actinobacteria* (16.0% in MC and 12.6% in IC, respectively; $p = 0.0488$), *Acidobacteria* (10.2% and 10.7%) and *Bacteroidetes* (8.4 and 8.9%) (Figure 6C).

Under leek, again *Proteobacteria* showed the highest relative abundance in MC and IC (33.3 and 33.6%, respectively), followed by *Actinobacteria* (15.5% and 15.6%), *Bacteroidetes* (12.3 and 12.2%) and *Acidobacteria* (9.1% and 9.2%) (Figure 6C). A crop effect emerged on *Bacteroidetes* relative abundance, highest under leek when compared with celeriac (12.2% and 8.6%, as MC vs. IC averages, respectively), independently from the CS ($p = 0.0029$).

The rDNA ITS fungal sequencing of ILVO rhizosphere soil samples revealed 3,496 different AVS describing the overall fungi community. The taxonomic assignment revealed 30% of unidentified ASV, while others were assigned to 14 different fungi Phyla, 190 Family and 332 Genera (Supplementary material 3), where *Ascomycota* dominated (36.8%), followed by *Basidiomycota* (15.5), *Chytridiomycota* (7.6%), *Glomeromycota* (3.6%), *Mortierellomycota* (2.0%.), *Aphelidiomycota* (1.3%), *Mucoromycota* (1.2%), and other Phyla with a percentage of ASV lower than 1%.

Under celeriac MC and IC, more than half of fungi population was constituted by *Ascomycota* (57.5 and 57.2%, respectively), followed by *Mortierellomycota* (22.9% and 21.6%) and *Basidiomycota* (16.0 and 16.2%) (Figure 6D). Under leek, *Ascomycota* Phylum was overrepresented (58.0 and 60.5%, in MC and IC, respectively), followed by *Mortierellomycota*, which decreased in IC (23.9 and 20.3%, respectively; $p = 0.0672$) and *Basidiomycota* (13.4 and 15.0%) (Figure 6D).

In AU site, reported NGS results refer only to cabbage and beetroot MC, describing the microbial community composition under the two companion crops used for field trial. Overall bacteria community obtained after bacterial rDNA 16S sequencing of rhizosphere soil samples revealed 38,541 different ASV. The taxonomic assignment revealed 855 (2.2%) unidentified ASV, while the others were assigned to 30 different bacteria phyla (SM1). One of them was the former candidate lineages frankia bacterial peritonitis (FBP) recently accepted as the novel phylum *Abditibacteraeota* (Tahon et al., 2018), and five of them belong to new candidates' phyla. After the taxonomic assignment in AU system, the highest number of ASV identified was of *Proteobacteria* (24.9 %), mostly constituted by gamma-proteobacteria (43.4% of the ASV identified as *Proteobacteria*), followed by delta-proteobacteria (39.3%) and alpha-proteobacteria (16.8%). The other Phyla identified were *Planctomycetes* (11.5%), *Acidobacteria* (11.5%), *Bacteroidetes* (10.1%), *Verrucomicrobia* (9.2%), *Patescibacteria* (7.7%), *Actinobacteria* (6.6%), *Chloroflexi* (4.5%), *Gemmatimonadetes* (2.9%), *Chlamydiae* (1.7%),

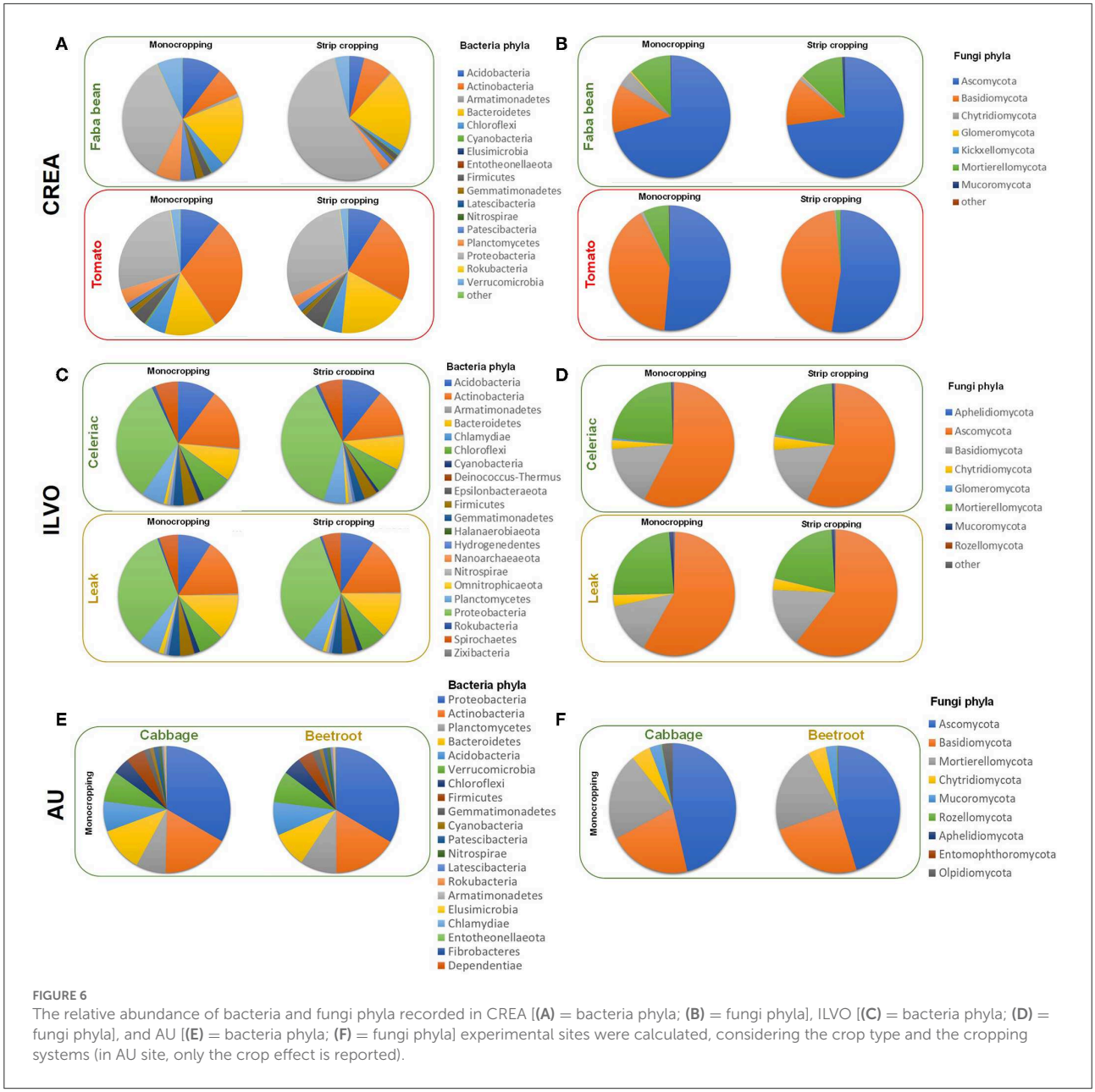


FIGURE 6
The relative abundance of bacteria and fungi phyla recorded in CREA [**(A)** = bacteria phyla; **(B)** = fungi phyla], ILVO [**(C)** = bacteria phyla; **(D)** = fungi phyla], and AU [**(E)** = bacteria phyla; **(F)** = fungi phyla] experimental sites were calculated, considering the crop type and the cropping systems (in AU site, only the crop effect is reported).

Firmicutes (1.4%), and *Latescibacteria* (1.0%), being unidentified phyla <1% of identified ASV.

All the ASV belongs to 292 different Family and 672 Genera. In AU experimental site, the relative abundance associated with beetroot and cabbage for *Proteobacteria* and *Actinobacteria* were similar (in beetroot: 33.5% and 16.5%; in cabbage: 33.3% and 17.0% respectively, Figure 6E). *Planctomycetes*, *Acidobacteria*, *Chloroflexi*, and *Gemmatimonadetes* gave slightly higher relative abundances under beetroot (9.3, 8.6, 4.7, and 1.9%, respectively) than under cabbage (7.6, 7.7, 4.3, and 1.6%, respectively). Relative abundance of *Verrucomicrobia* also was slightly higher under beetroot (8.1%) than under cabbage (7.9%) (Figure 6E).

The phylum *Bacteroidetes*, *Firmicutes*, and *Patescibacteria* showed a higher relative abundance under cabbage (11.4, 4.8, and 1.3%, respectively) then under beetroot (9.1, 3.8, and 1.0%, respectively, Figure 6E). The relative abundance observed for the other Phyla was lower than 1.0%.

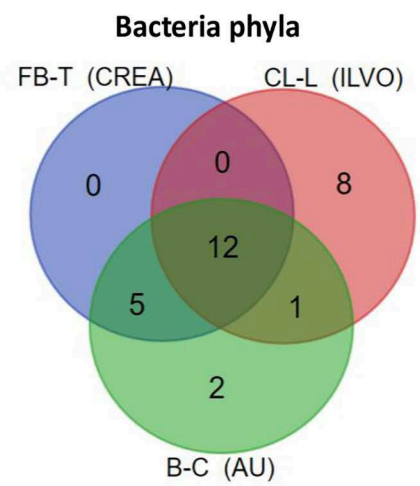
The overall community obtained after rDNA ITS fungal sequencing of rhizosphere soil samples revealed 6,546 different ASV. The taxonomic assignment revealed a very huge number of unidentified ASV (1870). The others were assigned to 14 different fungi Phyla (Supplementary material 3), particularly *Ascomycota* (39.6%), *Basidiomycota* (16.2%), *Chytridiomycota* (7.0%), *Glomeromycota* (3.5%), *Mortierellomycota* (2.0%),

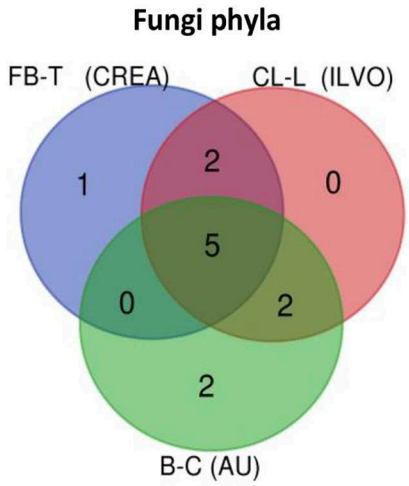
Aphelidiomycota (1.1) *Mucoromycota* (0.8%) *Rozellomycota* (0.5%), *Entomophthoromycota* (0.3%), *Olpidiomycota* (0.1%), and *Kickxellomycota* (0.2%), 251 Family and 481 Genera. The highest relative abundance was associated *Ascomycota* under cabbage (46.3%), being 45.2% under beetroot (Figure 6F). Opposite results were observed for *Basidiomycota* where the relative abundance was higher in beetroot samples (24.6%) than in cabbage samples (21.0%). The relative abundance of *Mortierellomycota* was 22.3% in beetroot and 21.8% in cabbage samples (Figure 6F). *Chytridiomycota* had a relative abundance of 4.6% and 4.9%, while *Mucoromycot*a 2.9% and 3.0% under beetroot and cabbage, respectively (Figure 6F). Regarding *Olpidiomycota* phylum, it was interesting that <36 counts only in two replicates of the samples were observed in beetroot samples, while over 550 counts for cabbage, corresponding to 0.0% of relative abundance under beetroot and 2.7 % under cabbage. For *Aphelidiomycota* and *Entomophthoromycota*, we observed an opposite trend compared to *Olpidiomycota*, with 0.0% of relative abundance under cabbage and 0.1% under beetroot for both phyla.

To better highlight the similarity–dissimilarity of bacterial and fungal populations in the investigated systems, in Figure 8. Venn diagrams of overall bacteria and fungi phyla recorded in CREA, ILVO, and AU experimental sites are reported.

Proteobacteria, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Armatimonadetes*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Chloroflexi*, *Firmicutes*, and *Rokubacteria* were found in all the tested sites, although at different relative abundance. On the opposite, in ILVO, *Entotheonellaeota*, *Patescibacteria*, *Elusimicrobia*, *Latescibacteria*, and *Verrucomicrobia* phyla were absent. *Chlamydiae* phyla was the unique phyla not found in CREA site, while *Nanoarchaeaeota*, *Halanaerobiaeota*, *Zixibacteria*, *Omnitrophicaeota, Spirochaetes*, *Hydrogenedentes*, *Deinococcus-Thermus*, and *Epsilonbacteraeota* were found only at ILVO experimental site, and *Dependentiae* and *Fibrobacteres* only at AU (Figure 7).

In relation to fungi phyla, *Basidiomycota*, *Ascomycota*, *Mucoromycota*, *Mortierellomycota*, and *Chytridiomycota* were ubiquitarian in all the tested systems, while *Kickxellomycota* were found only in CREA experimental site, being instead absent *Rozellomycota* and *Aphelidiomycota* phyla, which were recorded in ILVO and AU systems. In AU system, *Glomeromycota* very low relative abundance was not accounted under cabbage and beetroot MC (0.00071% under cabbage MC and 0.00443% under beetroot MC), although beetroot showed a certain mycorrhizal colonization intensity (<6%) (Figure 7).





Names	Total	Element
B-C (AU) CL-L (ILVO) FB-T (CREA)	12	Bacteroidetes, Armatimonadetes, Cyanobacteria, Acidobacteria, Gemmatimonadetes, Nitrospirae, Actinobacteria, Proteobacteria, Planctomycetes, Chloroflexi, Firmicutes, Rokubacteria
B-C (AU) FB-T (CREA)	5	Entotheonellaeota, Patescibacteria, Elusimicrobia, Latescibacteria, Verrucomicrobia
B-C (AU) CL-L (ILVO)	1	Chlamydiae
CL-L (ILVO)	8	Nanoarchaeaeota, Halanaerobiaeota, Zixibacteria, Omnitrophicaeota, Spirochaetes, Hydrogenedentes, Deinococcus-Thermus, Epsilonbacteraeota
B-C (AU)	2	Dependentiae, Fibrobacteres

Name	Total	Element
B-C (AU) CL-L (ILVO) FB-T (CREA)	5	Chytridiomycota, Mucoromycota, Ascomycota, Mortierellomycota, Basidiomycota
CL-L (ILVO) FB-T (CREA)	2	Glomeromycota, other
B-C (AU) CL-L (ILVO)	2	Rozellomycota, Aphelidiomycota
FB-T (CREA)	1	Kickxellomycota
B-C (AU)	1	Olpidiomycota, Entomophthoromycota

FIGURE 7
Venn diagrams of bacteria and fungi phyla in CREA, ILVO, and AU experimental field experiments. IN AU system, data are referred only to beetroot and cabbage MC systems.

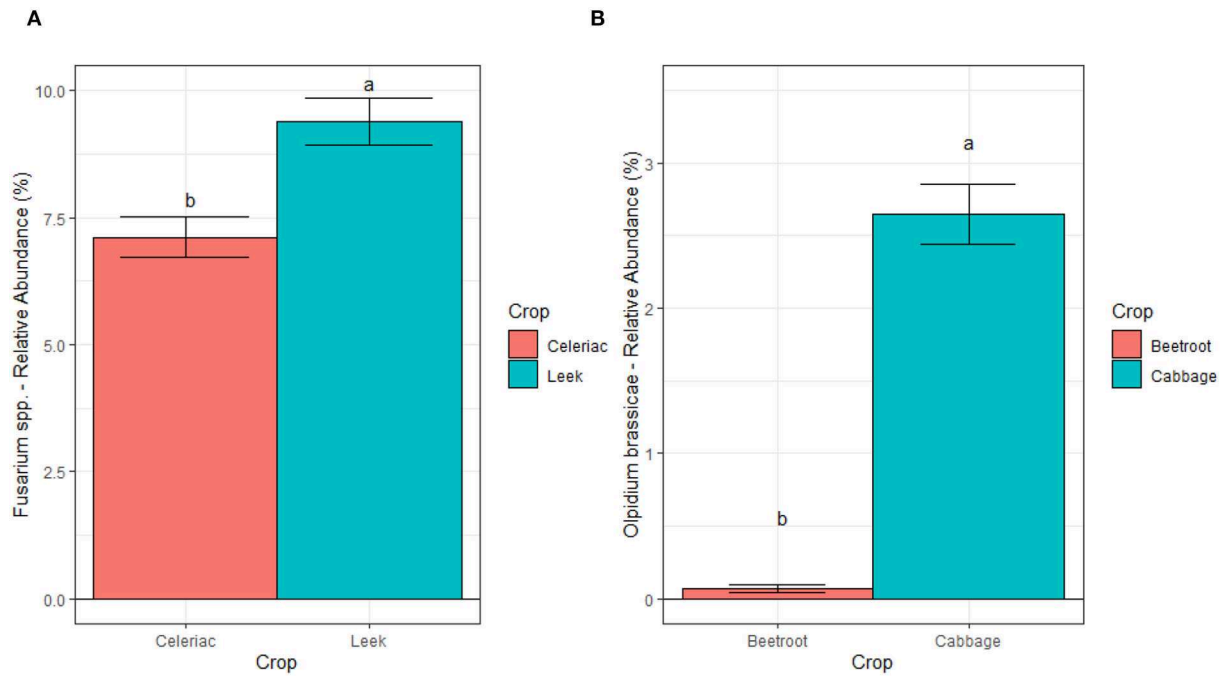


FIGURE 8
Relative abundances of *Fusarium* spp. at ILVO site **(A)** and of *Olpidium Brassicae* at AU site **(B)**, averaged across cropping systems and blocks. Bars denote standard errors of the mean. Different letters are significantly different at $p < 0.05$ (Tukey test).

We also investigated the effects of crop and CS on potential pathogens population through NMDS and PERMANOVA at CREA and ILVO sites. The NMDS did not yield a clear relationship between crop and CS on pathogens relative abundance and structure in both sites (Supplementary material 4). Table 3 shows the results of the PERMANOVA carried out on the relative abundance of the selected fungal pathogens population. At CREA the model explained about 38% of the total variability. Crop ($R^2 = 0.24$; $p \leq 0.05$) showed a significant effect on pathogens population. Similarly crop ($R^2 = 0.11$; $p \leq 0.001$) and block ($R^2 = 0.06$; $p \leq 0.01$) showed a significant effect on pathogens community at ILVO site, where the model explained about 22% of the whole variability.

We further studied the effect of crop and CS on *Fusarium* spp. at ILVO and CREA sites, and on *Olpidium brassicae* at AU through regression models. At ILVO, the analysis highlighted a significant effect of crop, with the higher relative abundance of *Fusarium spp.* under leek as compared to celeriac (Figure 8).

Similarly, the comparison across the different crop monocultures at AU showed a strong crop effect on *Olpidium brassicae* relative abundance. Here, the relative abundance of this plant pathogen was about eight times higher under cabbage compared to beetroot (Figure 8). A different scenario was observed at CREA site, where we found a significant interaction between crop and CS (Figure 9). We observed a lower relative abundance of *Fusarium spp.* in tomato rhizosphere under SC compared to MC, when the taxonomic attribution of the AVS identified *F. oxysporum*, *F. solani*, *F. nematophilum*, *F. proliferatum*, and 2 ASV of unidentified *Fusarium*. Nevertheless, the *post hoc* test did not highlight significant differences in relative abundance of this genus, potentially pathogenic, on tomato under the two CS.

TABLE 3 Results of the PERMANOVA carried out on the CREA and ILVO dataset on selected fungal pathogens.

	CREA		ILVO	
	R^2	Pr(>F)	R^2	Pr(>F)
Cropping system	0.07	n.s.	0.02	n.s.
Crop	0.24	*	0.11	***
Block	0.04	n.s.	0.06	**
Cropping system × crop	0.03	n.s.	0.03	n.s.

*, **, and *** significant at p ≤ 0.05, p ≤ 0.01, and p ≤ 0.001, respectively; n.s., not significant.

In Figure 10, a schematic representation of the main results obtained at CREA, ILVO, and AU experimental sites are described.

A linkage among the increase in relative abundance *of Proteobacteria,* the decrease in *Actinobacteria,* and the highest

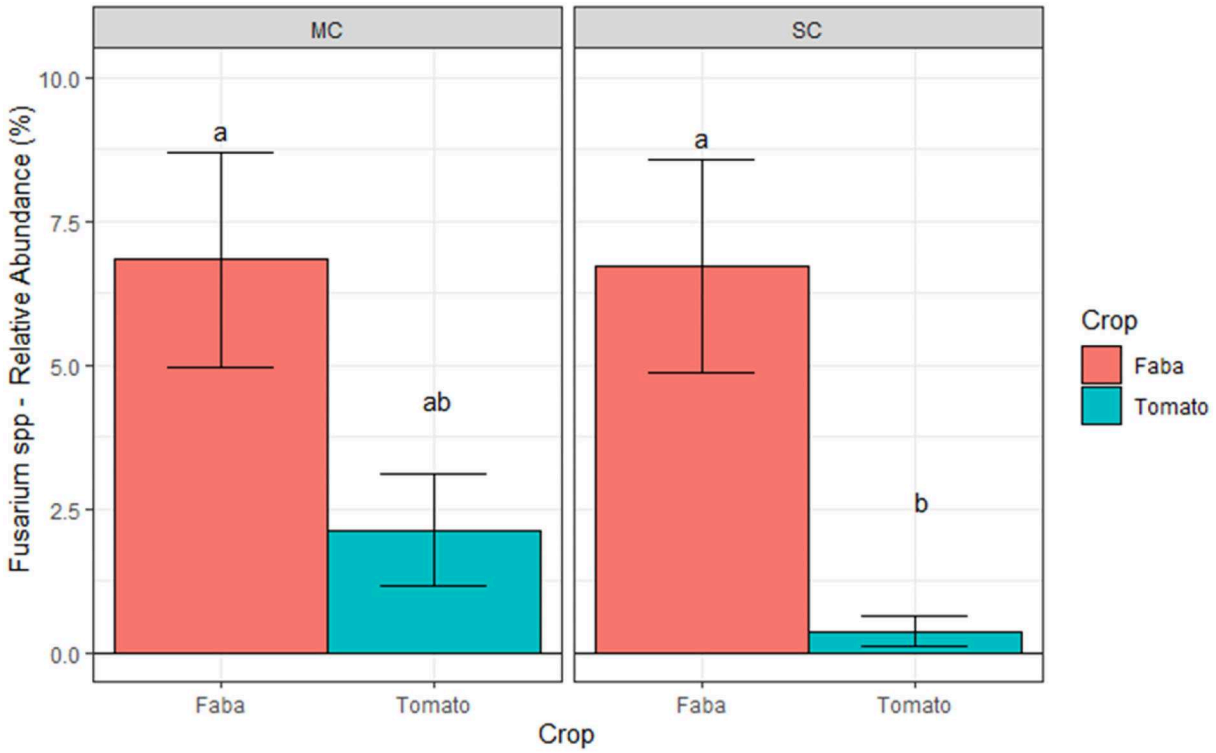


FIGURE 9
Relative abundances of *Fusarium spp.* at CREA site averaged across blocks. Bars denote standard errors of the mean. Different letters are significantly different at $p < 0.05$ (Tukey test). MC, Monocropping; SC, Strip cropping.

soil P_{av} was observed under SC faba bean at CREA, not recorded at ILVO celeriac–leak. At the same time, an increase in *Bacterioidetes* was found under both crops under SC at CREA, and under MC and IC leek at ILVO. On the opposite, a decrease of *Mortierellomycota* relative abundance was observed in tomato SC at CREA and leek IC at ILVO site, where P_{mic} was the highest. Interestingly, when C_{mic} was increased and P_{mic} was the highest, the mycorrhizal colonization of the crop increased, as at CREA and at AU.

Discussion

We hypothesized that, in studying multi-cropping vegetable systems, the plant diversity is able to shape soil microbial community supplying several ecosystem services, such as improved soil C-N-P cycles, increased plant mycorrhization, and reduced fungi soil-borne diseases. Actually, the coexistence in the field of plants with different belowground traits (Schmid et al., 2021) can lead to: i) a higher microbial community diversity in the rhizosphere, due to an additive interaction effect between the microbiota associated with both crops (complementary relationship among microbial species); ii) a lower microbial community diversity at the belowground, due to the migration of specific bacteria or fungi groups toward the rhizosphere soil, associated with the most "affine" crop (selection among microbial species). To verify the hypotheses, a multifunctional approach was applied.

Soil parameters and microbial stoichiometry

Soil organic carbon (SOC) and nutrient pools affect soil microbial stoichiometry by influencing bacteria or fungi dominance. Soil disturbance usually lowers TOC, organic N, and increases P_{av} content. This build up the diversity of low C_{mic}:P_{mic} fast-growing bacteria, which require high P_{av} to support their high growth rate (Delgado-Baquerizo et al., 2017). In natural or less disturbed soils, TOC and organic N increased, reducing fast-growing bacteria diversity in favor of fungi communities' dominance, particularly mycorrhizal fungi, which exploit soil P sink by the development of hyphal network (Schnepf et al., 2008; Chen et al., 2016). The soil pH is another key soil indicator affecting soil microbiota, working mainly on the relative abundance of principal decomposers groups of fungi and bacteria. A decrease in fungi/bacterial growth ratio was

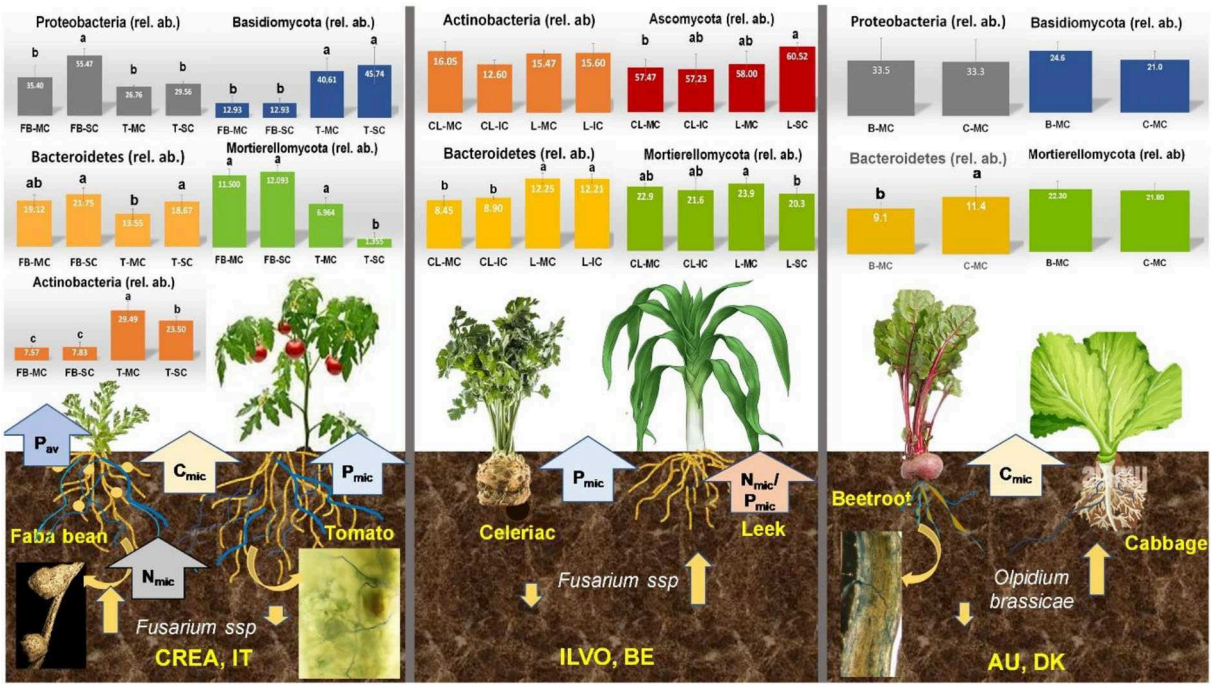


FIGURE 10
Synthesis of main relevant results obtained at CREA (IT), ILVO (BE), and AU (DK) experimental sites on belowground functional diversity. Bars denote standard errors of the mean. Different letters are significantly different at $p < 0.05$ (Tukey test). MC, monocropping; SC, strip-cropping; IC, intercropping.

observed going from pH 5.0 to 8.0 (Rousk et al., 2009). In our sites, overall pH was inversely correlated with bulk density, HWC, and HWP, and positively correlated with C_{mic}/TOC and P_{mic}, thereby influencing soil microbial biomass amount and quality (Table 2).

At CREA site, a shift toward bacteria community was found under faba bean SC (C_{mic}:N_{mic} >>5, >N_{mic}:P_{mic}, Rousk et al., 2009), while a net dominance of fungi communities under tomato SC was observed (C_{mic}:N_{mic} >5, < N_{mic}:P_{mic} ratio, Zhang and Elser, 2017) (Table 2). Since soil nutrient stoichiometry is one of the main predictors of microbial biomass composition (Strickland and Rousk, 2010; Delgado-Baquerizo et al., 2017), we argue that the lowest N_{mic} under faba bean SC was probably driven by the low availability of soil mineral N (7.3 mg kg^{-1}) at harvest under N-fixing legume crop, in absence of N-fertilization. Conversely, the highest soil mineral N (10.8 mg kg^{-1}) in tomato MC at harvest is the result of N-rich legume residue degradation by soil microbial communities, combined with the N input from fertilizer.

At ILVO, celeriac–leek IC induced a shift of soil microbes toward fungal communities. The very low N_{mic}:P_{mic} ratio suggests the dominance of saprotrophic groups in IC (Zhang and Elser, 2017). In contrast, beetroot–white cabbage IC in AU site apparently did not affect bacteria/fungi dominance, being microbial stoichiometry not changed by multi-cropping. However, the increase of C_{mic} and C_{mic}/TOC recorded under IC, compared to MC (Table 2), testifies to the ecosystem service provided by crop diversification on improving soil C stock by microbial biomass.

Crop root mycorrhization

Considering the ecosystem service provided by mycorrhizal fungi in supporting plant productivity and quality in organic systems (Gianinazzi and Gollotte, 2010; Verbruggen et al., 2010), we assert that multi-cropping can favor beneficial symbioses. As an example, the combination of different plants with supporting arbuscular mycorrhizal trait coexisting in the field supports the development of mycorrhizal hyphal network, thus boosting mycorrhizal colonization of the whole agroecosystem (Simard and Durall, 2004; Trinchera et al., 2016). We found evidence of the beneficial role played by crop diversification on root mycorrhization at the CREA site, where faba bean and tomato were more colonized by mycorrhizal fungi in SC than in MC (Figure 4A).

In AU site, we instead expected that the presence of intercropped cabbage would have completely contrasted root mycorrhizal colonization in field (Lebeis, 2015): in fact, *Brassicaceae* plants activate defense mechanisms based on glucosinolates hydrolysis (Lüthy and Matile, 1984), which generate a bacteria-dominated microbiome and no mycorrhiza harboring (Rumberger and Marschner, 2003). Although *Glomeromycota* phylum was not relevant in terms of relative

abundance (Figure 8), the coexistence of rhizosphere microbial communities under cabbage–beetroot IC apparently reduced the strong inhibiting effect played by cabbage on mycorrhizal fungi population at AU site. The presence of weeds species with supporting arbuscular mycorrhizal trait, such as *Capsella bursa-pastoris* L., *Senecio vulgaris* L., *Spergula arvensis* L., and *Plantago maior* L. (results not shown), recorded in beetroot IC only, evidently promoted the beetroot mycorrhization (Figure 4B), overruling the inhibiting effect played by the nonmycorrhizal cabbage (Hajiboland et al., 2020; Trinchera et al., 2021). This finding underpinned the hypothesis that different species of plant in the field play a selection of rhizosphere microbiome according to the plant functional traits: here, by promoting the migration of beneficial fungi population toward the beetroot (Trinchera et al., 2016).

Rhizosphere bacterial/fungal diversity

As far as the microbial functional diversity is concerned, multi-cropping did not translate into a highest bacteria/fungi community diversity, while a fungi phyla selection took place under SC at CREA site, mainly driven by legume species (Figure 5). In fact, faba bean showed a predominant role in shaping bacteria/fungi community at the belowground of both the companion crops: *Proteobacteria*, which also *Rhizobium* genus belong to, dominated the belowground diversity under the legume, being mainly α-, but also β- and γ-*Proteobacteria* associated with legume nodulation (Benhizia et al., 2004; Mus et al., 2016) (Figure 6). The presence of *Rhizobia* in soil as N-fixing bacteria largely depends on the secretions of the legume roots, which include both high and low molecular weight compounds acting as cues in plant–microbe signaling and recognition (Biate et al., 2014). In the late spring season, the presence of tomato roots growing close to faba bean ones in SC boosted the *Proteobacteria* accumulation around the faba bean roots. This is a first evidence of these bacteria selection induced by the legume root exudates, which promoted their migration toward legume roots and far from tomato ones, being this last one a *Rhizobia* nonhost plant. *Acidobacteria,* typically aerobes phyla, prefer low pH soils and operate by decomposing organic substrates and storing soil C as microbial biomass: they represent a core bacterial component among rhizobacterial assemblages, comprising 10.7% of the total observed operational taxonomic units (Na et al., 2019). They were well represented under faba bean MC, where the association with N-fixing bacteria was evidently favored but decreased rapidly in tomato SC rhizosphere soil, where the soil pH was the highest. *Actinobacteria* phyla instead were mainly associated with tomato, although a decrease was detected under tomato SC compared to MC. These phyla are mainly involved in fast degradation of low biodegradable organic compounds, such as hydrocarbons, lignin, and humus. Their decrease under tomato SC again indicated the selection effect played by legume crop, being these phyla underrepresented under faba bean. *Bacteroidetes* were well represented in CREA site regardless of CS. Generally, the impact of plant domestication process on rhizobacterial community composition leads to a decrease in *Bacteroidetes* relative abundance, while increasing *Actinobacteria* and *Proteobacteria* one (Pérez-Jaramillo et al., 2018). From an ecological point of view, soil *Bacteroidetes* thrive because of their ability to secrete diverse arrays of carbohydrate-active enzymes that target the highly varied glycans in soil, which carry out detritus decomposition (Larsbrink and McKee, 2020). The observed results evidenced the ability of crop diversification to modulate bacterial community composition in favor of those microbial groups generally reduced in agricultural, disturbed soil (Wolińska et al., 2017), due to the repeated mono-cultivation of highly domesticated varieties.

In relation to fungal community, *Ascomycota* were the predominant phyla under legume crop, while both *Ascomycota* and *Basidiomycota* were well-represented under tomato. The function of *Basidiomycota* is relevant in terms of organic matter stabilization in soil (microbial coefficient higher under tomato compared to faba bean). We stress that these fungi phyla play a key role in system ecology, due to their involvement in C cycling in temperate systems, as wood decomposers and ectomycorrhizal symbionts. For example, they form underground resource-sharing networks which support plant biodiversity in forest ecosystems (Taylor et al., 2015). Their lower relative abundance under faba bean at CREA highlights that the repeated introduction of legume species in crop rotations or in multi-cropping systems may reduce the relative abundance of *Basidiomycota* in bulk soil in the long term. This should be taken into consideration when designing organic multi-cropping systems, where SOM is subjected to high mineralization, such as those of Mediterranean region. Interestingly, *Mortierellomycota* showed the opposite behavior. They are non-saprotroph fungi phyla, living in soil on decaying leaves and other organic materials. Again, the plant made the difference, their relative abundance being highest under faba bean, independently from CS, and lowest under tomato SC. *Mortierellomycota*, and particularly of *Mortierella* genus (the most abundant genus in CREA system, see Supplementary material 3), promote plant growth across different types of crops, including herbaceous crops, so that to be considered a potential bioindicator and biocontrol agent for crop production and soil health (Zhang K. et al., 2020). *Mortierella* genus was associated with tomato rhizosphere at CREA site. The observed decrease in tomato SC was probably due to the proximity of tomato–faba bean roots and the presence of flattened legume residues as green mulch, which mostly favored the association of saprophyte, active decomposer fungi population (*Ascomycota*) with fungi groups involved in organic matter resynthesis (*Basidiomycota*). At last, we found that the increased mycorrhizal colonization observed under tomato SC did not correspond to an increase of *Glomeromycota* relative abundance in rhizosphere soil.

The decrease in *Actinobacteria* under celeriac IC at ILVO site, counteracted by the increase in relative abundance of *Proteobacteria* (the overrepresented bacteria phyla), again confirms the selection effect played by two different crops contemporary grown in field. *Bacteroidetes* relative abundance was the highest under leek: it was already found that *Bacteroidetes* increased as a response of crop diversification in a banana–leek rotation, compared to banana MC (Ouyang et al., 2011). Consequently, the fluctuation of *Bacteroidetes* relative abundance appeared a suitable microbial indicator to evaluate the effect of crop diversification on soil bacteria diversity. Concerning fungi, a transition toward *Ascomycota*-dominated community was observed in celeriac IC, indicating that fungal community shifted from slow-growing oligotrophic fungi groups to fast-growing copiotrophic fungi groups (Yang et al., 2017). Again, the decrease of *Mortierellomycota* in both IC celeriac and leek suggests that the shared roots between different companion crops exert a negative effect on relative abundance of this phyla. This result was already observed in other intercropped systems, where a decrease in *Mortierella* genus was detected. However, the reason for this reduction was not fully clear and calls for further investigations (Sen and Fengzhi, 2018).

In AU site, *Proteobacteria* and *Actinobacteria* dominated both cabbage–beetroot MC bacteria community, confirming that again a certain impact of domestication on rhizobacterial diversity took place. Unexpectedly, the crops did not change bacterial community composition, but a significant increase in *Bacteroidetes* relative abundance was observed under cabbage. In relation to fungi community composition, *Ascomycota*, the most representative phyla, *Basidiomycota*, and *Mortierellomycota* were predominant, again regardless of the companion crops. The high relative abundance of *Mortierellomycota* found in AU site is an evidence of good soil health, since *Mortierella*, the most represented genus among *Mortierellomycota*, can synthetize the arachidonic acid, recognized as an elicitor of phytoalexins in plants for suppressing plant disease (Eroshin et al., 1996; Tagawa et al., 2010). The absence of *Olpidiaceae* phyla in beetroot rhizosphere, instead detected in the cabbage one, has an important agroecological implication. Crop diversification has been often reported to be a viable strategy to decrease fungal pathogens accumulation with positive feedback on crop productivity (Maron et al., 2011). The mechanisms behind this effect include: (i) the exudation of allopathic compounds (e.g. Hao et al., 2010); (ii) reductions in the relative abundance of pathogens due to an increase diversity and physical occupation of pathogens niches (Mitter et al., 2013); (iii) reductions in pathogens host plant species due to higher crop diversity (He et al., 2019); and (iv) positive effects on antagonistic microbial communities (Latz et al., 2012). In this study, when we aggregated the data on fungal pathogens, we did not find a significant effect of the CS on pathogens communities at CREA and ILVO sites (Table 3). The relative abundance of specific *genera*, which also comprise pathogens (namely, *Fusarium* spp. and, particularly, *Olpidium brassica*) (Bolwerk et al., 2005), which are known to be cause of serious plant diseases, was significantly affected by crops (Figures 8, 9). From a field perspective, this result supports the idea that combining a nonhost with a host plant can limit the relative abundance of pathogens in long term, with positive effect on crop productivity. Only at CREA site, we also found an interaction effect of crop diversification in the rhizosphere, being *Fusarium* spp. less abundant under tomato when grown in SC compared to MC. Although the *post-hoc test* did not significantly discriminate across the different CS, this trend suggests a possible positive effect of multi-cropping system in diminishing *Fusarium spp* in tomato cultivation. The lack of a strong CS effect on the selected fungal pathogens was probably due to the sampling protocol: we indeed sampled the rhizosphere soils as a mean to investigate whether roots interactions under IC would have affected fungal pathogens populations. Probably, a bulk soil sampling could have yielded more solid information on the pathogens population under IC as compared to MC.

In conclusions, IC and SC may increase soil microbial biomass, N and P nutrient availability, thus shaping the microbial community toward predominance of bacteria or fungi community, in function of selected companion crops and pedoclimatic conditions. Multi-cropping does not increase the overall bacteria and fungi diversity, while we observed a crop selection effect on rhizosphere microbiota, rather than a complementary, additive effect among microbial species. In multi-cropping systems, increased *Bacteroidetes* and reduced *Mortierellomycota* relative abundance in rhizosphere soil suggest they can be considered as sensitive ecological indicators of improved agro-system functionality induced by plant diversity: *Bacterioidetes*, being able to testify the introduction of low-impact agricultural practices, and *Mortierellomycota* as undirect indicator of the reduced pressure made by pathogens. Multi-cropping favored the spontaneous mycorrhizal symbiosis between companion crops, leading to a corresponding increase of SOC accumulation in microbial biomass. Where companion crops were duly selected, multi-cropping also reduced the relative abundance of soil pathogens, with a potential positive effect on crop productivity in the long term. However, further studies are requested to understand the role played by fungal hyphae on SOC accumulation and by crop mycorrhization in reducing fungal pathogen in multi-cropping systems.

Data availability statement

Used bacteria and fungi primers and bacteria/fungi counts presented in this study are deposited in DRYAD repository "Supplementary materials 2, 3_Bac_Fungi primers and Bac_Fungi counts_Article: "Can multi-cropping affect soil microbial stoichiometry and functional diversity, decreasing potential soil-borne pathogens? A study on European organic vegetable cropping systems" _Frontiers in Plant Science". Accession number: https://doi.org/10.5061/dryad.kh1893296.

Author contributions

AT: first authorship, conceptualization, dataset elaboration, evaluation of root mycorrhization, results interpretation, and final revision. MM: DNA extraction, identification of bacteria/fungi rhizosphere populations from NGS dataset, and support to first authorship on Rhizosphere bacteria/fungi diversity section. DW statistical analysis and support to first authorship on paper revision. SO: DNA extraction and identification of bacteria/fungi rhizosphere populations from NGS data. JD: support on protocols for rhizosphere sampling and NGS analysis, NGS data interpretation, and paper revision. SS: stoichiometry determinations, sampling and evaluation of mycorrhizal colonization dataset, and paper revision. SD: sampling and evaluation of mycorrhizal colonization dataset and paper revision. JB: setting of experimental design and soil parameters determination. PK: determination of soil parameters and paper revision. HK: setting of experimental design and final paper revision. LL and TS: setting of experimental design and paper revision. GC: setting of experimental design. KW: senior authorship, conceptualization, and final revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.952910/full#supplementary-material

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A novel function of the key nitrogen-fixation activator NifA in beta-rhizobia: Repression of bacterial auxin synthesis during symbiosis

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Rhizobia fix nitrogen within root nodules of host plants where nitrogenase expression is strictly controlled by its key regulator NifA. We recently discovered that in nodules infected by the beta-rhizobial strain *Paraburkholderia phymatum* STM815, NifA controls expression of two bacterial auxin synthesis genes. Both the *iaaM* and *iaaH* transcripts, as well as the metabolites indole-acetamide (IAM) and indole-3-acetic acid (IAA) showed increased abundance in nodules occupied by a *nifA* mutant compared to wild-type nodules. Here, we document the structural changes that a *P. phymatum nifA* mutant induces in common bean (*Phaseolus vulgaris*) nodules, eventually leading to hypernodulation. To investigate the role of the *P. phymatum iaaMH* genes during symbiosis, we monitored their expression in presence and absence of NifA over different stages of the symbiosis. The *iaaMH* genes were found to be under negative control of NifA in all symbiotic stages. While a *P. phymatum iaaMH* mutant produced the same number of nodules and nitrogenase activity as the wild-type strain, the *nifA* mutant produced more nodules than the wild-type that clustered into regularly-patterned root zones. Mutation of the *iaaMH* genes in a *nifA* mutant background reduced the presence of these nodule clusters on the root. We further show that the *P. phymatum iaaMH* genes are located in a region of the symbiotic plasmid with a significantly lower GC content and exhibit high similarity to two genes of the IAM pathway often used by bacterial phytopathogens to deploy IAA as a virulence factor. Overall, our data suggest that the increased abundance of rhizobial auxin in the non-fixing *nifA* mutant strain enables greater root infection rates and a role for bacterial auxin production in the control of early stage symbiotic interactions.

KEYWORDS

root-nodule, legume, indole-acetamide, nitrogenase, infection

Introduction

Rhizobia are nitrogen (N_2)-fixing symbiotic bacteria that trigger the formation of root organs in leguminous plants, called nodules, where biological nitrogen fixation (BNF) takes place (Poole and Allaway, 2000; Masson-Boivin et al., 2009; Oldroyd et al., 2011). The establishment of this symbiotic interaction relies on a sophisticated molecular dialog between bacterial and plant partners (Lee and Hirsch, 2006; Ferguson et al., 2010; Oldroyd et al., 2011; Via et al., 2016). Plants first exude flavonoids in the rhizosphere, which chemoattract rhizobia to the roots (Lee and Hirsch, 2006; Cooper, 2007; Ferguson et al., 2010; Via et al., 2016). In response to flavonoids, bacteria induce the expression of the *nod* (nodulation) genes responsible for the biosynthesis of lipochitooligosaccharides known as nodulation factors (NFs). The perception of NFs leads to root hair deformation and curling that entrap the bacteria in an infection pocket (Gage and Margolin, 2000; Gage, 2004; Ferguson et al., 2010; Oldroyd et al., 2011). In parallel, NFs activate a signaling cascade in the plant cells that leads via a calcium spiking response to massive cell division in the root cortex. Then, multiplying bacteria penetrate the infection threads (ITs) formed toward the nodule primordia, where rhizobia are released into plant cells and differentiated into N_2-fixing bacteroids (Gage, 2004; Lee and Hirsch, 2006; Oldroyd et al., 2011; Poole et al., 2018). The expression of the rhizobial *nif* (nitrogen fixation) genes is nodule-specific and is activated by the alternative RNA polymerase sigma factor σ^{54} (or RpoN) together with its enhancer binding protein NifA (Hauser et al., 2007; Lardi et al., 2018). The impact of the loss of NifA function on nodulation, in addition to the absence of nitrogenase activity (Fix^-), has been described in several legume symbioses. Despite the different nodule types, the Fix^- nodules triggered by *nifA* mutant symbionts were often reported impaired in leghemoglobin production, to structurally decay, and/or eventually turn necrotic. For instance, the roots of *Lotus japonicus* colonized with the *Mesorhizobium loti* MAFF303099 *nifA2* mutant display minute white nodules (Nukui et al., 2006) while *Medicago truncatula* plants inoculated with the *Sinorhizobium meliloti* 1021 *nifA* mutant develop Fix^- mature nodules but, in contrast to wild-type symbionts, the bacteria prematurely die shortly after bacteroid elongation (Berrabah et al., 2015). A *Bradyrhizobium diazoefficiens* strain USDA110 *nifA* mutant showed an increased number of nodules all over the soybean root system and the induced nodules were necrotic (Fischer et al., 1986; Parniske et al., 1991). During symbiosis with *Aeschynomene americana*, the Fix^- *Bradyrhizobium* sp. DOA9 *nifAp nifAc* double mutant induced smaller nodules without impacting nodulation frequency (Wongdee et al., 2018). These typical Fix^- phenotypes led to suboptimal host plant development, hence indicating that NifA function is essential for symbiotic nitrogen fixation and nodule maintenance (Berrabah et al., 2015).

Phytohormones, especially auxin and cytokinin are also involved in coordinating the nodulation process (Ferguson et al., 2010; Oldroyd et al., 2011; Liu et al., 2018; Velandia et al., 2022). Auxins were first discovered as plant hormones playing crucial roles in plant growth, development, embryogenesis and tropism (Zhao, 2010). Several studies have shown that auxin transport and localization are important for nodule organogenesis during rhizobia-legume symbiosis since auxin influences nodule initiation, differentiation and vascular bundle formation (Mathesius, 2008; Kohlen et al., 2018; Shrestha et al., 2020; Torres et al., 2021). Interestingly, certain rhizobia are also capable of synthetizing auxins, mostly indole-3-acetic acid (IAA), which can directly interfere with plant auxin homeostasis (Mathesius, 2008; Spaepen and Vanderleyden, 2011) and functions as a signaling molecule (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011). The bacterial pathways for IAA synthesis either depend on or are independent of tryptophan (TRP) as precursor (Duca et al., 2014; Zhang et al., 2019). Although the TRP-independent pathway is not well studied yet, five pathways have been categorized as TRP-dependent: the indole-3-acetamide (IAM), the indole-3-pyruvic acid (IPA), the tryptamine, the indole-3-acetonitrile and the tryptophan side-chain oxidase pathways (Spaepen et al., 2007; Zhang et al., 2019). In microorganisms, the two most common and better studied pathways are the IAM and IPA pathways (**Supplementary Figure 1**; Spaepen et al., 2007; Morffy and Strader, 2020). In the IAM pathway that is mainly present and used by phytopathogenic bacteria, a monooxygenase (encoded by *iaaM*) converts TRP into IAM, which is then hydrolyzed to IAA and ammonia by the IAM hydrolase (encoded by *iaaH*). The IPA pathway converts TRP into IAA in three steps. First an aminotransferase converts TRP to IPA that is then decarboxylated to indole-3-acetaldehyde (IAAld) by the indole-3-pyruvate decarboxylase IpdC. IPA is finally oxidized to IAA by the IAAld dehydrogenase (Spaepen and Vanderleyden, 2011; Zhang et al., 2019; Morffy and Strader, 2020). Although this pathway can be found in some plant pathogens, it has been mostly associated with beneficial bacteria (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011; Patten et al., 2013). In rhizobia-legume symbiosis, bacteria contribute to the production of IAA in nodules (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011). Studies on *Rhizobium leguminosarum* bv. *viciae* LPR1105 genetically engineered to express *iaaM* from *Pseudomonas savastanoi* and *tms2* from *Agrobacterium tumefaciens*, suggested that IAA plays a role in the maintenance of a functional root nodule in *Vicia hirsute*, since nodules of this genetic variant exhibited a more efficient nitrogenase activity, a bigger size and a delayed senescence (Camerini et al., 2008).

Paraburkholderia phymatum STM815 is one of the main model organisms to study beta-rhizobia-legume symbiosis (Moulin et al., 2001) and stands out for its ability to establish N_2-fixing symbiosis with a wide range of legumes, including the economically important *Phaseolus vulgaris* (common bean)

(Elliott et al., 2006; Gyaneshwar et al., 2011; Mishra et al., 2012; Moulin et al., 2014; Lemaire et al., 2016; Lardi et al., 2017a). We previously showed that *P. phymatum* NifA is essential for nitrogenase activity during symbiosis and that a *P. phymatum nifA* mutant induced an increased number of *P. vulgaris* nodules compared to plants infected with the wild-type strain (Lardi et al., 2017b). Furthermore, a combined metabolomics and dual RNA-sequencing analysis of common bean nodules induced by a *P. phymatum nifA* mutant, revealed that NifA down-regulates the expression of *P. phymatum*'s auxin genes *iaaM* and *iaaH* thus leading to IAM and IAA accumulation. In contrast to the above-mentioned studies on legume symbioses with different rhizobial *nifA* mutants, the loss of NifA in *P. phymatum* did not trigger an immune response against the mutant bacteria (Bellés-Sancho et al., 2021a).

Here, we further investigate the effect in the absence of NifA on nodule architecture as well as the role of NifA in controlling the expression of *iaaMH* during different stages of symbiosis, from the plant-rhizobia recognition step until the development of a mature root nodule. Our data showed that nodules induced by the *P. phymatum nifA* mutant developed normally despite their clustered organization. We show that NifA represses the expression of the *iaaMH* genes in all stages of symbiosis. Furthermore, by constructing a mutant strain lacking the *iaaMH* operon, we demonstrated that *P. phymatum* produces IAA via the IAM pathway inside *P. vulgaris* root nodules. While this pathway does not seem to be essential for a functional symbiosis, the up-regulation of *iaaMH* in a strain lacking NifA was responsible for the increased number of nodule clusters. Finally, orthologs of the *P. phymatum iaaMH* genes were found in many strains of important phytopathogens such as *Pseudomonas syringae* and *Agrobacterium tumefaciens*, suggesting a related role in plant infection.

Materials and methods

Bacterial strains, media, and cultivation

Bacterial strains, primers and plasmids used in this work are listed in **Supplementary Table 1**. *Escherichia coli* and *P. phymatum* strains were routinely cultivated in Luria-Bertani (LB) (Miller, 1972) media and its modified version without salt (LB-NaCl), respectively (Liu et al., 2020). *Escherichia coli* strains were grown aerobically at 37°C and 220 rpm, while *P. phymatum* strains were incubated aerobically at 28°C and 180 rpm. When required, the growth medium was supplemented with the following antibiotics at the indicated concentrations: chloramphenicol (Cm, 20 μg/mL for *E. coli* and 80 μg/mL for *P. phymatum*), kanamycin (Km, 25 μg/mL for *E. coli* and 50 μg/mL for *P. phymatum*) or trimethoprim (Tm, 50 μg/mL for *E. coli* and 100 μg/mL for *P. phymatum*). For the *gfp*-expression analysis with germinated seeds, bacteria were grown on (A) BG medium [(A) B-minimal media supplemented with 0.3 mM NH_4Cl and 10 mM glucose as carbon source] (Liu et al., 2020).

Construction of *Paraburkholderia phymatum* GFP reporter strains and mutant strains

To construct the promotor fusion, the promotor region of Bphy_7769 was amplified by PCR with Bphy_7769p_*Eco*RI_F and Bphy_7769p_*Sal*I_R primers, using as a template *P. phymatum* STM815 genomic DNA (gDNA). The 207 bp-long product was digested with the restriction enzymes *Eco*RI and *Sal*I and cloned into the multiple cloning sites in front of the *gfp* gene in the vector pPROBE-NT using the same restriction sites as the insert. After transformation in *E. coli* Top10, the cloned sequence was verified by sequencing at Microsynth (Balgach, St. Gallen, Switzerland). The constructed plasmid was transferred into *P. phymatum* wild-type and a *nifA* insertional mutant (Lardi et al., 2017b) by triparental mating. The deletion mutant of the *iaaMH* genes was constructed by cloning an antibiotic resistance gene in between two external flanking sequences of the genes to be deleted. The primers Bphy7769_up_F_*Eco*RI and Bphy7769_up_R_*Nde*I were used to amplify the Bphy_7769 upstream fragment of 529 bp and the primers Bphy7768_dn_F_XhoI and Bphy7768_dn_R_*Eco*RI for the 525 bp-length to get the Bphy_7768 downstream fragment. A trimethoprim cassette *dhfr* followed by a transcription terminator was digested with *Nde*I from the p34E-TpTer plasmid (Shastri et al., 2017) and cloned in between the upstream and downstream fragments. The resulting 1,945 bp long DNA fragment was then inserted into the suicide pSHAFT2 vector, resulting in the pSHAFT2:Δ*iaaMH* plasmid, which was verified by sequencing. The pSHAFT2:Δ*iaaMH* plasmid was transferred into *P. phymatum* wild-type by triparental mating. Clones sensitive to Cm and resistant to Tm were selected and purified. The genomic integration of the construct was verified by PCR using Bphy7770_veri_F and Bphy7767_veri_R primers. The auxin deletion mutant was called Δ*iaaMH*. To construct the *nifA*-Δ*iaaMH* double mutant, the pSHAFT-nifA plasmid (Lardi et al., 2017b) was conjugated in *P. phymatum* Δ*iaaMH* and clones resistant to Cm and Tm were selected. To generate the complemented strain of the Δ*iaaMH* mutant and *nifA*-Δ*iaaMH* double mutant, both *iaaM* and *iaaH* genes with their native promoter were amplified by PCR using the primers Bphy7769_comp_F_*Xba*I and Bphy7768_comp_R_*Xba*I. The 3,246 bp-length product was then digested with *Xba*I and cloned in forward and reverse direction into the multicloning site of the vector pBBR1MCS2 with respect to the direction of *lacZ*α gene. These two *iaaMH* complemented constructs differ on their promoters: while the expression of *iaaMH* cloned in forward direction is driven by the promotor of

*lacZ*α (called pBBR1MCS2:*iaaMH*_*placZ*), the transcription of *iaaMH* genes cloned in reverse direction relies on their native promotor (pBBR1MCS2:*iaaMH*). The constructed Δ*iaaMH* or *nifA*-Δ*iaaMH* complemented strains were called Δ*iaaMH* + pBBR-*iaaMH* and *nifA*-Δ*iaaMH* + pBBR-*iaaMH* when *iaaMH* was expressed by their natural promoter or Δ*iaaMH*/*nifA*-Δ*iaaMH* + pBBR-*iaaMH*(*placZ*) when they were under the control of the strong promoter. The correct sequence was verified by sequencing using the LacZ_R and Bphy7768_seq_R primers.

Seedling preparation, plant infection, and growth condition

Phaseolus vulgaris, cv. Negro jamapa (common bean) seeds were surface-sterilized with absolute ethanol for 5 mins, 35% solution of H_2O_2 for another 5 mins and subsequently washed 10 times with sterile deionized water (Talbi et al., 2010). Seeds were placed on 0.8% agarose plates and incubated 48 h at 28°C in the dark for germination. Sprouted seeds were then transferred into yogurt-jars containing sterile vermiculite (VTT-Group, Muttenz, Switzerland) and 160 mL of nitrogen-free Jensen media (Hahn and Hennecke, 1984). Bacterial strains were grown in liquid LB-NaCl overnight as previously described. Then, cultivated cells were collected, washed twice with (A)B-minimal medium (Liu et al., 2020) and adjusted to a final OD_{600} of 0.025 (10^7 cells per mL) (Lardi et al., 2017b). One mL was inoculated directly on each germinated seedling. Plants were grown in the green-house with a day/night temperature of 25/22°C with 16 h of light in a constant humidity of 60%. Plants were watered twice a week with sterile deionized water and harvested after 21 days of incubation.

Characterization of symbiotic properties *in planta*

To assess the symbiotic properties, nodules of 21 days old plants inoculated with *P. phymatum* strains were harvested. The number of nodules, dry weight per nodule and nitrogenase activity were determined as previously described (Lardi et al., 2017a,b). To obtain the nodule occupancy, at least one nodule per inoculum was surface sterilized as previously described (Lardi et al., 2017a). Nodules were crushed and bacteroids were isolated by plating on LB-NaCl and counting of the colony forming units (CFU). For the quantification of the nodule-clustering phenotype, each root system (n = 15 plants) was completely washed with deionized water and individual basal roots were severed from the primary root and vertically aligned onto one polyester clear sleeve per plant. The individual root systems were scanned (300 dpi, ImageScanner III, GE Healthcare) and the following parameters were recorded from the images with ImageJ. We measured the primary and basal root lengths and numbers and recorded for every nodule of each root its dimensions (ROI area and shape descriptors) and its position on the root axis relative to the proximal extremity of the root.

Histological preparations

To obtain precise cytological features, the cell walls of *P. vulgaris* nodules were stained with Calcofluor White M2R (Sigma-Aldrich, Buchs, St. Gallen, Switzerland). In brief, fresh nodules midpoint transversal sections were obtained under the binoculars with sterilized fine blades and immediately covered with 20 µl of a filter-sterilized 1 mg/mL Calcofluor White solution in deionized water. After 5 mins of incubation, excess of dye, cell debris and free bacteria were removed by washing thrice with sterile tap water. The sectioned nodules were directly mounted in 10% glycerol on glass slides for acquisitions. Live/Dead staining was performed using the BacLight Bacterial Viability Kit (L7012, Invitrogen, Bleiswijk, Netherlands) according to the manufacturer guidelines. The procedure was applied to bacteroids extracted from fresh nodules after disrupting the tissue in 400 µl sterile 0.9% NaCl with a disposable micropestle and 2 mins centrifugation at 1,500 rpm, or to bacteria recovered from bacteroids into selective medium. For direct assessments of bacteroids viability into host cells, live/dead straining procedure was performed simultaneously to Calcofluor White preparations. All images were obtained from a confocal laser scanning microscope (DM5500Q; Leica, Wetzlar, Germany) fitted with a TCS SPE confocal unit (Leica, Wetzlar, Germany), an ACSAPO 40 × oil-immersion objective (NA = 1.15, Leica, Wetzlar, Germany) or an ACSAPO 10 × dry objective (NA = 0.3, Leica, Wetzlar, Germany), and laser lines set at 405, 488, and 532 nm with fixed detection windows corresponding to the maximum emission peaks of the respective fluorophores, using the LAS software (Leica, Germany). In each experimental set, the acquisition parameters were identical between samples. The obtained images were analyzed with ImageJ.[1]

In planta gfp-expression analysis

To visualize the induction of *iaaMH* expression on the surface of germinated bean roots, overnight cultures of *P. phymatum gfp*-reporter strains with empty pPROBE, *pnodB*, and *piaaMH* in the wild-type and *nifA* mutant strain were washed twice with 10 mM $MgSO_4$ and inoculated in melted 0.8% agarose (A)BG medium to a final OD_{600} of 0.05. Germinated seeds were placed in the middle of the plate with

1 https://imagej.nih.gov

the tip of the primary root submerged into the medium while the agarose medium was still liquid. After solidification, plates were incubated for 3 days at 28°C. Three independent biological replicates were prepared for this assay. The fluorescence was then quantified using a Leica M205 FCA fluorescent stereo microscope equipped with a DFC 7000 T CCD camera and the relative fluorescent signal was acquired through an ET GFP filter set (470/40 nm excitation, 525/50 nm emission). The GFP expression in bean inoculated with the *P. phymatum gfp*-reporter strains was monitored 10, 14, and 21 days after inoculation. Next, the sections were placed on a glass slide and observed under the microscope. Deeper inspection of the tissue sections was performed using the confocal microscope with additional Calcofluor White staining when required. The obtained images were analyzed using ImageJ.

Metabolite extraction and data analysis

The metabolite abundance in *P. vulgaris* root nodules inoculated with different *P. phymatum* auxin-mutant strains was compared. As previously described, approximately 30 mg of nodules or uninfected roots were immediately frozen with liquid nitrogen and metabolites were extracted using cold methanol (Lardi et al., 2016, 2018; Bellés-Sancho et al., 2021a,b). Three independent biological inoculants were processed with one or two plants per inoculum. Next, the extracts were injected twice and analyzed by non-targeted flow injection-time-of-flight mass spectrometry on an Agilent 6550 QTOF instrument (Agilent Technologies, Santa Clara, CA, USA), using the settings previously described (Fuhrer et al., 2011). A total of 285 ions were detected with distinct *m/z* within a tolerance of 0.001 Da and were matched to expected deprotonated molecules. The abundance of the metabolites involved in IAA production [according to the KEGG database (Kanehisa and Sato, 2020)] were estimated and compared according to the ion count values for each sample. The complete metabolomics data set, including a list of detected ions, annotations and intensities, is listed in the **Supplementary Table 2**.

Auxin production quantification

The estimation of the amount of auxinic compounds secreted by *P. phymatum* wild-type, *nifA* mutant, *iaaMH* mutant and the corresponding *iaaMH* complemented strains, was performed as previously described (Bellés-Sancho et al., 2021a). Briefly, overnight cultures were routinely grown in LB-NaCl and cells were collected by centrifugation (5 mins at 5,000 rpm) and washed twice with LB-NaCl. An initial cell density of OD_{600} = 0.05 was inoculated into 20 mL of LB-NaCl per strain and cultivated for 16 h at 28°C with 180 rpm shaking. Supernatants were collected by centrifugation and mixed with Salkowski's reagent in a volume ratio of 1:2 (Gordon and Weber, 1951). After 20 mins of incubation in the dark, the mixtures' absorbances were measured at 535 nm and compared to a standard curve of pure IAA (1003530010, Sigma-Aldrich, Buchs, St. Gallen, Switzerland). Three biological replicates were performed per strain.

Bioinformatic analysis

To identify bacteria that encode orthologs of the *iaaMH* genes and orthologs of *nifA*, *nifH*, and *nodA* (in *P. phymatum* STM815, these genes are all located on the 595,108 bp symbiotic plasmid pBPHY02), the protein sequences of IaaM (WP_012406795.1), IaaH (WP_012406794.1), NifA (WP_012406756.1), NifH (WP_012406781.1), and NodA (WP_012406745.1) were searched together against the NCBI's Identical Protein Groups (IPG) resource using cblaster (Gilchrist and Chooi, 2021). The maximum intergenic distance allowed between conserved hits was set to 600 kb and the presence of at least IaaM and IaaH was required, using cblaster's default BLAST parameters (e-value threshold of 0.01, minimum protein identity of 30% and query coverage of 50%). To visualize genes between and in the vicinity of these five query genes, the intermediate genes option was enabled and synteny plots of the 150 best-scoring clusters were generated using cblasters plot_cluster mode. By default, cblaster only reports orthologs that are assigned to a cluster, meaning they cannot exceed the specified maximum intergenic distance and must occur on the same contig, a limitation for scaffold-level assemblies. Therefore, the cblaster session file was parsed using a custom Python script to extract both BLAST hits that could be assigned to a cluster as well as those that could not. Specifically, for each strain identified by cblaster, each query protein on top of IaaM and IaaH was checked for having a hit located in a cluster. If at least one hit was part of a cluster, the most similar hit to the query in the cluster was chosen. Otherwise, the hit with overall highest similarity was chosen, regardless of its genomic position. Similarity was defined as the product of sequence coverage and sequence identity, both normalized to 1 (range 0.15–1.0). Strains were grouped by species and the minimal and maximal cluster score assigned by cblaster (based on the number and similarity of genes in the cluster), as well as the minimal and maximal similarity for each gene was calculated. The resulting summary table (**Supplementary Table 3**) was grouped by genus and species and ordered by their respective maximum cluster scores. The table was then filtered to only contain assigned species (all "sp." entries were removed) and some additional entries were removed (having a contamination, not being a genome assembly or failing NCBI's taxonomy check). A phylogenetic tree based on the IaaH and IaaM orthologs in the species listed in

Supplementary Table 3 was generated. A single genome was chosen to represent each species: The NCBIs representative genome was chosen if it was identified by cblaster, otherwise the best genome was selected based on NCBIs taxonomy check, completeness of the genome, and the number of contigs reported. The orthologs of both proteins were separately aligned using Clustal Ω 1.2.4 (Sievers et al., 2011) and the automatic model selection mode of RAxML 8.2.12 (Stamatakis, 2014) (PROTGAMMAAUTO) was used to determine the best protein selection model for each protein. The alignments of both genes were concatenated using BioPython 1.79 (Cock et al., 2009) and RAxML was used to create 100 bootstrap trees with partitions defined such that the best model is used for each protein (LG for IaaH and JTT for IaaM, still in PROTGAMMA mode). The resulting bootstrap trees were concatenated and bootstrap values were added to the best tree using RAxML with -f b flag. The tree was visualized using FigTree. To look at conservation on the DNA level, the nucleotide sequence from Bphy_7758 to Bphy_7769 (NC_010627.1:528492-542282) was blasted against NCBIs nt database (downloaded 05.05.2022) using blastn 2.12.0 + with the default settings from NCBIs website. The results were converted to GFF format using the NCBIXML module from BioPython and inspected in IGV (Thorvaldsdóttir et al., 2013). The GC content in the region of the predicted *P. phymatum iaa* operon (Bphy_7767-9; Burkholderia genome database[2]; NC_010627:532782-547282) was analyzed using a custom Python script as a sliding window analysis with a window size of 2 kb and a step size of 0.4 kb. A second sliding window analysis with a window size equal to the operon length of 4,500 bases was used to determine if the GC content of the predicted *iaa* operon significantly deviated from the average GC content of windows of similar size from the plasmid pBPHY02 (NC_010627). The GC content of the predicted operon was calculated separately and its location among all sorted window GC contents was determined. The results were visualized with Matplotlib 3.5.1 (Hunter, 2007; Caswell et al., 2021).

Statistical analysis

The statistical analysis for the characterization of the *gfp* expression data, symbiotic properties, Salkowski's indolics quantification and the metabolite's ion count analysis of the nodules was performed with GraphPad Prism 6.0 using an ordinary one-way ANOVA with Tukey's multiple comparison (p-value $\leq$ 0.05). 1D clustering of the pooled nodules positions was performed according to the Jenks natural breaks classification method with 10 classes and 25,000 iterations. A population of 500 randomized values ranging from 0 to 250 mm was used as negative control.

2 https://burkholderia.com/

Results

Paraburkholderia phymatum lacking *NifA* (Fix$^-$) does not develop abnormal nodules in common bean

The Fix$^-$ *P. phymatum* STM815 *nifA* mutant triggers hypernodulation in *P. vulgaris* (Lardi et al., 2017b), a phenotype we associated with the repression of the autoregulation of nodulation (AON) system (Bellés-Sancho et al., 2021a). To evaluate the structural effects on common bean nodules induced by the *P. phymatum nifA* mutant, 11-days old bean plants inoculated with the *nifA* mutant were compared to inoculations with wild-type and *nifA* complemented symbionts. The examined bean plants infected with the *nifA* mutant harbored an increased number of nodules that often clustered in mature parts of the roots (Figure 1A) and unusual structures. This phenotype was reverted by genetic complementation of the *nifA* mutant. Closer inspection of the mature atypical structures under the microscope revealed that they were in fact composed of manifold nodules that seem fused with each other (Figure 1B). Transversal sections of single young, round nodules and larger, flatter mature nodules displayed no notable histological differences between plants infected with wild-type and *nifA*, and their overall organization remained consistent with established determinate nodule architectures (Figures 1C,D). However, the sections of clustered *nifA* nodules showed various levels of fusion of cortical, parenchymal and endodermal tissues, shared vasculature but distinct infection zones, *i.e.,* originating from discrete ITs (Figures 1E,F). We did not find any *nifA* nodule with necrotic or disorganized internal architecture. Mature wild-type, *nifA* and *nifA* complemented nodules indistinctively exhibited the coloration characteristic of leghemoglobin. The organogenesis of nodules occupied with the *nifA* mutant strain was thus not impaired and the observed cluster-like structures seem to be derived from multiple primordia initiated at very close sites.

In situ live/dead staining of mature nodule transversal sections confirmed that wild-type, *nifA* and *nifA* complemented bacteroids occupying infected plant cells showed comparable survival rates, with a similar distribution of live/dead cells (Figure 2A and Supplementary Figure 2). However, we observed that nodules colonized by the *nifA* mutant showed a decreased number of infected cells and an overall lower occupancy of the nodular tissues. Further, plant cells infected by the *nifA* mutant appeared bigger, longer and rounder than wild-type infected cells (Figure 2B). Interestingly, these alterations were not totally mitigated in nodules occupied by the *nifA*

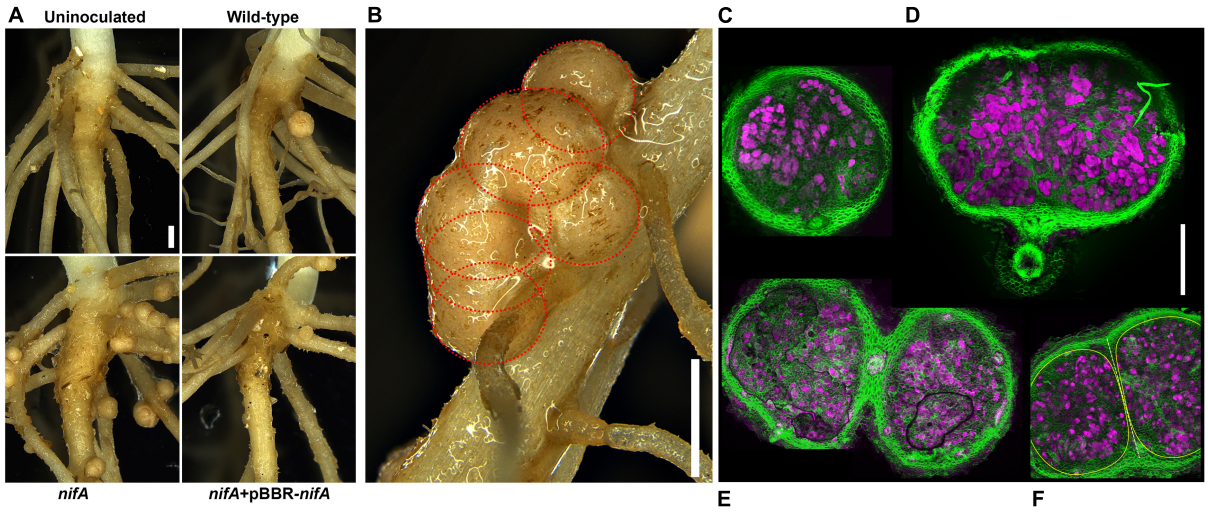

FIGURE 1
nifA-triggered nodules that fuse during development. **(A)** Representative root-shoot junctions of 11 days post inoculation (dpi) *Phaseolus vulgaris* inoculated with or without the indicated strains. Bar = 1 mm. **(B)** Close-up view of an unusual structure formed by *Paraburkholderia phymatum nifA*-occupied nodules. The red dotted circles delineate the boundaries of six independent but intertwining nodules. Bar = 1 mm. **(C–F)** Confocal laser scanning micrographs of nodular transversal sections. Maximum z-projections of 30 μm × 1 μm optical sections. Calcofluor white staining of plant cell walls (green) and Syto9 DNA staining of bacteroids (magenta). Single wild-type or *nifA*-triggered young (**C**, here colonized by wild-type *P. phymatum*) or mature (**D**, here colonized by *P. phymatum nifA* mutant) nodules are indistinguishable at macroscopic scale, respectively. **(E)** Two closely developing *nifA* nodules sharing cortical and vascular tissues (black lines delineate air bubbles produced in the mounting of the sample). **(F)** Fused *nifA* nodules (yellow outlines) separated by a cortical wall (dashed line). Bar = 500 μm.

complemented strain. As cell death of *nifA* bacteroids occurs in elongating cells (Berrabah et al., 2015), we sought to compare the morphologies of the wild-type and mutant bacteroids obtained from surface-sterilized, crushed nodules. *P. phymatum* STM815 bacteroids appeared as a mixture of rod-shaped and spherical cells, irrespective of the strain identity, with a significant number of bacteroids undergoing cell division (**Figure 2C**). We also recovered the bacteroids by growing them on fresh medium with appropriate antibiotic selection and subjected them to the same analysis. All strains grew as rod-shaped bacteria with similar dimensions to the corresponding bacteroids, with a slight increase in dividing cells number for the *nifA* mutant. It is noteworthy to mention that live/dead staining of these cells did not show significant differences between strains (data not shown). Our data suggest that *P. vulgaris* nodules occupied by *nifA* do not drastically differ from wild-type colonized nodules. The only noticeable difference we could associate with NifA loss were disturbances in infected plant cell size and frequency.

Paraburkholderia phymatum iaaMH are expressed in presence of germinated seeds and repressed by NifA

To examine the expression of the *iaaMH* genes in the early stages of symbiosis, 2 day-old germinated *P. vulgaris* seeds were placed on soft-agar nitrogen-limited medium plates inoculated with *P. phymatum* STM815 wild-type and a *nifA* mutant containing the *iaaMH-gfp* reporter plasmid (hereafter WT-$_p$*iaaMH* and *nifA*-$_p$*iaaMH*, respectively). *P. phymatum* wild-type containing the empty pPROBE vector (WT-pPROBE) was used as negative control. As a positive control, the *P. phymatum* reporter strain $_p$*nodB*, containing the promotor of the *nodB* gene (Bphy_7722) fused to *gfp*, was employed (WT-$_p$*nodB*) (Hug et al., 2021). The gene *nodB* is involved in the synthesis of the backbone of NFs, and its expression is known to be induced by flavonoids secreted by the host plant (Guerreiro et al., 1997). After 3 days of incubation at 28°C, the *gfp* expression of the reporter strains co-inoculated with the germinated seeds was examined under the fluorescence microscope. While the negative control only displayed root autofluorescence, the *iaaMH-gfp* reporter in the wild-type strain was expressed at a similar level as the *nodB-gfp* fusion in the vicinity of the root apex (**Figure 3A**). GFP signals consistently decreased with increasing distance to the root to reach background levels at approx. 3 cm from the apex (**Figure 3B**). This indicates that *nodB* and *iaaMH* promotor activities depended on diffusible root exudates in our assays. In the *nifA* mutant, *iaaMH-gfp* signals were two-fold up-regulated compared to wild-type levels close to and also 3 cm away from the root apex (**Figure 3B**). These results suggest that NifA represses the expression of *iaaMH* and that root exudates induce *iaaMH* expression.

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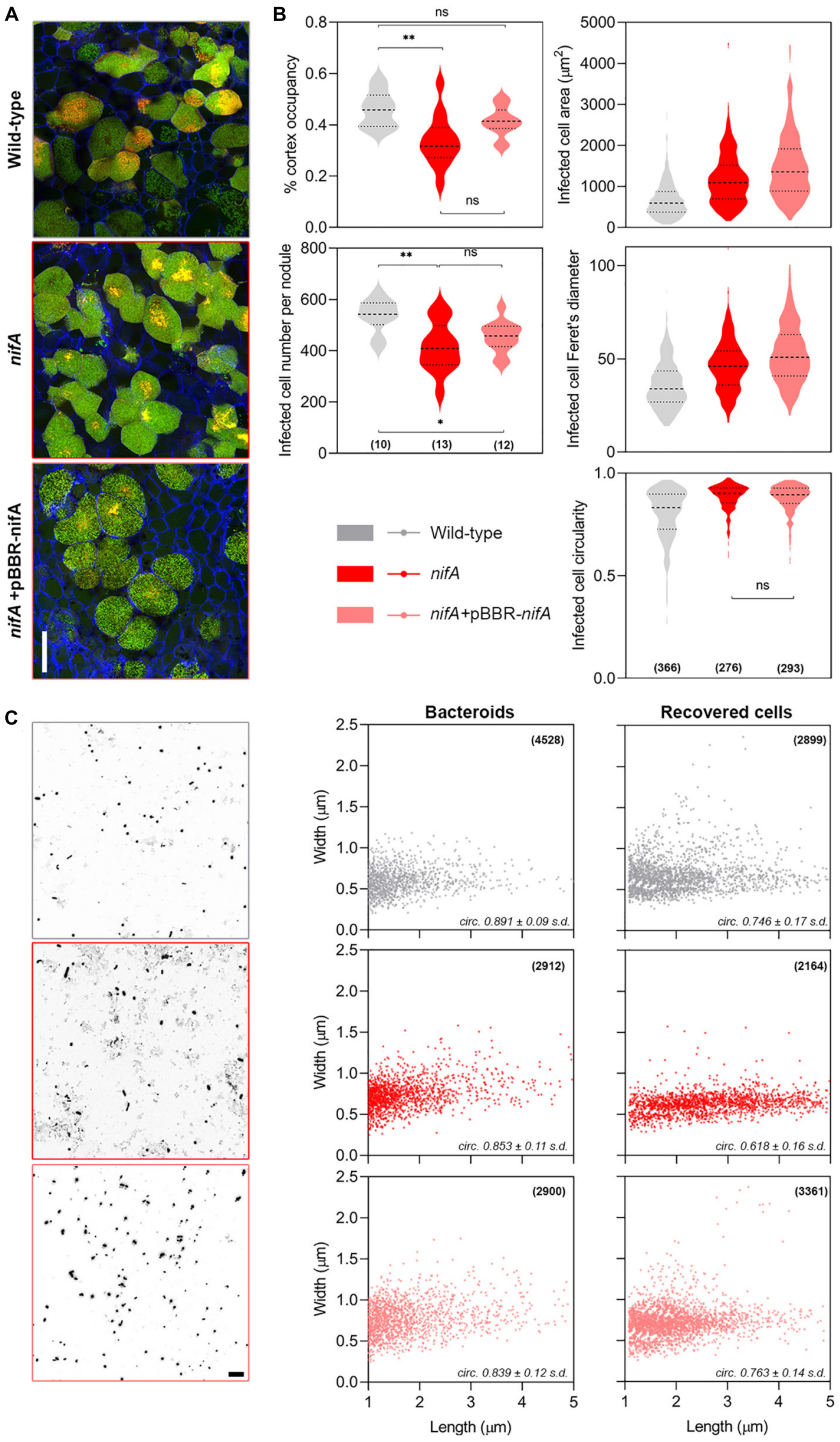

FIGURE 2

Phenotypic characterization of *Phaseolus vulgaris* nodules occupied by *Paraburkholderia phymatum* wild-type, *nifA* and *nifA*-complemented strains. **(A)** Live/Dead staining of infected cells. Confocal laser scanning micrographs of nodular transversal sections. Maximum z-projections of 10 µm × 0.5 µm optical sections. Propidium iodide (red), Syto9 (green), and Calcofluor White (blue). Note the similar distribution of red/green signals between the different samples. All samples are shown at the same magnification, bar = 50 µm. **(B)** Nodules and plant cells parameters. Numbers in brackets indicate the number of independent samples. Data represent two independent assays. Ordinary one-way ANOVA with Tukey's *post hoc* test. The absence of annotation between samples implies $p < 0.001$. Otherwise $^{**}p < 0.01$, $^{*}p < 0.05$, ns, not significant. **(C)** Bacterial cells parameters. Left, high-contrast bright field micrographs of bacteroids preparations obtained from crushed fresh nodules. Bar = 10 µm. Right, cytometric properties of bacteroids obtained from crushed nodules and bacteroids cultured in selective medium. Cell dimensions were extracted from CLSM micrographs with MicrobeJ. Numbers in brackets indicate sampled cells number. The calculated cell circularity is indicated in italics. *s.d.,* standard deviation of mean.

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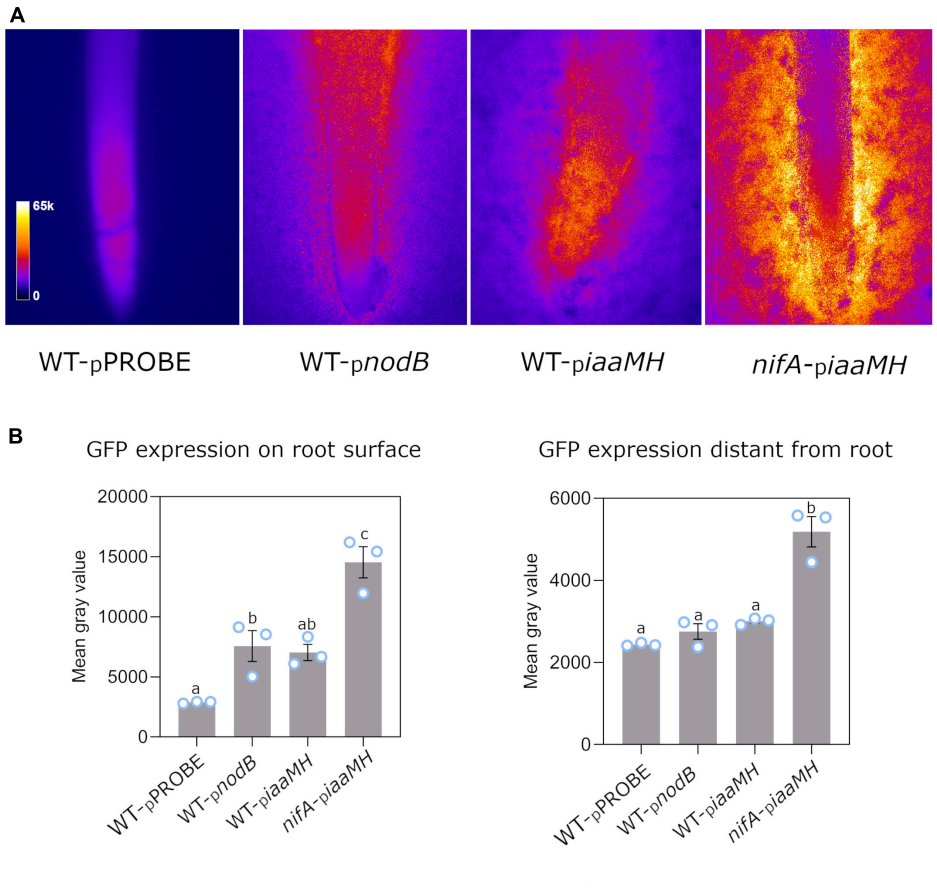

FIGURE 3
NifA-dependent *iaaMH-gfp* expression on germinated *Phaseolus vulgaris* roots in *Paraburkholderia phymatum* STM815 wild-type (WT-$_p$*iaamH*) and in a *nifA* mutant (*nifA*-$_p$*iaaMH*). A *gfp* reporter construct was used. *P. phymatum* wild-type containing the empty pPROBE (WT-$_p$PROBE) and the *nodB* promoter sequence (WT-$_p$*nodB*) were used as a negative and a positive control, respectively. **(A)** Representative stereomicrographs of one of the three biological replicates inoculated per strain. Fire lookup table (LUT) indicates fluorescent signal intensity (see insert, left panel). **(B)** Quantitative analysis of *gfp* expression on *P. vulgaris* root surface and at 3 cm distance from the root. Blue circles represent individual data points. Error bars indicate the standard error of the mean (SEM). Significant differences between samples were analyzed with one-way ANOVA with Tukey's test (p-value $\leq$ 0.05). Same letters indicate that samples are not statistically significantly different (a–c).

Paraburkholderia phymatum iaaMH expression is constitutive and controlled by NifA during the establishment of *Paraburkholderia phymatum–Phaseolus vulgaris* symbiosis

To investigate in which step(s) of *P. vulgaris* nodule organogenesis the *P. phymatum iaaMH* genes are expressed and controlled by NifA, we used the reporter strains previously described to track the changes in *iaaMH* expression and bacterial colonization patterns during the different development stages of the symbiosis. Germinated seedlings of *P. vulgaris* were inoculated with the *P. phymatum iaaMH* reporter strain in a wild-type (WT-$_p$*iaaMH*) and *nifA* mutant background (*nifA*-$_p$*iaaMH*). The plants were incubated for 10, 14 (during the formation of young nodules), and 21 days (optimal nitrogen fixation activity) and the GFP fluorescence signals in developing and mature nodules were microscopically monitored, either non-invasively or from transversal sections. As expected, negative control plants containing the empty pPROBE vector did not show specific GFP signals, although auto fluorescence occurred in mature nodules (Figure 4A). However, the *iaaMH* promoter was active at all tested time points during the infection process. This is congruent with the data presented in the previous section, pointing to an early activation of bacterial auxin production. Additionally, in absence of NifA, the *iaaMH* genes were expressed at greater levels compared to the wild type in all stages of nodule organogenesis, suggesting that NifA is tightly controlling auxin biosynthesis throughout the symbiosis. Although we cannot exclude that *P. phymatum* expressing low levels of *iaaMH-gfp* were present in our samples and initiated infection sites, we observed a plethora of specific

GFP signals strongly associated with the plant tissues. This *gfp* expression was not due to surface-attached bacteria, as the thorough washing steps we applied to the roots easily removed such microcolonies. We could readily visualize *iaaMH-gfp* expression from nascent primordia to mature nodules in plants colonized with both strains (**Figure 4A**). Roots infected with WT-$_p$*iaaMH* occasionally showed fusing infected primordia and numerous scattered GFP *punctuae*. We found this pattern strikingly enhanced on *P. vulgaris* roots inoculated with *nifA*-$_p$*iaaMH* (**Figure 4A** and **Supplementary Figure 3**), where *iaaMH-gfp* promotor activity was regularly observed in multiple adjacent developing nodule primordia. By closely inspecting the distribution of the GFP signals outside of the nodular outgrowths, we located them in direct proximity to root hair cells (**Figure 4B**). Confocal microscopy established that the majority of these fluorescent signals represent *P. phymatum* ITs. We could partition these infection sites into three groups: (1) elongated ITs that failed reaching the cortical layer, (2) branching cortical ITs with emerging nodular primordia and (3) large and diffuse ITs occupying infected epidermal cells (**Figure 4B**). We could not determine if the colonized epidermal cells were preferentially root hairs in the latter group. At day 21 post infection, both plants colonized with WT-$_p$*iaaMH* or *nifA*-$_p$*iaaMH* showed nodules with leghemoglobin coloration and equivalent GFP signals in their respective infected cells (**Figure 4C** and **Supplementary Figure 4**).

Paraburkholderia phymatum iaaMH genes are essential for the production of indole-acetamide and indole-3-acetic acid in *Phaseolus vulgaris* nodules

The involvement of the *P. phymatum iaaMH* genes in the production of indole-acetamide (IAM) and indole-3-acetic acid (IAA) was confirmed using a metabolomics analysis on nodules infected by *P. phymatum* wild-type, the *nifA* insertional mutant, an *iaaMH* deletion mutant (Δ*iaaMH*) and a *nifA*-Δ*iaaMH* double mutant strain. Metabolites of *P. vulgaris* nodules induced by these different *P. phymatum* strains were extracted 21 days after inoculation and were compared to those extracted from uninfected roots. Three independent biological replicates inoculated per strain were analyzed using a non-targeted metabolomics approach by flow injection time-of-flight mass spectrometry (Lardi et al., 2016, 2018; Bellés-Sancho et al., 2021a,b). A total of 285 ions were matched to deprotonated metabolites and were annotated based on the accurate mass. Out of all detected metabolites, we focused on the ion counts belonging to IAM and IAA. As we previously reported, plants inoculated with *P. phymatum* wild-type produced significantly higher amounts of IAM and IAA than non-inoculated plants (Bellés-Sancho et al., 2021a), while the levels of these two auxins in the *nifA* mutant were found elevated compared to the wild-type nodules (**Figure 5**; Bellés-Sancho et al., 2021a). The amount of IAM and IAA in nodules induced by the single Δ*iaaMH* and double *nifA*-Δ*iaaMH* mutant decreased to the same levels as observed in non-inoculated roots, suggesting that bacterial auxin production accounted for the main portion of IAA and IAM found in wild-type and *nifA* nodules (**Figure 5**). We therefore conclude that the presence of the *iaaMH* genes lead to IAA and IAM production and confirm that the key regulator of nitrogen fixation, NifA, negatively regulates IAA and IAM amounts in *P. phymatum-P. vulgaris* nodules. Only few metabolites were found to be differentially abundant in nodules infected with a Δ*iaaMH* mutant. In addition to IAA and IAM, significantly lower amounts of the metabolites xanthine, linamarin, 4-phospho-L-aspartate and pantothenic acid were found in the nodules infected with the Δ*iaaMH* mutant compared to the wild-type (**Supplementary Table 4**). Moreover, in nodules formed by the Δ*iaaMH* mutant, the intermediate of the lysine degradation pathway saccharopine, the precursor of jasmonic acid alpha-linoleic acid, the serotonin degradation product 5-hydroxylacetic acid and 1-nitronaphthalene-5,6-oxide were detected at higher levels.

NifA-dependent clustering *of Phaseolus vulgaris* nodules is induced by the presence of *Paraburkholderia phymatum iaaMH* genes

To evaluate the possible contribution of *P. phymatum*'s auxin production in the NifA-dependent hypernodulation and nodule clustering phenotype in *P. vulgaris* (Lardi et al., 2017b; Bellés-Sancho et al., 2021a), the symbiotic properties of *P. phymatum* wild-type, *nifA* mutant, Δ*iaaMH* and the *nifA*-Δ*iaaMH* double mutant were characterized. Compared to the plants inoculated with *P. phymatum* wild-type, plants in symbiosis with Δ*iaaMH* did not show any significant difference in any of the properties tested (number of nodules, dry weight per nodule, normalized nitrogenase activity), suggesting that the absence of bacterial auxin alone did not significantly affect symbiosis with the common bean (**Figure 6** and **Supplementary Table 5**). Moreover, the Δ*iaaMH* strain complemented with *iaaMH* expressed from its natural promoter also showed no difference in the symbiotic properties compared to the mutant strain. The hypernodulation phenotype of the *nifA* mutant was reverted to wild-type levels when the *nifA* gene was complemented on a plasmid. Similar to the phenotype observed in plants inoculated with the *nifA* mutant, the *nifA*-Δ*iaaMH* double mutant showed a significantly higher number of nodules compared to the plants inoculated with the wild-type. However, the nodule number in the double mutant was slightly reduced compared to the *nifA* mutant, although this difference was not significant (**Figure 6**). This suggests that

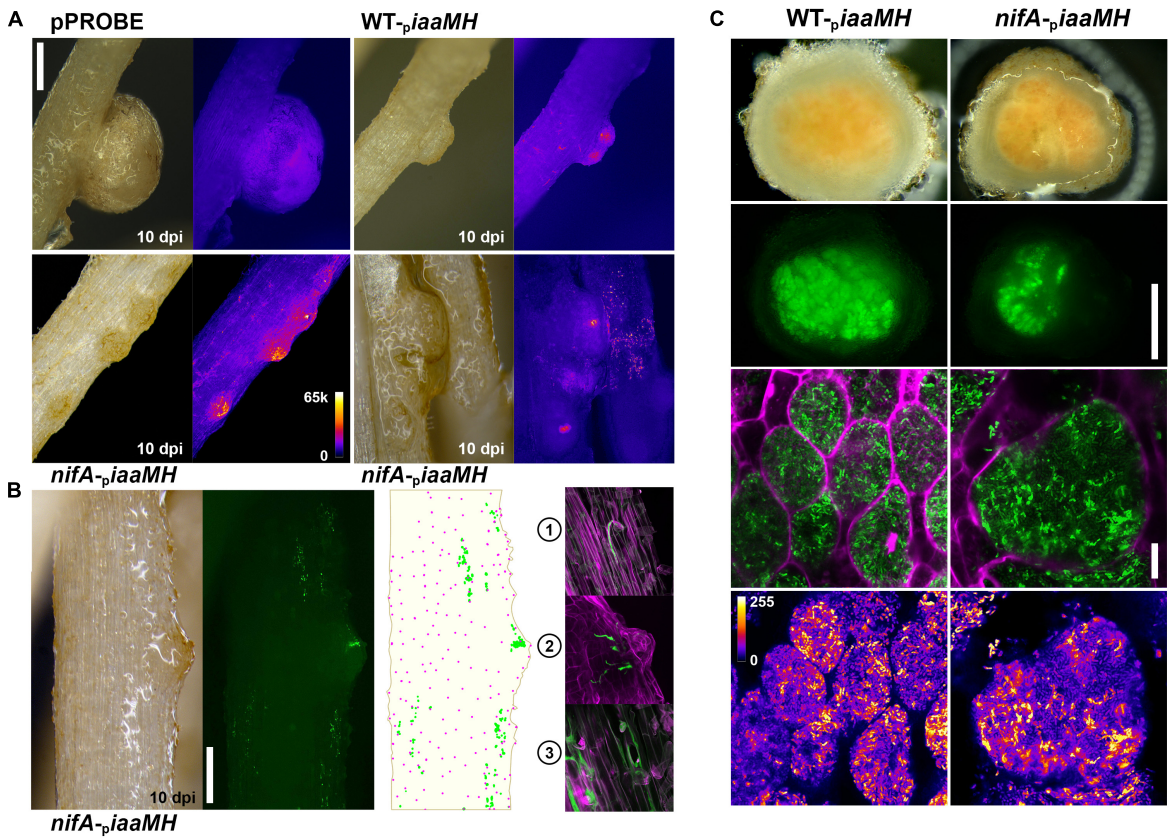

FIGURE 4
In situ monitoring of *iaaMH* promotor activity. **(A)** $_{p}$*iaaMH*-driven *gfp* expression in *Phaseolus vulgaris* roots in the given conditions. Upper-left, note the absence of specific fluorescence in mature nodules occupied by pPROBE-only inoculants. Upper-right, wild-type *Paraburkholderia phymatum* occasionally triggers fusing nodules. Note the fluorescent signals scattered on the root epidermis. Lower left, note the higher GFP fluorescence in single and fused developing nodules or on the epidermis infected by the *nifA* mutant. Lower right, two *nifA*-occupied roots displaying either developing nodules or heavily colonized epidermal area. All micrographs were acquired with the same acquisition settings, Fire LUT, 16-bit depth. Bar = 500 μm. **(B)** GFP signals distribution on a *nifA*-colonized root. The cartoon depicts the overlapping of GFP signals (green) and identified root hair positions (magenta) from the micrograph. Bar = 500 μm. (1) An arrested IT. (2) A branching IT and nodular outgrowth. (3) ITs and densely colonized epidermal cells. **(C)** Upper panel, transversal sections of $_{p}$*iaaMH*:*gfp* expressing 21 dpi mature nodules colonized by wild-type and *nifA* strains. Note the coloration indicating leghemoglobin production in both nodule types. *nifA*-$_{p}$*iaaMH* infected cells lose more frequently the fluorescence provided by the pPROBE construct. Bar = 500 μm. Lower panel, $_{p}$*iaaMH*-driven bacteroids *gfp* expression in infected cells (green). Calcofluor staining (magenta). Maximum z-projections of 10x × 0.5 μm LSCM optical sections. Lower images display the green signal quantitatively. Fire LUT, 8-bit depth. Bar = 10 μm.

bacterial auxin contributes to increase hypernodulation when NifA is absent. Like the *nifA* mutant, the *nifA*-Δ*iaaMH* mutant showed no nitrogenase activity, indicating that NifA controls expression of the nitrogenase independently of the presence of the auxin biosynthesis genes. We next determined the number of bacteroids inside nodule occupied by the wild-type and the different *nifA* and *iaaMH* mutant strains and found that the nodule occupancy was similar for each strain (Supplementary Figure 5). Remarkably, plants inoculated with Δ*iaaMH* and the *nifA*-Δ*iaaMH* double mutant complemented with *iaaMH* under control of the strong *lacZ* promotor induced abnormal, thicker roots without nodules, presumably due to an excess of exogenous auxin production (Supplementary Figure 6). These results confirm that *P. phymatum iaaMH* is not essential for a functional symbiosis with *P. vulgaris*. However, auxin seems to influence the nodulation frequency observed with the *nifA* mutant.

To determine the impact of NifA and IaaMH functions and their interplay at a finer resolution, we conducted a precise phenotypical characterization of common bean plants 21 days post inoculation (dpi) by monitoring their root parameters in presence of different strains. We first recorded the position of each individual nodule along the axis of each tap and basal root from 15 plants. At this stage, all plants presented comparable statures and apparent health. Figure 7A shows the first centimeters of representative basal root colonization. We used this positional information to build a map of nodule frequency distribution for the entire root system of the individual

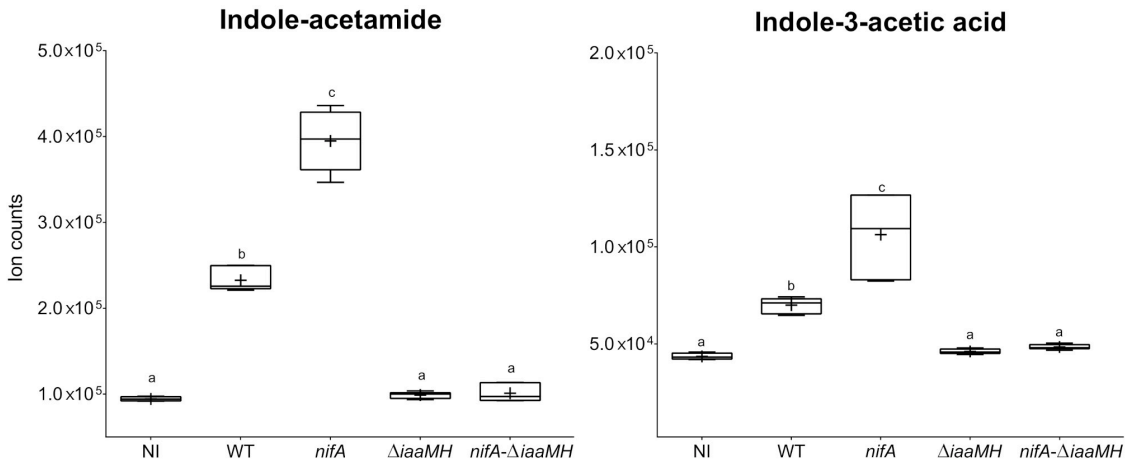

FIGURE 5
Ion counts observed for indole-acetamide (IAM) and indole-3-acetic acid (IAA). The respective levels are indicated for *Phaseolus vulgaris* non-inoculated roots (NI) and nodules occupied by *Paraburkholderia phymatum* wild-type (WT), *nifA* mutant (*nifA*), as well as Δ*iaaMH* and *nifA*-Δ*iaaMH* double mutant strains. Plus-symbols (+) indicate the arithmetic mean while whiskers represent minimum and maximum values. Differences between samples were analyzed with one-way ANOVA with Tukey's test (p-value ≤ 0.05). Statistically significant differences are indicated with different letters (a–c).

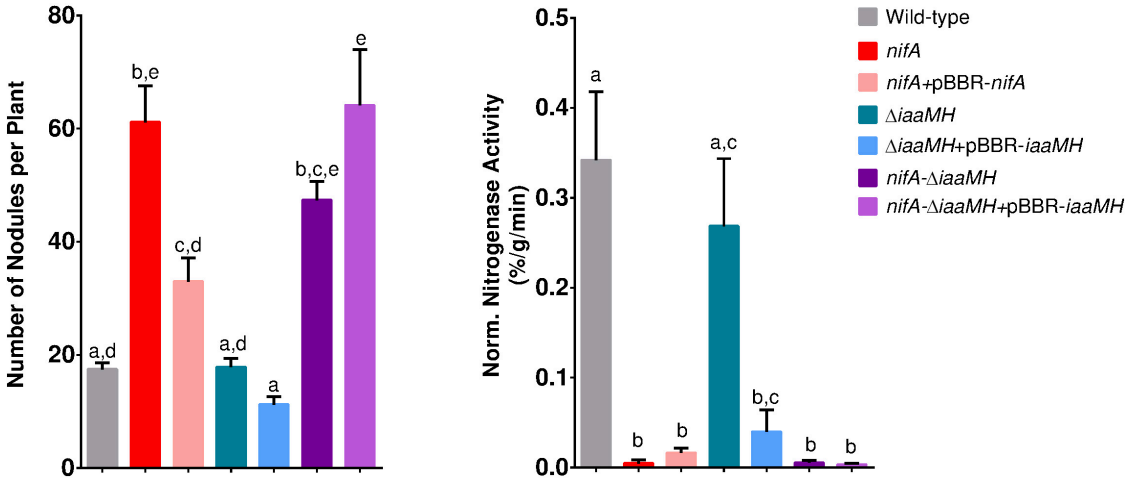

FIGURE 6
Symbiotic properties of *Paraburkholderia phymatum* wild-type, *nifA* mutant, *nifA* complemented strain, Δ*iaaMH*, Δ*iaaMH* complemented strains, *nifA*-Δ*iaaMH* double mutant and *nifA*-Δ*iaaMH* double mutant complemented with *iaaMH*. At least four biological replicates were used, consisting of a minimum of four plants per replicate. Error bars indicate the standard error of the mean (SEM). For each histogram, values with the same letter are not significantly different (a–e) (ANOVA, Tukey's test with p-value ≤ 0.05).

samples (Figure 7B). This dataset allowed us to conclude that, irrespective to the tested bacterial strain, the bulk of *P. vulgaris* nodulation occurred within the first 75 mm of the root system, with a large portion of nodules developing in the few first centimeters of the roots. This indicated us that the changes in auxin production measured in *nifA* and *iaaMH* mutants did not dramatically influence nodule positioning along the root developmental axis. As an imbalance in auxin homeostasis has often been reported to alter root development and growth, we sought to measure the status of the root system in our experiment. The number of basal roots developed per plant were not significantly different between the tested conditions (Figure 7C). We also observed no significant differences in the lengths of tap roots or basal roots between samples, respectively (Figure 7C). Although we did not measure these parameters, the lateral root and root hair densities also appeared homogeneous in all tested plants. Thus, we found no compelling evidence of a direct bacterial auxin effect on the systemic root system architecture, indicating that its contribution to the symbiosis might be locally restricted. In contrast, the loss of NifA function

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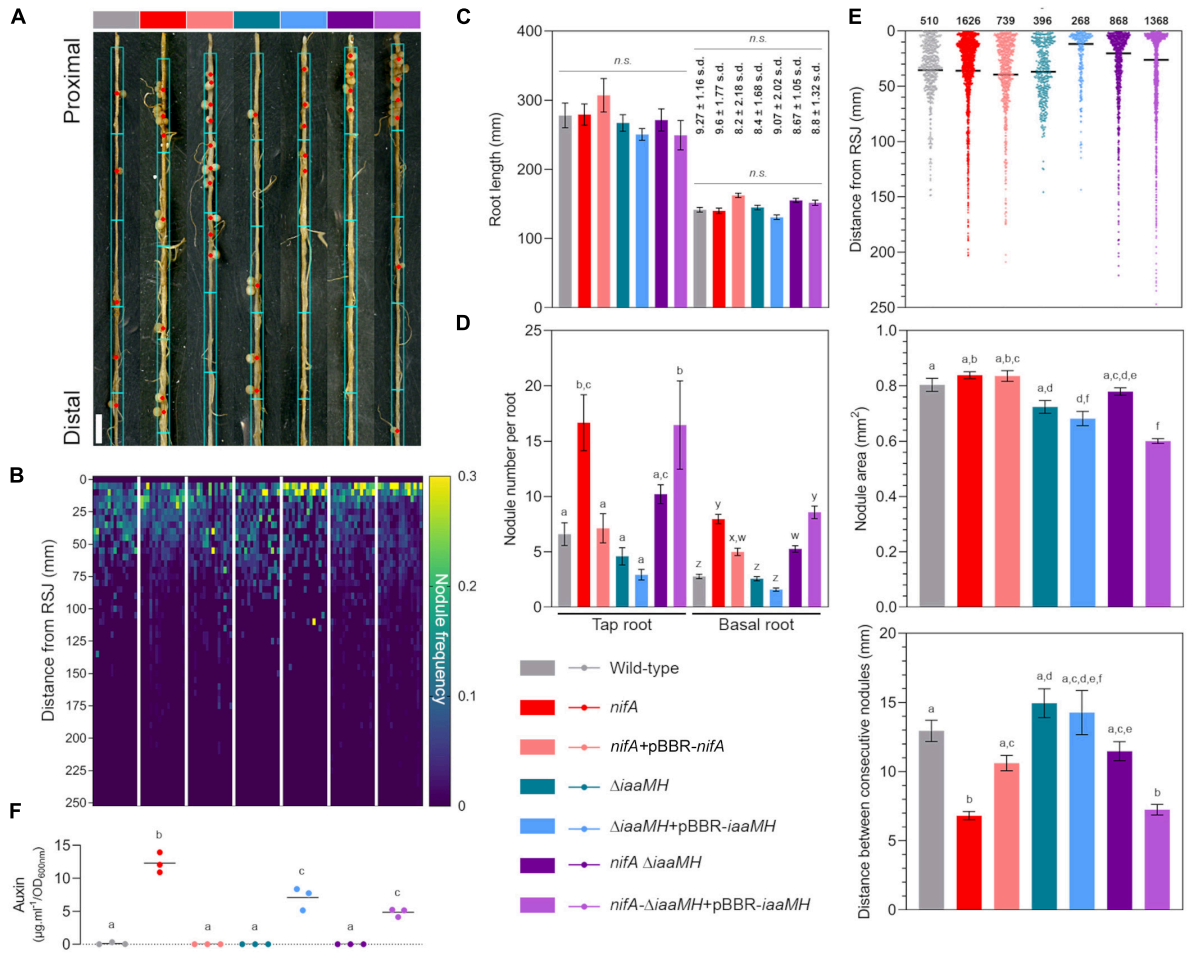

FIGURE 7
Detailed characterization of *Paraburkholderia phymatum*-*Phaseolus vulgaris* symbiosis nodulation patterns in the presence of discrete strains. **(A)** Representative 21 dpi *P. vulgaris* basal roots infected with the indicated strain. Red dots indicate the centroid of each single or clustered nodules. Cyan boxes illustrate the suggested nodulation windows along the root axis. Bar = 5 mm. **(B)** Frequency of nodulation in the entire root system of 15 individual plants in response to the given inoculants. The positions of individual nodules relative to the root-shoot junction were pooled into 5 mm bins. **(C)** Mean tap and basal root lengths of the sampled plants. Whiskers represent standard error. Bold numbers indicate the mean number of basal roots and standard deviation of the mean. Significant differences between samples means were analyzed with one-way ANOVA with Tukey's *post hoc* test (p-value ≤ 0.05). Same letters indicate that means are not statistically significantly different when required. *n.s.*, not significant. **(D)** Mean nodule number per root type. Whiskers represent standard error. **(E)** Total nodules parameters for the sampled plants. Individual dots represent single nodule position on the root axis. Black bars represent means. Whiskers represent standard error. Bold numbers indicate the total number of sampled nodules. Significant differences between samples means were analyzed with one-way ANOVA with Tukey's *post hoc* test (p-value ≤ 0.05). Same letters indicate that means are not statistically significantly different. **(F)** Quantification of indolics production in liquid cultures of the given strains in $\mu g \cdot ml^{-1}$ in relation to the cultures' optical density OD_{600}. Individual dots represent each different biological replicate. Different letters account for significantly different values.

nearly tripled the number of developed nodules on tap and basal roots (Figure 7D). This increase was restored to wild-type levels in plants colonized with the complemented *nifA* strain, although the number of nodules per basal root remained significantly greater than wild-type numbers. Interestingly, the Δ*iaaMH*-colonized plants displayed a nodule number per root close to wild type, even slightly lower, a tendency that seemed more pronounced in inoculations with the functionally complemented strain Δ*iaaMH* + pBBR-*iaaMH*. Finally, the inactivation of *iaaMH* in the *nifA* mutant background resulted in a clear mitigation of the *nifA* nodulation phenotype, the latter being restored in plants inoculated with a *nifA* strain harboring the complemented *iaaMH* mutant. Taken together, our data strongly suggest that NifA governs root nodular density via negative control of *iaaMH* expression in *P. phymatum*. However, by visualizing the 1D distribution of the positions of the whole population of nodules on the roots of each condition tested here, it appeared that the obtained dot plots displayed comparable shapes and local densities. We then hypothesized that the hypernodulation triggered in *nifA*-colonized plants

might solely be explained by an enrichment of developed nodules in pre-defined root areas (**Figure 7E**). We first verified that the sizes of the nodules colonized by the various strains did not significantly differ (**Figure 7E**). To our surprise, the nodules occupied by the *iaaMH* mutant appeared smaller. Nevertheless, we retained a mean diameter of 1 mm for all nodules. By calculating the distance between consecutive nodules along the axis of individual roots, the clustering of *nifA* nodules was manifest (**Figure 7E**). This phenotype was alleviated by either the complementation of the NifA function or by disrupting IaaMH-mediated auxin synthesis in the *nifA* background. Restoring *iaaMH* functionality in the *nifA* strain led to the *nifA* phenotype. Single manipulations of the *iaaMH* genes did not influence nodule clustering when compared to roots colonized with the *P. phymatum* wild-type. We next categorized the frequency of consecutive nodules spacing into 1 mm bins (**Supplementary Figure 7A**) and uncovered that 46.3% of all *nifA*-occupied nodules aggregated into root sections under 3 mm in length. In comparison, nodules occupied with the *nifA* complemented strain (31.0%), Δ*iaaMH* (23.3%), Δ*iaaMH* + pBBR-*iaaMH* (36.4%), or *nifA*-Δ*iaaMH* (37.7%) showed similar clustering to wild-type-colonized nodules (21.5%) in such segments. Within this window, the nodules hosting the *nifA*-Δ*iaaMH* + pBBR-*iaaMH* strain displayed a clustering frequency of 53.8%, congruent with *nifA* values. To establish if the observed nodular clusters were indiscriminately scattered onto the root axis, we therefore subjected our samples' 1D root nodular coordinates to Jenks natural breaks optimization (**Supplementary Figure 7B**). Our computations broke the different datasets into 10 classes with goodness of variance fits over 98%. We deduced from the classes limits the root windows in which nodulation occurred for all tested conditions. Regardless of the inoculants and the number of nodules, *P. vulgaris* nodulation occurred on roots in discrete windows consistently increasing in size while progressing toward the root apex, a pattern remarkably reminiscent of the lateral root emergence developmental process. Within the first 60–75 mm of roots, all inoculated plants displayed a uniform windowing ranging from 10 to 20 mm, approximatively (blue boxes in **Figure 7A**). In younger parts of the roots, nearer the apices, the nodules of roots colonized by the *nifA*, *nifA* complemented, *nifA*-Δ*iaaMH* and *nifA*-Δ*iaaMH* + pBBR-*iaaMH* strains displayed larger nodulation windows. Consistent with the rest of our data, plants hosting wild-type, Δ*iaaMH* or Δ*iaaMH* + pBBR-*iaaMH* shared very similar distributions. In comparison, a population of randomized values were evenly distributed in 10 windows of about 20 mm along a theoretical root axis of 250 mm.

Taken together, our data suggest that the clustered hypernodulation observed in *nifA*-colonized plants results from a strong increase in developed nodules originating from discrete, facilitating root sites. In analogy to lateral root development, such sites might be genetically or hormonally primed to respond to internal auxin signaling. To confirm that the nodular levels of auxinics we previously measured (Bellés-Sancho et al., 2021a) originated from bacterial production under NifA-IaaMH regulation, we estimated the amount of indolics in our strains after planktonic growth (**Figure 7F**). In comparison to wild-type *P. phymatum* that produced traces of indolics, the *nifA* mutant synthetized considerable amounts (37.9 μg/ml). This trait was returned to wild-type levels in the *nifA*-complemented and *nifA*-Δ*iaaMH* strains. As expected, the Δ*iaaMH* was unable to produce auxinics. However, we found that the presence of pBBR-*iaaMH* in either the Δ*iaaMH* or *nifA*-Δ*iaaMH* backgrounds displayed similar elevated levels of auxinics, thus implying that the production of IAA in these strains is not entirely controlled by NifA. This might, at least partially, explain the lower nodule number observed in plants hosting Δ*iaaMH* + pBBR-*iaaMH* and the smaller nodule sizes triggered by strains expressing the pBBR-*iaaMH* plasmid. We conclude that the *P. phymatum* IaaMH-dependent auxin synthesis is directly involved into the initiation and development of *P. vulgaris* nodules in predetermined susceptible zones along the root axis.

The *iaaH and iaaM* genes are present in the genus *Paraburkholderia* and are related to auxin production genes from phytopathogenic species

To identify orthologs of *P. phymatum* STM815's *iaaMH* genes in other bacterial species, an *in silico* analysis using the respective *P. phymatum* protein sequences as query for a cblaster search was carried out (see section "Bioinformatic analysis" in "Materials and methods"). Strains retrieved from this analysis were required to have both orthologs located adjacent to each other and to have a full species name (sp. hits were not allowed as they might bias the results). Among the 1,001 strains satisfying these criteria, we found 174 *Pseudomonas syringae,* 134 *Agrobacterium tumefaciens*, 121 *Burkholderia vietnamensis,* 93 *Pseudomonas chloroaphis*, 72 *Dickeya dicanthola*, 65 *Agrobacterium rhizogenes*, and 62 *Agrobacterium vitis* strains. These top seven species accounted for more than 70% of all hits. Overall, the strains belong to 79 different species many of which were represented by a single strain, including several *Paraburkholderia* species (**Supplementary Table 3**). To create a phylogenetic tree, we selected one representative strain per species, preferentially including NCBIs representative genome, reference genomes or complete genomes over fragmented Illumina assemblies (see section "Bioinformatic analysis" in "Materials and methods"). The phylogenetic analysis revealed several distinct groups of strains containing IaaM and IaaH (**Figure 8A**). In order to investigate potential functional links between proteins related to auxin production with other rhizobial traits (such as

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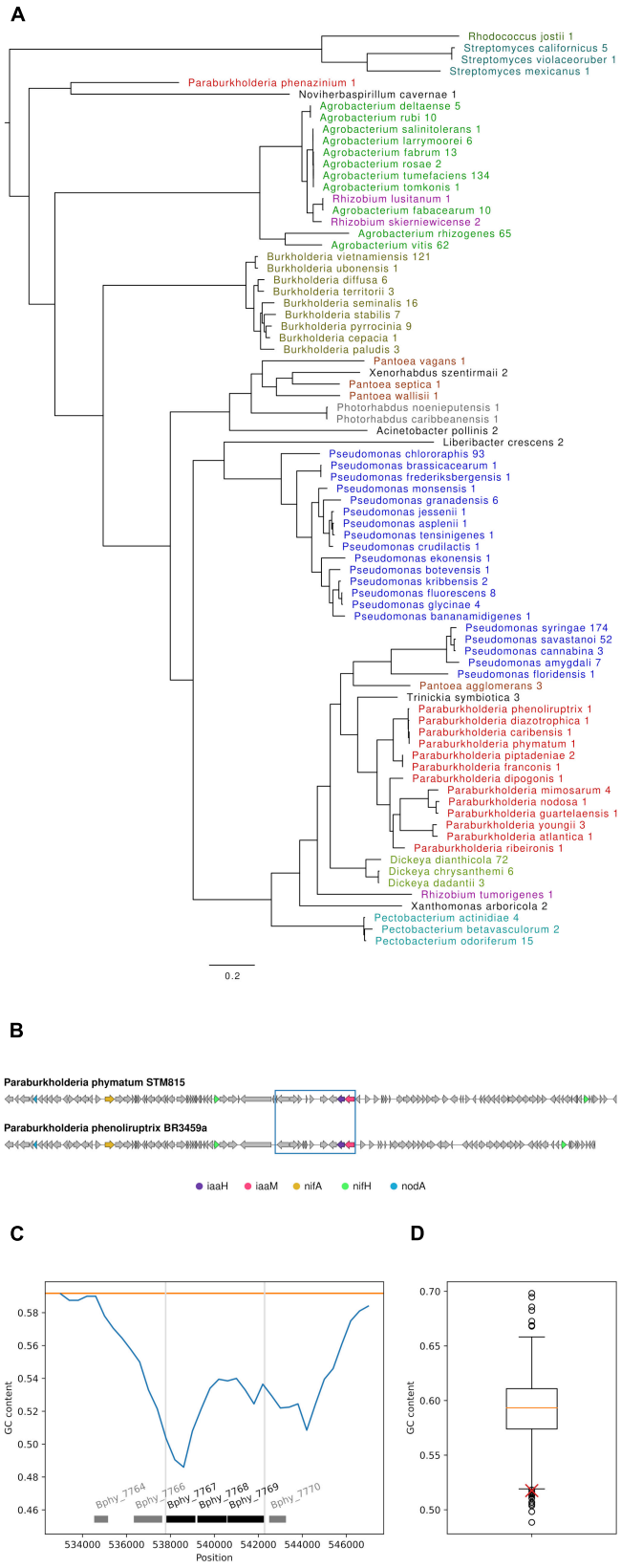

FIGURE 8

In silico analysis of *Paraburkholderia phymatum iaaMH* genes. **(A)** Maximum likelihood phylogenetic tree of co-occuring orthologs of IaaH and IaaM in other species identified by cblaster. The numbers indicate how many strains per species were found. When more than one strain was

(Continued)

FIGURE 8 (Continued)
identified for a species, only the genome best representing the species was chosen **(B)** Synteny of *iaaM* (pink), *iaaH* (purple), *nifA* (yellow), *nifH* (green), and *nodA* (blue) between *P. phymatum* STM815 (top) and *P. phenolirupterix* BR3459a (bottom). Note that in both strains, two copies of *nifH* are present. A BLAST of the DNA sequence from Bphy_7758 to Bphy_7769 (blue box) identified *P. phenoliruptrix* BR3459a as the only other strain containing the whole sequence (100% sequence coverage of 13,791 nucleotides, 99.4% nucleotide identity). **(C)** GC content (blue line) of the *P. phymatum iaaMH* genes and the surrounding genomic region on the symbiotic plasmid, analyzed with a sliding window analysis (see "Bioinformatic analysis" section in "Materials and methods"). The mean GC content of the plasmid is shown as orange line, the extent of the predicted auxin operon is shown by gray vertical lines and the genes in black. A potential IS5 family transposase gene (Bphy_7770) is located to the right side of *iaaM*. **(D)** Boxplot showing the GC content distribution of all windows of similar size on the symbiotic plasmid (NC_010627.1). The GC content of the window containing the three predicted auxin operon genes (red cross) shows a significantly lower GC content.

nitrogen fixation and nodulation) at a genomic level, we analyzed the co-occurrence of *nifA, nifH* and *nodA* genes in bacterial strains that encode the *iaaMH* genes (**Supplementary Table 3**). Orthologs of all five genes were only present in 30 strains belonging to several species from the genus *Paraburkholderia,* namely *P. phenoliruptix, P. ribeironis, P. atlantica, P. youngii, P. dipogonis, P. nodosa, P. guartelaensis, P. mimosarum, P. caribensis*, *P. diazotrophica, P. franconis, P. piptadeniae* and *P. phenazinium,* and three strains of *Trinickia symbiotica.* Notably, *P. phenoliruptix* was the strain with the highest homology score for all five gene products present on the same contig (minimum and maximum score 3.21 and 8.48, respectively). The homologous region in this strain showed 100% sequence coverage and 99.4% nucleotide identity with the one of *P. phymatum* (**Figure 8B**). Three strains belonging to *Dickeya* genus (*D. chrysanthemi, D. dianthicola,* and *D. dadantii*) presented, in addition to *iaaMH*, the *nifA* and *nifH* genes. Among the strains that only carried orthologs of the *P. phymatum iaaMH* genes (without *nif* or *nod* genes), high similarity was found in species belonging to the genera *Agrobacterium, Pantoea, Pseudomonas, Rhizobium, Xanthomonas, Pectobacterium, Acinetobacter, Photorhabdus, Xenorhabdus, Liberibacter, Noviherbaspirillum, Streptomyces,* and *Rhodococcus.* Outside of the *Paraburkholderia* group, the IaaMH orthologs of the phytopathogenic strains *A. vitis*, *A. rhizogenes,* and *A. tumefaciens* showed the highest similarity with the *P. phymatum* genes. Orthologs of *P. phymatum* IaaMH (maximum and minimum score 3.08) were also found in *Burkholderia* strains belonging to the Burkholderia cepacia complex (Bcc) that are opportunistic human pathogens, with the addition of *B. vietnamiensis* and *B. ubonensis* which also carry *nif* genes (Eberl and Vandamme, 2016). The region containing the *P. phymatum iaa* operon is located on the symbiotic plasmid and showed a significantly lower GC content (a difference of 0.074) compared to the rest of the plasmid (0.592) (**Figures 8C,D**). Furthermore, our bioinformatic analysis predicted that Bphy_7770 (the gene upstream of *iaaM*), codes for a putative IS5 family transposase. This result together with the phylogenetic reconstruction analyses suggests that the *iaaMH* genes in *P. phymatum* and other *Paraburkholderia* strains may have been acquired horizontally from a phytopathogenic ancestor.

Discussion

We present here the first study of the nodule architecture and organization induced by a beta-rhizobial *nifA* mutant and associate it to beta-rhizobial auxin synthesis during symbiosis with *P. vulgaris*.

As reported earlier (Bellés-Sancho et al., 2021a), the *P. phymatum nifA* mutant triggers nodule clustering (**Figures 1A,B**). Other than this phenotype, no abnormalities were found in *nifA* nodules (**Figures 1C–F**) or root traits. We reasoned that this phenotype might originate from the deregulation of early nodule proliferation, presumably from primary cortical initiation sites. Plant cells infected with the *nifA* mutant were found larger than the ones infected with the wild type (**Figure 2B**), a subtle change that we can attribute to the effects of auxin, as the hormone drives cell elongation (Majda and Robert, 2018). No difference was observed between wild-type and *nifA* bacteroids (**Figure 2A**), which is in contrast to reports showing that the lack of NifA provokes bacteroid death in *S. meliloti* 1021-*M. truncatula* symbiosis (Berrabah et al., 2015) seemingly sanctioning inefficient Fix$^-$ symbionts via a plant immunity response.

Interestingly, in alpha-rhizobia, NifA was reported to positively control the production of rhizobial hormones (Nukui et al., 2006; Murset et al., 2012; Nett et al., 2022). For instance, NifA in *B. diazoefficiens* USDA 110 has been shown to control the synthesis of gibberellin, a plant hormone also produced by bacteria, which induced bigger nodules in soybean in later stages of the symbiosis (Nett et al., 2022). We showed here that during the early phase of the *P. phymatum-P. vulgaris* symbiosis, the bacterial *iaaMH* genes are induced by root exudates (**Figure 3**). In line with this observation, a previous transcriptomics analysis had shown that *P. phymatum iaaMH* expression was 1.8-fold upregulated when exposed to *Mimosa pudica* root exudates (Klonowska et al., 2018). Root exudates are composed of a blend of primary and secondary metabolites, including the amino acid TRP, a precursor of IAA (Spaepen et al., 2007; Vives-Peris et al., 2020). In other strains, TRP induces the expression of IAA biosynthetic genes from either the IPA pathway in *Enterobacter cloacae* UW5 (Ryu and Patten, 2008) or the IAM pathway in *P. syringae* pv. *syringae* and *D. dadantii* 3937 (Duca and Glick, 2020). Flavonoids are

also compounds excreted by the roots, which can induce the expression of genes related to IAA synthesis, as shown in *Rhizobium* sp. NGR234, where the IPA pathway is induced by the transcriptional regulator NodD (Theunis et al., 2004). In addition, flavonoids not only influence bacterial auxin production, but can also inhibit plant auxin transport in roots during nodule formation, leading to auxin accumulation in the infection zone (Mathesius et al., 1998; Brown et al., 2001; Wasson et al., 2006; Peer and Murphy, 2007; Breakspear et al., 2014). A previous transcriptome analysis performed by our group had shown that the expression of the *iaaHM* operon, at the time annotated as an amine oxidase, was upregulated in *P. vulgaris* nodules compared to free-living conditions (Lardi et al., 2017b), which would suggest a possible role during symbiosis. However, in this study no obvious symbiotic role of IAA and IAM were observed (**Figure 6**). We cannot exclude that the absence of a symbiotic phenotype is related to the plant host and the type of nodules (determinate or indeterminate) the plant forms, since legumes use auxin to achieve the initial buildup in different layers of the cortex (Kohlen et al., 2018). For instance, in studies where the bacterial auxin balance was disrupted by overproducing IAA in *R. leguminosarum* RD20, no difference was observed in determinate *P. vulgaris* nodules compared to wild type, while in *Vicia sativa* and *M. truncatula* (both forming indeterminate nodules) less nodules were formed by this strain (Pii et al., 2007; Camerini et al., 2008). A future characterization of the *P. phymatum iaaMH* mutant using other plant hosts that form indeterminate nodules will help to further decipher the role of rhizobial auxin in distinct nodule types. In addition, it is important to mention that auxin induces different effects depending on concentration and plant sensitivity toward auxin, *i.e.*, concentrations outside the optimal range lead to an inhibition of plant growth (Persello-Cartieaux et al., 2003; Remans et al., 2008). Noteworthy, abnormal root development and no nodule formation were observed when plants were inoculated with a strain artificially over-expressing *iaaMH* (**Supplementary Figure 6**).

Although the *P. phymatum iaaMH* mutant was not impaired in its symbiotic abilities, the overexpression of the *iaaMH* genes in a *nifA* mutant was responsible for the formation of clusters of nodules in adjoining nodule initiation sites on the plant root (**Figures 1**, **7**). This phenotype might arise from the increased number of ITs in roots infected by a *nifA* mutant compared to the wild type (**Figure 4B** and **Supplementary Figure 3**). In line with the current hypothesis that auxin positively regulates epidermal infections (Velandia et al., 2022), auxins overexpression in the *P. phymatum nifA* mutant seems to act locally and promote the occurrence of infection events. This is reminiscent of the *L. japonicum-M. loti* symbiosis, where auxin was proposed to be a promotor of the initiation or elongation of the IT formation. In fact, the inhibition of the plant TAR-YUC auxin biosynthesis pathway impaired the IT elongation (Nadzieja et al., 2018). In *M. truncatula* infected with *S. meliloti,* it has been shown that a local auxin accumulation correlates with nodule numbers (Roy et al., 2017; Kohlen et al., 2018). In fact, the *M. truncatula* supernodulation mutant *sunn* (supernumerary nodules, an AON mutant) (Schnabel et al., 2005), showed an increased level of local auxin transport and expression of the auxin response gene *GH3* at the zone of nodule initiation (van Noorden et al., 2006, 2007). Nevertheless, we suggest that *P. phymatum* NifA activity mainly interplays with the *P. vulgaris* AON system, finally leading to a nodule number increase in predetermined root zones. In addition, the overproduction of bacterial auxin when NifA is absent is rather altering colonization frequency than being essential for nodulation. The induced infectivity driven by auxin could be associated with an increased competitive fitness of the strain, since *P. phymatum* is known to outcompete other *Paraburkholderia* species in nodulating common bean (Lardi et al., 2017a).

Interestingly, the *P. phymatum iaa* operon shares a high degree of homology with the *iaa* operon of phytopathogens (**Figure 8** and **Supplementary Table 3**) such as *Agrobacterium tumefaciens* Q15/94 and *P. syringae* pv. *savastanoi.* While *A. tumefaciens* uses the IAM pathway as a virulence factor to induce crown gall tumors in plants (Zupan et al., 2000), in *P. syringae* IAA induces virulence (Djami-Tchatchou et al., 2020, 2022) and in *P. savastanoi* the *iaaMH* genes are involved in the colonization process of *Mytus communis* (Schiff et al., 2019). Orthologs of the *P. phymatum* STM815 *iaaMH* genes were also present in *Burkholderia* species belonging to the Bcc group, which includes human opportunistic pathogens (**Supplementary Table 3**). In *P. phymatum* and other nitrogen-fixing and nodulating *Paraburkholderia* strains, in addition to the close related *Trinickia symbiotica* but not in alpha-rhizobia, the *iaaM* and *iaaH* genes co-occur with nitrogen fixation (*nif*) and nodulation (*nod*) genes (**Figure 8A** and **Supplementary Table 3**), suggesting that the IAA-production could be a specific trait of plant-colonizing *Paraburkholderia* strains. The only exception was the endophytic *P. phenazinium,* which only presented the *iaaMH* (Sexton et al., 2020). Based on our phylogenetic analysis (**Figure 8A**) and the low GC content of the predicted *P. phymatum iaa* operon compared to the GC content average of the symbiotic plasmid (**Figures 8C,D**), the *iaaMH* from *Paraburkholderia* may have been horizontally acquired from a common phytopathogen ancestor. A common evolutionary origin of these genes in *A. tumefaciens*, *A. rhizogenes*, *P. savastanoi*, and *P. agglomerans* pv. *gypsophilae* has already been suggested, as well as their horizontal transfer between *P. savastanoi* pv. *savastanoi* NCPPB 3335, *P. syringae* pv. *aceris* M302273PT and *P. syringae* pv. *syringae* B728a (Morris, 1995; Patten et al., 2013; Aragón et al., 2014). Moreover, in the plant-associated bacterium *P. agglomerans* pv. *gypsophilae*, which causes opportunistic human infections, the *iaaMH* genes are located on a pathogenicity island with other virulence genes

(Barash and Manulis-Sasson, 2009). Further analysis on whether the *iaaMH* operon is also associated with pathogenicity islands in other pathogenic species will provide valuable information on the evolution of these genes as virulence factors.

In summary, our data suggest that under the optimal control of NifA, IaaMH-driven auxin production might contribute to the successful establishment and progression of nodular primordia in response to cortical IT invasion. In absence of *nifA*, the unrestricted, exogenous bacterial auxin levels might intensify root hair infection and bypass the developmental check-points normally imposed by the host. This concept is supported by the extended symbiotic defects observed in plants inoculated with our *iaaMH* complementation strains. Circumventing the NifA fine tuning of bacterial auxin input in these strains presumably led to an improper homeostasis of the phytohormone and triggered root swellings, underdevelopment and poor nodulation frequency. Exploring the molecular mechanisms behind the mode of action of NifA as a negative regulator and the possible role of bacterial auxin as a symbiotic signal could grant deeper insights into the role of auxin biosynthesis in nitrogen-fixing and nodulating *Paraburkholderia* strains and will provide valuable information to improve competitiveness of rhizobia in the soil. We envision the potential pathogenic origin of the *iaaMH* genes as an evolutionary advantage trait used by root-colonizers to promote infection events in early symbiosis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

PB-S, BH, CHA, AB, and GP contributed to conception and design the experiments. PB-S, YL, and AB performed the experiments. PB-S, BH, ES, CHA, LE, NZ, AB, and GP analyzed the data. PB-S, CHA, AB, and GP wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

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Plant growth-promoting microorganisms as biocontrol agents of plant diseases: Mechanisms, challenges and future perspectives

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Plant diseases and pests are risk factors that threaten global food security. Excessive chemical pesticide applications are commonly used to reduce the effects of plant diseases caused by bacterial and fungal pathogens. A major concern, as we strive toward more sustainable agriculture, is to increase crop yields for the increasing population. Microbial biological control agents (MBCAs) have proved their efficacy to be a green strategy to manage plant diseases, stimulate plant growth and performance, and increase yield. Besides their role in growth enhancement, plant growth-promoting rhizobacteria/fungi (PGPR/PGPF) could suppress plant diseases by producing inhibitory chemicals and inducing immune responses in plants against phytopathogens. As biofertilizers and biopesticides, PGPR and PGPF are considered as feasible, attractive economic approach for sustainable agriculture; thus, resulting in a "win-win" situation. Several PGPR and PGPF strains have been identified as effective BCAs under environmentally controlled conditions. In general, any MBCA must overcome certain challenges before it can be registered or widely utilized to control diseases/pests. Successful MBCAs offer a practical solution to improve greenhouse crop performance

with reduced fertilizer inputs and chemical pesticide applications. This current review aims to fill the gap in the current knowledge of plant growth-promoting microorganisms (PGPM), provide attention about the scientific basis for policy development, and recommend further research related to the applications of PGPM used for commercial purposes.

KEYWORDS

biofertilizers, biopestcide, crop yield, disease suppression, pathogen suppression, plant growth-promoting rhizhobacteria

Introduction

Plant pathogens and pests can have a large impact on agricultural productivity. Plant diseases reduce yields by 21–30% in several important crops worldwide (Savary et al., 2019). Meanwhile, certain plant pathogens have developed long-term resistance against chemical management (Lucas, 2011). Some economically important plant diseases have become more prevalent. Dependence on chemical pesticides has become one of the most pressing challenges to global environmental sustainability and public health (Fones et al., 2020). Because many of insecticides are difficult to break down into simpler components that are less dangerous, toxic residues remain in the soil; thus, posing health concerns (Gilden et al., 2010). Awareness of the environmental and health risks associated with synthetic chemical pesticides is highly recommended for sustainable crop management and less used chemicals (Donley, 2019).

Synthetic agrochemicals have been considered unsustainable, causing the quest for more environmentally friendly alternatives. The focus of modern agriculture research has turned to farm practices. Plant growth-promoting rhizobacteria (PGPR) are effective, environmentally safe, and non-toxic naturally occurring microorganisms than can serve as a promising alternative to chemical pesticides. Besides, environmental factors can affect agricultural productivity; thus, this may worsen the scenario in a variety of ways. We have many reasons to take serious actions toward plant disease control management to improve our health and reduce the effects of environmental stresses (Chaloner et al., 2021). Biological control provides one of the most economical and long-term effective strategies for managing plant diseases and reducing crop loss.

Recent advances in our understanding to plant growth-promoting microorganisms (PGPMs) warrant a proper scientific evaluation of the relationship between the properties of PGPMs and their impact on plant growth, yield, and resistance/tolerance to biotic and abiotic stresses. In addition, this review study builds on a growing body of literature concerning some potential implementations of PGPMs in sustainable agriculture. Here, the aim is to provide a state-of-knowledge review reporting the effects of PGPMs on plants and finding solutions to the challenges that face microbial biological control agents (MBCAs) when applied on a large scale compared with those of chemicals.

PGPR as promising biocontrol agents

Soil is a complex ecosystem containing various groups of microorganisms, including bacteria, fungi, protists, and animals (Müller et al., 2016). These microorganisms play key roles in plant development, nutrient regulation, and biocontrol activities. They settle in the rhizosphere and endo-rhizosphere of plants, where they use a variety of direct and indirect processes to support plant growth. Lyu et al. (2020) have stated that the phytomicrobiome (plant-associated microorganisms) can provide competitive, exploitative, or neutral alliances with plants; thus, affecting crop yield. Recently, scientists have looked deeply into employing beneficial PGPR to inhibit phytopathogens and promote plant growth (Qiao et al., 2017; Alwahshi et al., 2022). A key part of this might be attributed to the enhancement of target specificity between PGPR and the plant species (Lommen et al., 2019).

According to Zgadzaj et al. (2016), rhizosphere microbiome refers to bacterial, archaeal and fungal communities as well as their genetic material closely surrounding plant root systems. Microorganisms can indirectly impact crop health and phenotypic plasticity by influencing the growth of plants and defense responses due to their co-evolution with plants on a large scale (Goh et al., 2013). The rhizosphere is home to various microorganisms that provide steady PGPR supplies (Antoun and Kloepper, 2001). The phytomicrobiome includes the bacterial population that colonize the rhizosphere, on the root surface, and between the root cortex cells (Inui Kishi et al., 2017). Since plants can first colonize the terrestrial environments, PGPR have co-evolved with related plants; resulting in synergistic host plants' relationships (Gouda et al., 2018). The effects, methods, and possibility for successfully applying PGPR to agricultural plant production in controlled situations have been the subject of numerous studies. This is critical for developing more widely used methods of biological control that consider field settings.

Safety and quality control are more crucial in vegetable cultivation since we use them less processed or unprocessed, and they have impact on health. PGPR are more achievable under greenhouse conditions. Because of the controlled environment, a significant number of prospective BCA has been discovered, and maybe ready for placement (Singh et al., 2017); thus, they have been confirmed to be successful in greenhouse investigations (Liu et al., 2018). *Bacillus subtilis, Bacillus amyloliquefaciens* and *Pseudomonas stutzeri* are among those shown to achieve success in root colonization as well as prevention of the pathogen *Phytophthora capsici* in cucumber (Islam et al., 2016). At the post-harvest stage, *B. subtilis* can protect tomato fruits from infection by *Penicillium* sp. and *Rhizopus stolonifer* (Punja et al., 2016).

Under greenhouse conditions, *B. amyloliquefaciens* isolates, diminish *Fusarium oxysporum* causing *Fusarium* wilt disease (Gowtham et al., 2016). In controlled situations, PGPR -as BCAs- are effective, indicating their role in greenhouse production systems and their efficacy in commercial horticulture. It is not necessary to distinguish the indirect PGPR pathways for pathogen infection avoidance and plant growth promotion under abiotic stresses. In addition, PGPR with biocontrol activities that also enhance plant growth would be more effective in practice. Plant tolerance to abiotic conditions and resistance to phytopathogens causing plant diseases can be improved by PGPR (Bhat et al., 2020; Leontidou et al., 2020). Some strains benefit plants' coping with stress and flourishing in abiotic environments (Goswami and Deka, 2020).

While most researchers have reported PGPR under these controlled conditions, few of them have investigated their effectiveness as BCAs, especially when combined with an abiotic stress. This is a critical factor in field biocontrol, and when climate change affects the ecosystem. The long synergism between PGPR and plant may deliver various benefits to the host plant (Fan et al., 2020).

Biocontrol mechanisms using PGPR

PGPR can enhance the availability of certain nutrients [phosphate solubilization and nitrogen (N_2) fixation], or synthesize the phytohormones [indole-3 acetic acid (IAA), ethylene (ET), jasmonic acid (JA), gibberellic acid (GA), and cytokinins (CKs); Mengiste et al., 2010; Vejan et al., 2016; Gouda et al., 2018; Sham et al., 2019].

PGPR colonizing a host plant can stimulate its growth through direct and indirect mechanisms (Figure 1). Direct mechanisms include the production of plant hormones, solubilization of phosphates, and increased uptake of iron. Indirect effects include antibiotics production, nutritional competition, parasitism, pathogen toxin inhibition, and induced resistance (Elnahal et al., 2022). The attitude of "PGPR" in creating phytohormones, molecules of signaling metabolites, and related substances describe how plants protect themselves from drought as an example of abiotic stress and salinity (Jochum et al., 2019). According to Abbas et al. (2019), PGPR may also alter the shape of the roots, resulting in increased root surface and improved root performance. In addition, PGPR can compete with other bacteria by colonizing rapidly and accumulating a greater supply of nutrients, preventing other organisms from growing (Salomon et al., 2017, Abd El-Mageed et al., 2020). PGPR have different strategies to colonize, of which each is tied to a particular host (Choudhary et al., 2011). In general, pathogen infections can be suppressed by using antibiotics and antifungal metabolites; thus considered a well-known direct biological control strategy (Raaijmakers et al., 2002). Bacteriocins, antibacterial proteins, and enzymes are examples of antimicrobial peptides (Compant et al., 2005). Antibiotics are small antimicrobial molecules produced by PGPR that can inhibit the process of metabolic or growth activities of microbial pathogens (Duffy et al., 2003). These antibiotics, which are mostly strain-specific, can target the ribosomal RNA (rRNA), alter the membrane structure, and damage the cell walls of bacterial pathogens (Abriouel et al., 2011; Maksimov et al., 2011; Nazari and Smith, 2020).

Many bacteria produce bacteriocins, where some have a greater variety of inhibitory activities than others (Abriouel et al., 2011). Siderophores are specialized chelating agents of ferric iron that inhibit phytopathogens from gaining access to iron; thus, maintaining plant health particularly in iron-deficient environments (Shen et al., 2013). PGPR can manage various plant diseases by depriving pathogens of iron, thereby reducing disease development and generating extracellular siderophores (Radzki et al., 2013). Bacteriocins, siderophores, and antibiotics have thus been identified as the three supreme operative approaches for potential biocontrol prior to *in vivo* applications (Kloepper et al., 1980). Several studies have investigated PGPR as a potential plant disease management tool to synthesize plant-beneficial metabolites such as siderophores (Subramanian and Smith, 2015).

PGPR can indirectly increase crop stress tolerance. Signal chemicals, such as phytohormones and specialized signal molecules, enable plant-to-microbe and microbe-to-plant communication (Lyu et al., 2020). The control and regulation of activities in the holobiont include the host plant and the "specific" phytomicrobiome (the plant-phytomicrobiome interaction). Two microbe-to-plant signals, lipo-chitooligosaccharides (LCOs) and thuricin17 (TH17), enhance stress tolerance in different plant species (Lyu et al., 2020). Resistance-inducing and antagonizing PGPR might be useful as new inoculants with combinations of different mechanisms of action, leading to a more efficient use for biocontrol strategies and plant growth promotion (Glick, 1995).

PGPR can also produce volatile organic compounds (VOCs) that play a significant role in plant growth and induced systemic resistance (ISR) to pathogens (Raza et al., 2016). Beneduzi et al. (2012) found that PGPR can trigger ISR as a strategy to improve disease resistance of plants. Roots colonization by arbuscular mycorrhizal fungi (AMF) and certain strains of non-pathogenic bacteria can improve plant resistance to biotic stresses

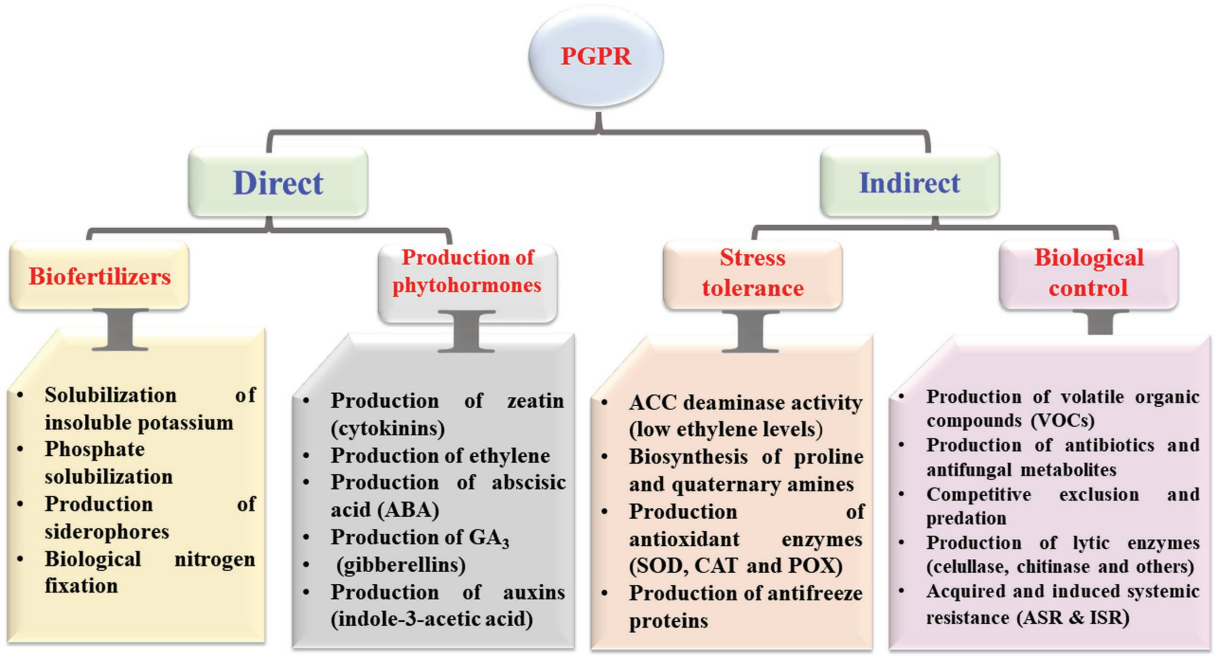

FIGURE 1
Direct and indirect mechanisms mediated by plant growth-promoting rhizobacteria (PGPR) with beneficial effects on host plants. ACC, 1-aminocyclopropane-1-carboxylic acid; SOD, superoxide dismutase; CAT, catalase; and POX, peroxidase.

(Mauch-Mani et al., 2017; Pérez-de-Luque et al., 2017). ISR triggered by PGPR and plant growth-promoting fungi (PGPF) can be found in a wide range of plant taxa (Bhattacharyya and Jha, 2012). Systemic acquired resistance (SAR); however, can be activated by a pathogen infection (Gao et al., 2015). Salicylic acid (SA) signaling is associated with the production of pathogenesis-related (PR) genes. Unlike SAR, ISR functions independently of SA, but requires responses to ET and JA. This can be achieved by the induction of defense-related gene expression; although is not always associated with induced PR proteins (Mathys et al., 2012).

Previous studies have identified ISR to stimulate PGPR via the SA-dependent pathway rather than the JA/ET-dependent pathway (Takishita et al., 2018). Other plant hormones, such as auxins, GA, CKs and brassinosteroids, may also contribute to plant immunity (Nakashita et al., 2003; Kazan and Manners, 2009; Giron et al., 2013; Rady et al., 2021). Hormonal crosstalk is thought to allow the cultivation and exert their immunological growth and defense reactions (Pieterse et al., 2014; AbuQamar et al., 2017; Sham et al., 2017).

PGPR are involved in diverse mechanisms to enhance plant growth and/or act as BCAs. Crop production promotion and disease management could be investigated together to ensure sustainability and cost-effectiveness of agricultural systems. Thus, effective PGPR strains can promote stress tolerance and nutrient absorption, plant development, and battling fungal/bacterial diseases. Thus, this appears to be a win-win situation to the PGPR strain and the host plant.

Challenges of employing PGPR as BCAs

PGPR-based biocontrol provides effective and long-lasting disease management. Europe and the United States are the most promising marketplaces for biocontrol products, followed by South America (Barratt et al., 2018). Although many PGPR have been tested *in vitro* and commercially proven as BCAs, new biocontrol products have been released from research activities carried out in the United States and Europe (Glick, 2012; O'Brien, 2017; Rosier et al., 2018). In general, the market of BCAs and their products is growing; yet, it is not well-adopted compared with chemical pesticides as the most common crop management method (Mishra et al., 2015).

Before being publically accepted/registered as a commercial BCA, there are certain requirements/needs that have to be taken into consideration (Bashan et al., 2014). As such, researchers should improve the efficacy of BCAs to manage certain disease(s). This can be achieved by having a BCA that has as many beneficial characteristics and mechanisms of action as possible. Such characteristics may include, but not limited to, the ability of the BCA to grow fast *in vitro*, produce a wide range of bioactive metabolites, possess high rhizosphere competence abilities, enhance plant growth performance, be environmentally safe, have the compatibility with other rhizobacteria/fungi, and be tolerant to abiotic stresses (Lyu et al., 2020). Successful colonization of root tissues and/or the rhizosphere is a critical component for any

PGPR strain to be an effective BCA; thus, to perform well against plant pathogens. On the other hand, performance of the inoculated PGPR may vary, depending on the survival rate in the soil, crop compatibility, interaction with other local microbial species and the environmental factors (Vejan et al., 2016). Survival and colonization are major components when identifying effective BCA isolates. *In vitro* antagonism experiments are often used to investigate the effect of bacterial isolates on certain diseases, prior to greenhouse and/or field trials (Bashan et al., 2014).

Performance of PGPR is generally assessed according to the geographical areas, soil types, host crop species, and under various environmental conditions (Choudhary et al., 2011). BCA growth is often easier to monitor under controlled conditions, i.e., greenhouses. The preference of this stage by most researchers could be attributed to the stability of environmental conditions. Greenhouse experiments evaluating the performance of BCAs under controlled conditions can provide strong theoretical and practical support for the application of PGPR in the field. Thus, this ensures the feasibility and efficacy of PGPR for commercial horticulture production, disease management and climate change conditions such as those found under field conditions.

PGPR stability is also influenced by the method, formulations, transportation, and storage conditions. To achieve high levels of the BCA survival (McIntyre and Press, 1991), one should improve the formulation technology (Lobo et al., 2019), increase the shelf-life of the BCA product (Carrasco-Espinosa et al., 2015), optimize the production of targeted microbial types (Zhang et al., 2019) and achieve low-cost production at large scales (Kang et al., 2017). Many scientists have attempted to extend the shelf-life of PGPR by decreasing the storage temperature and/or modifying the combinations of additives (Lee et al., 2016; Berger et al., 2018). Extensive research on the risks and benefits of BCAs is also required, because agricultural disease management approaches rely on this balance.

Due to the diverse modes of action, identification, characterization, the registration of promising PGPR strains take time and require academic-industry collaborations. Using natural sources (e.g., BCAs) to control pathogens also poses a set of legal and ethical issues that may threaten the local biodiversity (Hajek et al., 2016). In that regard, new species/populations of BCAs have been restricted from entering specific countries. For commercial uses, the application of PGPR in protected environments such as greenhouses is much easier, due to a more isolated and controlled environment delivery and potentially less negative ecological consequences. Another challenge that is linked to the widespread implementation of PGPR-based biocontrol is the regulatory problems. Currently, each country has its regulatory system that greatly vary among them (Bashan et al., 2014).

High development costs for new commercial BCAs, for example, have been identified as a barrier to the BCA industry's expansion in Australia (Begum et al., 2017). The high regulatory expenses of importing new BCAs into Australia is one of the most serious challenges. BCA registration requies tight coordination among governmental institutes, universities, and industrial sectors to facilitate the assessment and commercialization of new BCAs and their products. The shortage of programs for financial and ecological benefits can also be added as a challenging problem (Heimpel et al., 2013). For global marketing and local practical applications, commercialization should follow international legislation. The International Biological Control Organization (IOBC) have gathered academicians, researchers and practitioners from different sectors/fields to identify the barriers and provide recommendations to overcome these limitation (Barratt et al., 2018).

When compared to chemical pesticides, which are more reliable and predictable, farmers could notice little or no economic gain. Such programs, including local seminars, training workshops and free conferences may increase awareness about the application of BCA in specific farming areas. Finally, PGPR-based biocontrol can hold a lot of promises to reduce agrochemicals use in agriculture. The widespread use of PGPR as BCAs requires massive effort from regulatory bodies and crop growers to convince the public and earn their trust in the capacity of the new BCA products to manage diseases and increase crop yields. High-value crop production in greenhouses could be an ideal place to test the efficacy of PGPR as BCAs in response to different abiotic stresses. Based on recent successful greenhouse trials, BCAs can be used in the field for managing disease and associated agricultural plant growth enhancement (Alwahshi et al., 2022).

Rhizobacteria as BCAs

In the past few decades, rhizobacteria have gained attention when applied to grains, seeds, roots, and/or soils to help the plant grow and develop. Rhizobacteria are important for N_2 fixation, promotion of plant growth, and biological control of plant pathogenic microorganisms. Recently, various microbial species are presently used in bacterization, containing *Azospirillum*, *Azotobacter*, *Bacillus*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bradyrhizobium*, *Frankia*, *Pantoea*, *Pseudomonas*, and *Thiobacillus* (Whipps, 2001). Many plant diseases associated with nematodial, bacterial and fungal infections have been reported to be managed by PGPR. The use of BCAs has been controversial in suppressing nematode populations because other soil microorganisms and the host plant can be adversely affected. To manage diseases associated with plant-parasitic nematodes a combination of biological management, nematicides, organic soil amendments, and crop rotation have been used (Timper, 2011). *In vitro* culture filtrates of a strain of *Pseudomonas* sp. can suppress juvenile mortality of *Meloidogyne javanica*; thus, considerably reduce root gall and nematode population, and enhance plant development and yield (Nasima et al., 2002). Furthermore, inoculations with *Bacillus* spp. affect nematode behavior and feeding (Viaene et al., 2006). *Pseudomonas striata*, *Pseudomonas fluorescens*, and *B. subtilis* strains also overturn the population of nematodes (Table 1; Khan et al., 2012). Root-knot nematode

TABLE 1 Bacterial and fungal plant growth-promoting strains used as biocontrol agents against plant pathogenic microorganisms.

Host	Pathogen	Disease	PGP strains Bacteria/Fungi	References
I. Bacteria				
Soybean	*Fusarium solani, Macrophomina phaseolina*	Root rot	*Bradyrhizobium* sp.	Parveen et al., 2019
	Sclerotinia sclerotiorum	White mold	*Butia archeri*	Vitorino et al., 2020
Pigeon pea	*Fusarium udum*	*Fusarium* wilt	Rhizobacteria spp.	Dukarea and Paulb, 2021
	Erwinia tracheiphila		*Glutamicibacter* spp. FBE-19	Fu et al., 2021
Apple	*Mucor piriformis*	*Mucor* rot	*Pseudomonas fluorescens*	Wallace et al., 2018
Rice	*Meloidogyne incognita*	Root-knot nematode	*Trichoderma citrinoviride* Snef1910	Tariq et al., 2020
	Magnaporthe oryzae	Blast disease	*Pseudomonas putida* BP25	Ashajyothia et al., 2020
	Xanthomonas axonopodis pv. glycines	Bacterial pustule	*Pseudomonas parafulva* JBCS1880	Kakembo and Lee, 2019
	Phytophthora capsici	NA	*Pseudomonas*, *Burkholderia*	Khatun et al., 2018
	Xanthomonas oryza	Bacterial leaf blight	*Bacillus subtilis strain* GBO3	Faizal Azizi and Lau, 2022
Strawberry	*Macrophomina phaseolina*	Charcoal rot disease	*Azospirillum brasilense*	Viejobueno et al., 2021
	Botrytis cinerea	Gray mold	*Bacillus amyloliquefaciens* Y1	Maung et al., 2021
Cotton	*Macrophomina phaseolina*	Charcoal rot disease	*Pseudomonas aeruginosa* and *Sargassum ilicifolium*	Rahman et al., 2017
	Colletotrichum gossypii	Ramulosis disease	*Bacillus amyloliquefaciens,* and *Bacillus velezensis*	Ferro et al., 2020
Citrus fruit	*Penicillium digitatum*	Blue mold	*Bacillus megaterium*	Mohammadi et al., 2017
Oil seed rape	*Sclerotinia sclerotiorum*	*Sclerotinia* stem rot	*Trichoderma atroviride*	Hidayah et al., 2022
Brassica campestris L.		Sclerotiniose	*Bacillus thuringiensis*	Wang et al., 2020
Canola		Sclerotinia stem rot	*Paenibacillus chlororaphis*	Savchuk and Fernando, 2004
Maize	*Fusarium graminearum*	Stalk rot	*Bacillus methylotrophicus*	Cheng et al., 2019
Wheat	*Stagonospora nodorum*	*Stagonospora nodorum* blotch	*Bacillus subtilis* 26DCryChS	Maksimov et al., 2020
	Rhizoctonia solani AG-8	Wheat root pathogen	*Bacillus subtilis*	Zhang et al., 2021a
Pepper	*Phytophthora capsici*	Blight and fruit rot	*Bacillus licheniformis* BL06	Li et al., 2020
Tomato	*Fusarium oxysporum* f. sp. *lycopersici*	*Fusarium* wilt	*Brevibacillus brevis*	Liu et al., 2022
	Rhizoctonia solani	Damping-off	*Burkholderia cepacia* BY	Al-Hussini et al., 2019
Mango	*Lasiodiplodia theobromae*	Dieback	*Streptomyces samsunensis* UAE1, *Streptomyces cavourensis* UAE1, *Micromonospora tulbaghiae* UAE1	Kamil et al., 2018
Tea	*Colletotrichum* sp,	Shoot necrosis	*Trichoderma camelliae*	Chakruno et al., 2022
Date palm	*Fusarium solani*	Sudden death syndrome	*Streptomyces polychromogenes* UAE2, *Streptomyces coeruleoprunus* UAE1	Alblooshi et al., 2022
			Streptomyces tendae UAE1, *Streptomyces violaceoruber* UAE1	Alwahshi et al., 2022
	Thielaviopsis punctulata	Black scorch	*Streptomyces globosus* UAE1	Saeed et al., 2017
Royal poinciana	*Neoscytalidium dimidiatum*	Stem canker	*Streptomyces rochei* UAE2, *Streptomyces coelicoflavus* UAE1 and *Streptomyces antibioticus* UAE1	Al Raish et al., 2021
			Streptomyces griseorubens UAE2	Al Hamad et al., 2021
Banana	*Fusarium* spp.	Postharvest diseases	*Trichoderma* spp.	Snehalatharani et al., 2021
II. Fungi				
Rice	*Helminthosporium oryzae, Bipolaris oryzae*	Leaf brown spot	*Trichoderma viride, Trichoderma harzianum, Trichoderma hamatum*	Khalili et al., 2012; Mau et al., 2022

(Continued)

TABLE 1 (Continued)

Host	Pathogen	Disease	PGP strains Bacteria/Fungi	References
Scorzonera	*Alternaria scorzonerae, Fusarium culmorum*	Root and stem rot	*Trichoderma harzianum T-22*	Patkowska, 2021
			Trichoderma spp.	Bilesky-José et al., 2021
	Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium solani, Fusarium cucurbitae, Pythium aphanidermatum, Rhizoctonia solani, Mycosphaerella melonis		*Trichoderma aggressivum*	Sánchez-Montesinos et al., 2021
Tobacco	*Fusarium, Rubrobacter,* and *Talaromyces* spp.	Root rot	*Paenibacillus polymyxa*	Yao et al., 2021
			Trichoderma harzianum	
Okra	*Meloidogyne incognita*	Root-knot disease	*Trichoderma virens*	Tariq et al., 2018
Beans	*Botrytis cinerea*	Chocolate spot	*Trichoderma atroviride*	Yones and kayim, 2021
	Sclerotinia sclerotiorum	Wild mold	*Trichoderma asperellum*	Zapata-Sarmiento et al., 2020
Onion	*Sclerotium cepivorum*	White rot		Rivera-Méndeza et al., 2020
Tomato	*Colletotrichum gloeosporiodes*	Crop loss	*Trichoderma longibranchiatum*	De la Cruz-Quiroz et al., 2018
Cabbage	*Fusarium oxysporum*	Cabbage *Fusarium* wilt	*Rhizobactrin*	Khafagi et al., 2020
	Sclerotium sclerotiorum	Cabbage wilt	*Trichoderma hamatum*	Jones et al., 2014
Cocoa	*Phytophthora Palmivora*	Black pod	*Aspergillus fumigates*	Adebola and Amadi, 2010
Tomato	*Fusarium oxysporum f.* sp. *lycopersici*	Wilt	*Penicillium oxalicum*	Murugan et al., 2020
	Rhizophagus intraradices	*Verticillium* wilt	*Penicillium pinophilum*	Ibiang et al., 2021
	Meloidogyne javanica	Root-knot disease	*Paecilomyces lilacinus*	Hanawi, 2016
Vigna radiata	*Meloidogyne incognita*		*Purpureocillium lilacinum*	Khan et al., 2019
Pineapple	*Meloidogyne javanica*		*Purpureocillium lilacinum*	Kiriga et al., 2018
Carrot			*Pochonia chlamydosporia*	Bontempo et al., 2017
Kiwi	Postharvest diseases	Kiwi fruit wound	*Debaryomyces hansenii*	Sui et al., 2021
	Soil-borne pathogens		Rhizosphere	Tsegaye et al., 2018
Tomato	*Sclerotium rolfsii*	Southern blight	*Stenotrophomonas maltophilia* PPB3	Sultana and Hossain, 2022
	Phytophthora infestans	Late blight	*Rhizopus* spp.	Agbor et al., 2021
Peaches	*Monilinia laxa*	Postharvest fruit decay	*Aureobasidium pullulans*	Di Francesco and Baraldi, 2021
Sweet potato	*Ceratocystis fimbriata*	Black rot disease	*Pseudomonas chlororaphis* subsp. *aureofaciens* SPS-41	Zhang et al., 2021b
Rice	*Pyricularia oryzae*	Rice blast fungus	Rhizobacteria	Nabila and Kasiamdari, 2021
Chickpea	*Rhizoctonia bataticola*	Chickpea dry root rot	*Bacillus subtillis*	Chiranjeevi et al., 2021

(RKN) infestations have been successfully managed via biological management using *Bacillus* isolates (Lee and Kim, 2016). Few studies have reported the endophytic *P. fluorescens* and *Bacillus* spp. to promote systemic resistance in crops against nematodes owing to the increased activities of phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase, as defense-related enzymes for producing antagonistic chemicals and altering explicit root exudates such as amino acids and polysaccharides (Abbasi et al., 2014). *P. fluorescens* isolates increased defense enzymes in tomatoes resistant to RKN (Kavitha et al., 2013). In comparison with the control, the application of *P. fluorescens* and *Paecilomyces lilacinus* resulted in low nematode community in roots, tubers and soils (Mohan et al., 2017).

According to Jha et al. (2015), losses resulting from post-fresh fruit and vegetable harvest in India ranged between 4.6% and 15.9%. Although fungicides can inhibit the growth of phytopathogens, their use causes problems to the environment as well as the human and animal health (Nunes, 2012). The most environmentally acceptable practice to control post-harvest fungal diseases is by using BCAs. In general, BCAs can protect plants from fungal diseases, and are currently a viable option to manage post-harvest diseases associated with plant pathogens (Ghazanfar et al., 2016). In agriculture, BCAs can offer a number of advantages, including the reduction in the causing agents, farming preservation, minimum labor, soil, water plant contamination, and waste management difficulties (Torres et al., 2016). Fungal species, such as *Alternaria*, *Aspergillus Penicillium*, and *Fusarium* producing mycotoxins, are harmful to green vegetables and cause post-harvest diseases. Mycotoxins, such as fumonisin, ochratoxins, aflatoxins and other toxins, are released in vegetables and fruits infected with the fungal pathogens, *Fusarium*, *Alternaria*, and *Aspergillus* (Sanzani et al., 2016). The use of biopriming and pelletizing techniques of *Serratia plymuthica* HRO-C48 alongside *Verticillium dahliae* in canola plants revealed a significant

biocontrol (Muller and Berg, 2008); thus, providing evidence of the ability of BCAs to manage diseases comparable to chemical fungicides.

Bacillus spp. produce a variety of compounds involved in the biocontrol of phytopathogens on various plants, including potato, rice, tomato, wheat, groundnut, brinjal, chickpea, and cucumber (Peng et al., 2014). *Bacillus* sp. BS061 isolate can mitigate the effect of *Botrytis cinerea* to reduce the occurrence of gray mold and powdery mildew diseases in strawberry and cucumber (Kim et al., 2013). Park et al. (2013) found that *Pectobacterium carotovorum* SCC1 can manage soft rot disease in tobacco plants when conjugated with *B. subtilis* strain B4 and BTH fungicides. The root-knot and root rot pathogens are often suppressed when *Pseudomonas* spp. are used as BCAs (Habiba et al., 2016). Farhat et al. (2017) revealed that PGPR isolates had antifungal activities in mungbean plants against *Rhizoctonia solani*, *Macrophomina phaseolina*, *F. solani*, and *F. oxysporum*. These isolates can also be used to prevent fungal infections that cause root rot disease. The application of the bacterial BCA, *Pseudomonas aeruginosa*, can manage anthracnose in the chili pepper against the causal pathogen *Colletotrichum capsici* (Jisha et al., 2018). *P. aeruginosa* can also induce systemic resistance of chili pepper to anthracnose.

Synthetic chemical pesticides are mainly used for post-harvest disease management. Thus, this may lead to plant pathogen resistance, soil deterioration, and toxicological hazards for the humans and the environment. Nowadays, a general trend, as a result, has been shifted toward finding an alternative to the use of agrochemicals in plant disease management. Compared to synthetic chemical fungicides, the use of microbial antagonists or BCAs has become a "hot" topic due to the numerous advantages as non-hazardous, green, economical and feasible applications to control post-harvest pathogen infections (Bonaterra et al., 2012).

Fungi as BCAs

Fungal BCAs are able to antagonize plant pathogens and protect their host plants. For example, several strains of *Trichoderma* have been developed as BCAs against the fungal pathogens *Penicillium*, *Fusarium, Aspergillus*, *Alternaria*, *Pythium*, *Rhizoctonia*, *Phytophthora*, *Pyricularia*, *Botrytis*, and *Gaeumannomyces* (Pal and Gardener, 2006; Adebola and Amadi, 2010; Agarwal et al., 2011; Alam et al., 2011; Nally et al., 2012). As a BCA, *Trichoderma* can suppress various air- and soil-borne plant pathogens; thus, can be conceivably used as biopesticides in greenhouse and/or field trials. According to Silva et al. (2017), certain strains of nematophagous fungi can manage the populations of *Meloidogyne enterolobii* in an integrated pest management (IPM) approach. AMF could also protect crops against soil-borne pathogens, including RKN, albeit the unclear mechanisms of antagonism (Vos et al., 2012).

The use of nematophagous and endoparasitic fungi has been deployed as antagonists to suppress RKN (Pendse et al., 2013). The talc-based formulation of the fungal BCA, *Paecilomyces lilacinus*, was found to be more active in reducing the population of *Meloidogyne incognita* in soils cultivated with tomato plants (Priya and Kumar, 2006). The efficiency of *P. lilacinus* in controlling nematodes was observed in several horticultural crops, including tomato, okra, and capsicum (Rao, 2007). The most widely used BCA for plant-parasitic nematodes is the fungus *P. lilacinus*, which has shown an appropriate replacement to synthetic chemical control in pre- and post-planting applications (Atkins et al., 2005). *P. lilacinus* infects eggs, juveniles and females of *M. javanica* by direct hyphal penetration (Esfahani and Pour, 2006). *P. lilacinus* can boost tomato yield while reducing the population of *M. incognita* in the soil and on the roots (Kalele et al., 2010). RKN management can also be achieved by using *P. lilacinus* and *Bacillus firmus* either individually or in combination. However, the mixture of *P. lilacinus* and *B. firmus* applied in soils 2 weeks prior to tomato transplantation showed the best practice to control *Meloidogyne* spp. (Anastasiadis et al., 2008).

Coating the seed with *Trichoderma viride* and *P. lilacinus* effectively reduced the nematode population in the soil. Species of *Aspergillus* and *Paecilomyces* were found to be antagonistic to *M. incognita* when compared to the single bio-agent treatment; thus, resulting in enhanced plant growth (Table 1; Bontempo et al., 2017). Kerry and Hidalgo-Diaz (2004) developed a management technique using the nematophagous fungus *Pochonia chlamydosporia* to manage RKN for the purpose of organic vegetable production. Okra seeds treated with *Trichoderma harzianum*, *T. viride*, *P. lilacinus*, *P. chlamydosporia*, and *P. fluorescens* at 20 g kg^{-1} seed signifcantly reduced the nematode population in the soil and promoted plant growth development (Kumar et al., 2012). Sharf et al. (2014) have reported that *P. chlamydosporia* exhibited nematicidal effects against *M. incognita* on infected common bean under greenhouse condition. *Trichoderma* spp. synthesizing chitinases, lytic enzymes, proteases, and glucanases were found to manage vegetable crop diseases (Punja and Utkhede, 2003). *T. harzianum*, *T. viride*, and *T. hamatum* have nematocidal properties when they colonize the roots of host plant and enhance their growth performance (Girlanda et al., 2001; Siddiqui and Shaukat, 2004; Zhang and Zhang, 2009). Because crop yield is mainly influenced by climatic conditions, agronomic factors, pests, and nutrient availability in the soil (Harman et al., 2004; Elrys et al., 2019a, 2020a), researchers must consider these factors in the selection of fungal BCAs.

Likewise, *Trichoderma* spp. can prevent nematode penetration and development in plants through the regulation of metabolites (Bokhari, 2009). Usman and Siddiqui (2012) have shown that the *M. incognita* and other RKN are more affected by the culture filtrate of *Trichoderma*. Species of *Trichoderma* can produce viridin, a nematicidal chemical (Watanabe et al., 2004). Gliotoxin and acetic acid, have also been reported as nematicidal substances in the culture filtrates of *T. virens* and *T. longibrachiatum,* respectively (Anitha and Murugesan, 2005). In response to *M. incognita*, *T. polysporum* has the ability to synthesize cyclosporine, the peptide that has a nematicidal action (Li et al.,

2007). The efficacy of *P. lilacinus*, as bioagents or bioproducts in mixtures, significantly decreased the number of *M. incognita* on eggplant (Hanawi, 2016). It has been shown that different sources of N and carbon affect the growth and antagonism of *Trichothecium roseum* and *T. viride* (Arya, 2011). Although fructose and lysine were mostly effective against *T. viride*, rhamnose and glycine were more effective against *T. roseum*. There is an adverse effect of fungal culture filtrates on the egg hatching and juvenile mortality of RKN. Plants treated with the fungal BCA, *Lecanicillium muscarium*, decreased the number of galls in plants, eggs, juveniles (J2) and the reproduction factor (Rf) of *Meloidogyne hapla* compared to control plants (Hussain et al., 2017). In addition, plant growth was greatly improved when treated with *L. muscarium*. *Trichoderma* and *P. lilacinum* isolates dramatically reduced nematode egg number and mass, minimized root gal injury, and improved plant root mass development when compared to control plants without the fungal BCAs (Kiriga et al., 2018). Overall, more than 30 genera and 80 species of fungi can parasite RKN (Gaziea-Soliman et al., 2017). Prince et al. (2011) have demonstrated that the fungus, *Colletotrichum falcatum*, has antagonistic potential against the fungal pathogens *Penicillium citrinum*, *Botrytis cinerea*, and *Trichodermum glaucum*. Moreover, other fungi, such as *Ampelomyces speciosus* and *Acremonium alternatum*, have the ability to degrade the mycelia of fungal pathogens, indicating that not only rhizobacteria, but also fungi can serve as BCAs (Kiss, 2003).

Mechanisms used by MBCAs

Understanding the appropriate conditions for implementing proper programs against plant pathogens requires collaborations between different research groups focusing on the mechanisms associated with MBCAs to manage diseases on plants. In the last two decades, extensive research has focused on the antifungal effect, rhizosphere colonization, and crop benefits linked to MBCA (Compant et al., 2010; Al Hamad et al., 2021; Al Raish et al., 2021; Alwahshi et al., 2022). Thus, the products of MBCA on plant fungal pathogens and their impacts on plants are illustrated in Figure 2. The primary strategy of MBCA are summarized as antibiosis, competition for micronutrients such as iron, mycoparasitism, production of hydrolytic enzymes, and induction of ISR in host plants (Figure 3). In addition, the production of metabolites that are inhibitory to plant pathogenic rhizosphere microorganisms is considered one of the major biocontrol activities in many MBCA (Haas and Keel, 2003).

In several microorganisms, antibiosis, also known as secondary metabolites, results in the production of various toxic chemicals to pathogenic microorganisms; thus, they are suitable for the plant growth and development. An antibiotic-producing microorganism must manufacture the antibiotic in the correct microniche on the root surface to effectively control plant diseases (Lugtenberg and Kamilova, 2009). Actinobacteria (8,700 distinct antibiotics), bacteria (2,900), and fungi (4,900) can produce massive amounts of antibiotics (Bérdy, 2005). Mutagenesis has been reported to be successful in determining the role of antibiotics generated by bacterial BCA isolates to control pathogens associated with plant infections (Liu et al., 2007).

Ongena and Jacques (2008) have investigated the lipopeptides (surfactin, iturin, and fengycin) in *Bacillus* spp. It has also been reported that pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), and phenazine can be potential antibiotic metabolites in *Pseudomonas* (Raaijmakers and Mazzola, 2012). *Pseudomonas* spp. have the ability to generate pyoluteorin, siderophores, and cyanide, among other antimicrobial chemicals (Compant et al., 2010). In addition, the enzymatic activity of cellulase, proteases, β-glucanase, and chitinase can lyse fungal cells (Hernandez-Leon et al., 2015). Antibiotic metabolites produced by *Pseudomonas* spp. are regulated by complex regulatory networks and high number of transcription factors (Berry et al., 2014). Significant classes of antifungal antibiotics are lipopeptides or peptides that are produced by the ribosomes or non-ribosomes of *Bacillus* spp. (Figure 2). Arseneault and Filion (2017) have discussed that antibiotics can be generated by BCA strains in soil.

Bacillus spp. have the ability to produce various biologically active chemicals that hinder the development of several crop diseases (Zhao et al., 2013). An investigation by Chowdhury et al. (2015) revealed that the quantity of antibacterial or antifungal chemicals produced by *Bacillus* spp. in the rhizosphere is somewhat little, causing doubts on the role of rapid management of plant diseases. Several isolates of *P. fluorescens* were found to generate cyclic lipopeptides (CLPs), such as viscosinamide, amphisin and tensin, that were effective against fungal pathogens, *R. solani* and *Pythium ultimum* (Nielsen et al., 2002).

Biological control is an application of beneficial organisms, genes, and their products in the form of metabolites (Glare et al., 2012). Several *in vitro* metabolites of microorganisms were utilized to control pathogenic infections (Köhl et al., 2019). As a result, these secondary metabolites can be utilized as products of a BCA; and thus, they are effective to ameliorate the negative impact of other pathogenic microorganisms while also being environmentally friendly. Antimicrobial activities of some fungal BCAs may also exhibit antagonistic effects against fungi. For example, *Trichoderma* spp. are commonly found in soil and provide a variety of volatile and nonvolatile compounds. Volatile compounds, such as cyanide, hydrogen, ET, aldehydes, ketones and alcohols; and nonvolatile substances, such as peptides, can inhibit the mycelial growth in some pathogenic fungi. Many antifungal compounds, such as gliovirin, gliotoxin, viridiol, heptelidic acid, valinotrocin, and viridin can be produced by *Gliocladium virens*, which acts as a MBCA. Singh et al. (2005) demonstrated that gliotoxin can effectively reduce the fungal pathogens, *Pythium aphanidermatum*, *M. phaseolina*, *Pythium debaryanum*, *R. solani*, *Sclerotium rolfsii*, and *Rhizoctonia bataticola*. Vinale et al. (2009) have stated that the production of 1-hydroxy-3-methylanthraquinone, 1,8-dihydroxy-3-methyl-anthraquinone, T22 azaphilone, harzianolide, T39butenolide, and

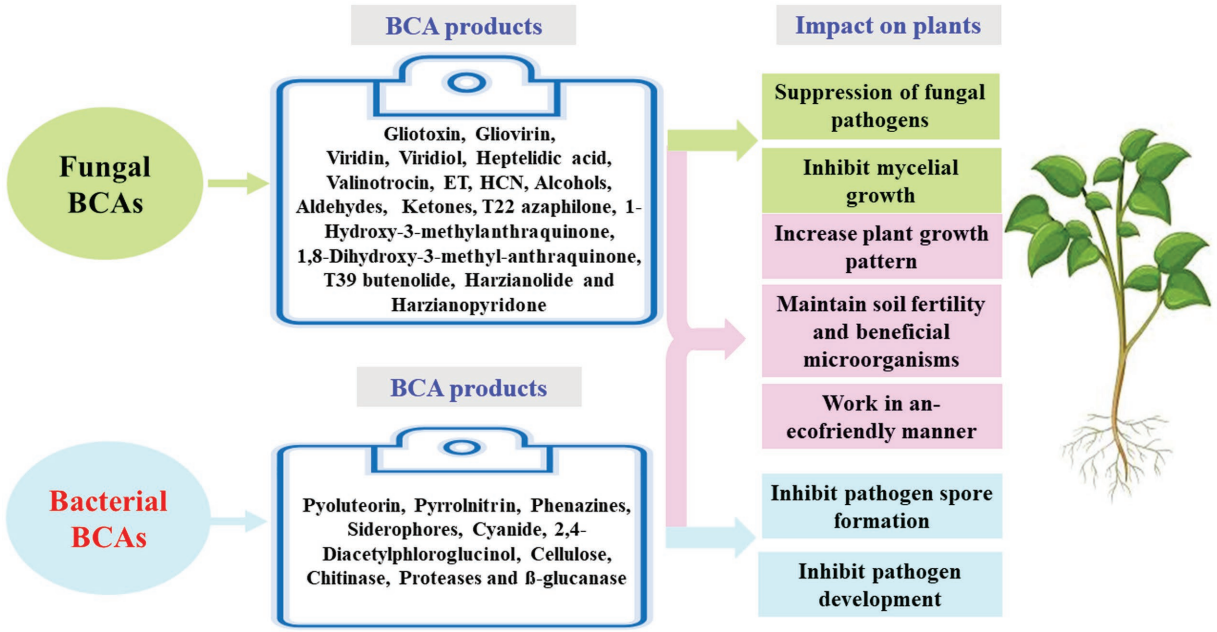

FIGURE 2
Fungal and bacterial biocontrol agents (BCAs) and their impact on plant. ET, ethylene; HCN, hydrogen cyanide.

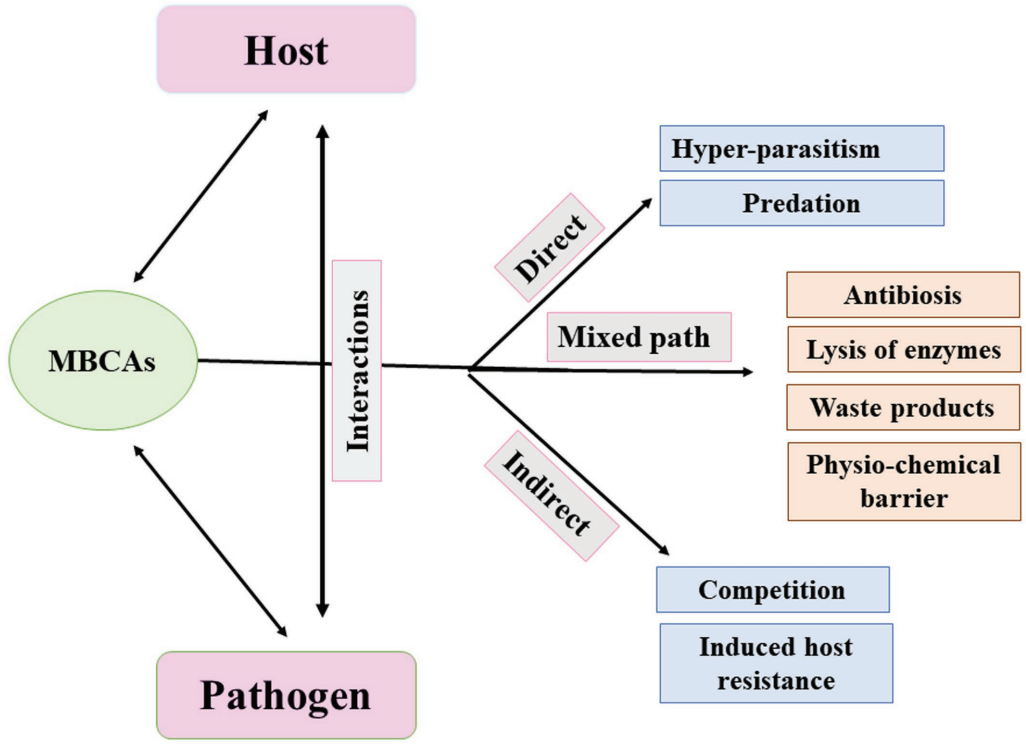

FIGURE 3
Pathways of microbial biocontrol agents (MBCAs).

harzianopyridone by *T. harzianum* strains T22 and T39 has the ability to control the plant fungal pathogens *Leptosphaeria maculans*, *Phytophthora cinnamomi*, *R. solani*, *Botrytis cinerea*, and *P. ultimum*. Several secondary metabolites have been isolated and recognized by different methods such as high-performance liquid chromatography (HPLC) and gas chromatography–mass

spectrometry (GC–MS). Shanthiyaa et al. (2013) have investigated three isolates, Cg-5, Cg-6, and Cg-7, that produce the secondary metabolite, chaetoglobosin A, in the culture filtrate detected by the UV spectrum at 250 nm. The antimicrobial compounds released by fungi may also control phytopathogens during post-harvest infections. The post-harvest infection causes excessive damages in vegetables and fruit (Figures 4, 5).

Competition in the rhizosphere

Co-existence of two living microorganisms occurs when a population of a particular microorganism strives to achieve something greater, such as space or food supply (Stirling, 2017). Pathogenic and non-pathogenic microorganisms compete for food and resources in the rhizosphere. It has been known for a long time that non-pathogenic plant-associated bacteria are usually protected by colonizing plants and, as a result, this debilitate the limited available substrates and prevent the spread of the pathogens. The abilities of any microorganism to compete with others for essential nutrients and exudates secreted by the plant roots and their capability of colonizing into the root surface of host plants are termed rhizosphere competence. In the rhizosphere, the beneficial interactions between plants and microorganisms can regularly occur; thus, promoting growth and/or enhancing tolerance to biotic and abiotic stresses in plants (Zamioudis and Pieterse, 2012).

Rhizosphere competence can successfully establish microbial communities on or near the plant roots. Plant root colonization by PGPM can protect plants against pathogens and promote plant growth, and chemotaxis to root exudates is considered as an essential prerequisite for efficient root colonization (de Weert and Bloemberg, 2006). Microbial community in the rhizosphere is found to be important shortly after planting, but regularly decreases during the cropping season (Weller, 2007). Adesina et al. (2009) have reported *in vitro* antagonism of 15 *Pseudomonas* strains to *R. solani* in the rhizosphere. Only *Pseudomonas jessenii* RU47 has been effective to bottom rot disease on lettuce caused by *R. solani*. Tryptophan can stimulate the growth of adventitious roots and root hairs of the IAA-producing *B. subtilis* SRB28, which can colonize root tissues in sorghum, produce microcolonies, and persist in the rhizosphere (Das et al., 2010).

Rhizosphere microorganisms promoting plant growth, represent a wide range of species. PGPM are categorized according to their ability to colonize roots, survive, increase their numbers in the microhabitats on the root surface, compete with indigenous microorganisms, and increase resistance in host crops (Gamalero et al., 2004). PGPM can not only promote plant development, but also they are often used as BCAs to suppress plant diseases. The plant-associated *Bacillus*, *Pseudomonas*, *Lactobacillus* and actinobacteria strains are used as biofertilizers

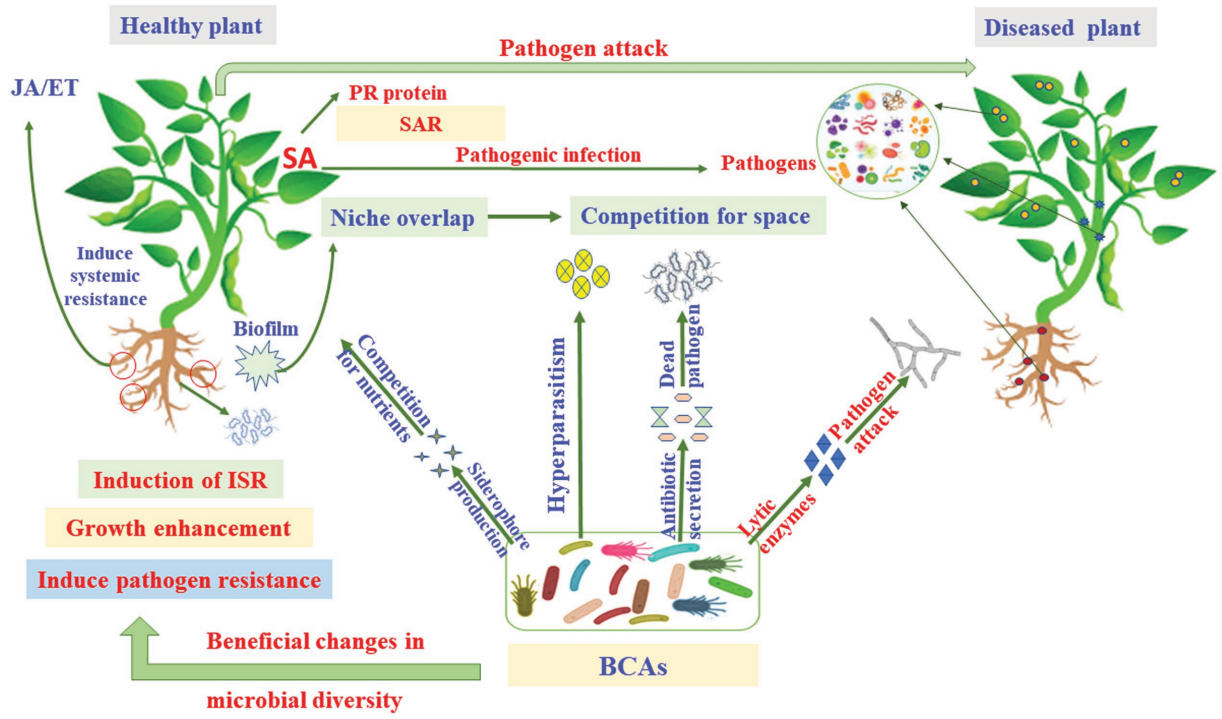

FIGURE 4
Pathways and modes of action of microbial biocontrol agents (MBCAs). ET, ethylene; ISR, induced systemic resistance; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance.

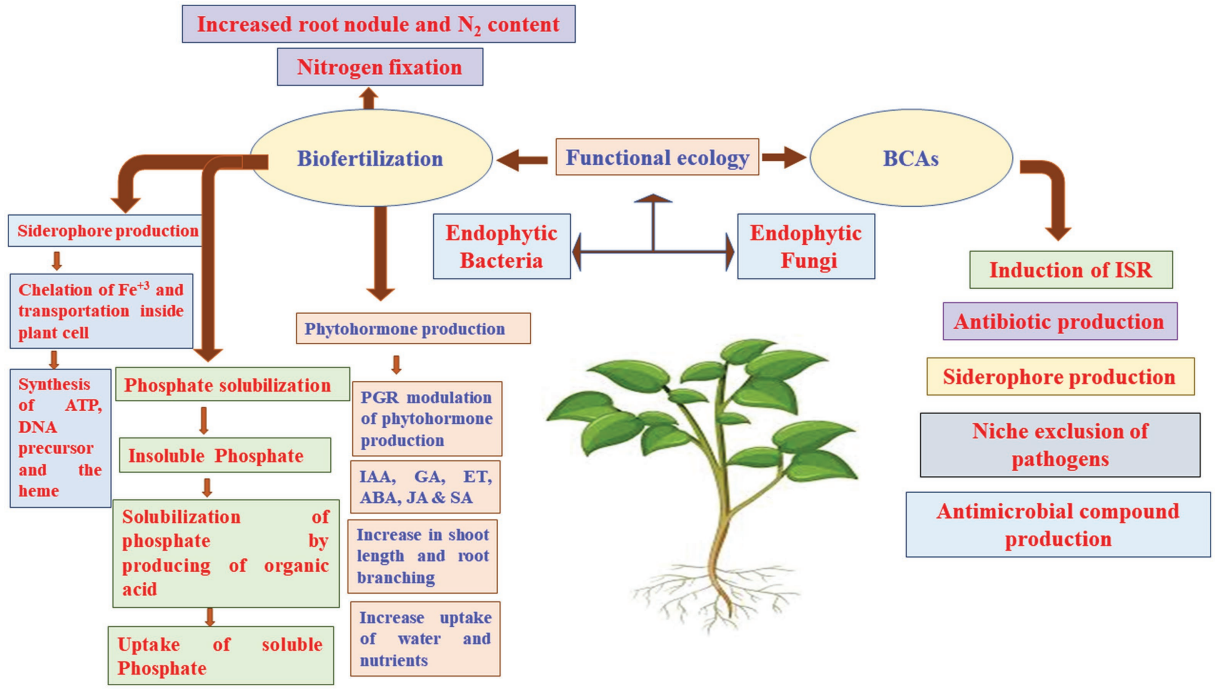

FIGURE 5
Dynamic microbial ecologies. ABA, abscisic acid; BCA, biocontrol agents; ET, ethylene; GA, gibberellic acid; IAA, indole acetic acid; ISR, induced systemic resistance; JA, jasmonic acid; PGR, plant growth regulators; SA, salicylic acid.

and BCAs in agriculture (Borriss, 2011; Sivasakthi et al., 2014; Lamont et al., 2017; Shivlata and Satyanarayana, 2017). Furthermore, *Acetobacter*, *Serratia*, *Azospirillum*, *Paenibacillus Burkholderia*, *Herbaspirillum*, and *Rhodococcus* can also enhance growth in crop plants (Babalola, 2010). Chakraborty et al. (2013) reported that a number of PGPR traits, such as production of siderophore, solubilization of phosphate, synthesis of IAA, and antagonism against fungal pathogens, were found to stimulate growth in tea plants. This has been linked with an increase in the number of shoots and leaves under greenhouse and field conditions. In general, soils with active microbial ecosystems and high organic matter require less fertilizer than soils without any microorganisms (Bender et al., 2016).

Biofertilizers made from microorganisms that help plants obtain their nutrients can colonize plant roots, to solubilize P, produce siderophore and HCN, and fix N_2 (Figure 5; Pii et al., 2015; El-Sobky et al., 2022). N_2 fixation by PGPR provides a considerable amount of N to the farming systems worldwide, with estimations ranging from 20 to 22 Tg N annually (Herridge et al., 2008), which may reach in some years to up to 40 Tg N (Galloway et al., 2008). Moreover, it has been reported that the biological N_2 fixation may provide the African countries approximately 12 Tg N $year^{-1}$ (Elrys et al., 2019b, 2020b). Crop yields might be limited by other nutritional elements, such as Fe and Zn. Similar to P, Fe is highly abundant in soils; yet, it is not available to plants in most cases. The synthesis of organic acids or siderophores by various PGPR strains increases Fe accessibility (Ahmed and Holmstrom, 2014).

Auxins are produced by a variety of PGPR (Gupta et al., 2015) that is involved in plant growth and development (Jha and Saraf, 2015) and plant architecture (Vacheron et al., 2013). The auxin, IAA, produced by PGPR has received much of attention. It is highly involved during PGPR-plant interactions (Afzal et al., 2015). Auxin-producing PGPR have been reported to cause transcriptional alterations in the hormone levels, resistance/tolerance to biotic/abiotic stress, and regulation of cell wall-linked genes (Spaepen et al., 2014). IAA may also increase root length (Hong et al., 1991), enhance root biomass, while reducing the size and density of stomata (Llorente et al., 2016). Plant growth and development can also be stimulated by the induction of auxin-response genes (Ruzzi and Aroca, 2015).

In addition, PGPRs can produce GA and CKs (Gupta et al., 2015), although the exact process remains unknown (Kang et al., 2009). A limited number of PGPR strains can produce huge amounts of GA; thus, significantly increase the shoot growth in plants (Jha and Saraf, 2015). Exudates are expected to contain organic acids, sugars, and amino acids, which are highly abundant in the cytoplasm of plants, but low quantities of complex secondary metabolites, including flavonoids, terpenes, and phenolic substances, which may attract certain rhizosphere microorganisms (Musilova et al., 2016). Plant health and physiology could be improved due to PGPR colonization of roots, resulting in more seeds and blooms (Kumar et al., 2016). According to Nivedhitha et al. (2008), actinobacteria isolated from the rhizosphere of bamboo was found to be capable to

suppress the fungal pathogen, *Fusarium* sp., while boosting plant growth and development. Harzianic acid produced by *T. harzianum* not only promoted plant growth, but also showed antifungal effects against *Pythium irregulare*, *Sclerotinia sclerotiorum*, and *R. solani*, even at very low doses (Vinale et al., 2009). MBCA are important to the advancement and improvement of plant growth development, as well as the prevention of the attack of plant pathogens.

Future perspectives

Biological control management is one of the most promising applications for sustainable agriculture. It is a proven to be eco-friendly agricultural pest control approach. This strategy uses living microorganisms to reduce the pest populations in a conservative, dependable, and ecologically amicable manner. In the developed countries, biological control is a remarkable tool to achieve sustainable, less expensive, and safe pest control management; thus, offering benefits to breeders and consumers when compared to synthetic (chemical) pest management. This review has provided an overview of antagonistic modes of action of MBCA, which are regarded as practical substitutions to synthetic fungicides as well as stimulation of plant growth and development for post-harvest purposes. Researchers working in the field of MBCA must anticipate new and distinct questions, in order to provide solutions that help in the development of novel biocontrol technologies/applications. Bioinformatics, molecular biology, analytical chemistry, and biostatistics have also shed lights on new research areas aimed at defining the MBCA-pathogen-plant interaction (Spadaro and Gullino, 2005).

One should not neglect the environmental conditions that also play a crucial role in the process of antagonism and the mode(s) of action of MBCA. The following conditions should be taken into consideration, when researchers isolate, identify and characterize a MBCA strain:

1. The spread of the infection associated with nematodes, fungi, and bacteria, as well as the potential antagonists in the micro- and macro-environment of the interaction.
2. The best conditions for the application of BCA.
3. The reaction of MBCA to the local communities and to various management strategies.
4. The limiting factors of effective colonization and articulation of biological control characteristics.
5. The plant components and dynamics that induce host defense.

Conclusion

Many crops are affected by various pathogens. PGPM of pests and diseases in crops are generally regarded as a sustainable alternative for conventional chemical plant protection. These PGPR and PGPF acting as MBCAs are a safe, effective, and environmentally friendly form of pest management that do not harm the environment or the human health. PGPR/PGPF are antagonistic microorganisms that could be exploited as biopesticides and biofertilizers for better plant health and growth improvement. Adoption of PGPR/PGPF-based biopesticides/biofertilizers on a commercial scale may substantially contribute to sustainable agriculture and safe environment. This review has provided an overview on the research related to PGPMs, their benefits and effects as potential bioinoculants for plant growth and biological control. The increased use of PGPMs requires the achievement of accurate selection of beneficial PGPR/PGPF strains and consortia, the mechanisms underlying PGPM-plant interactions, and the ability to prepare for future agricultural challenges.

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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Conflict of interest

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

Publisher's note

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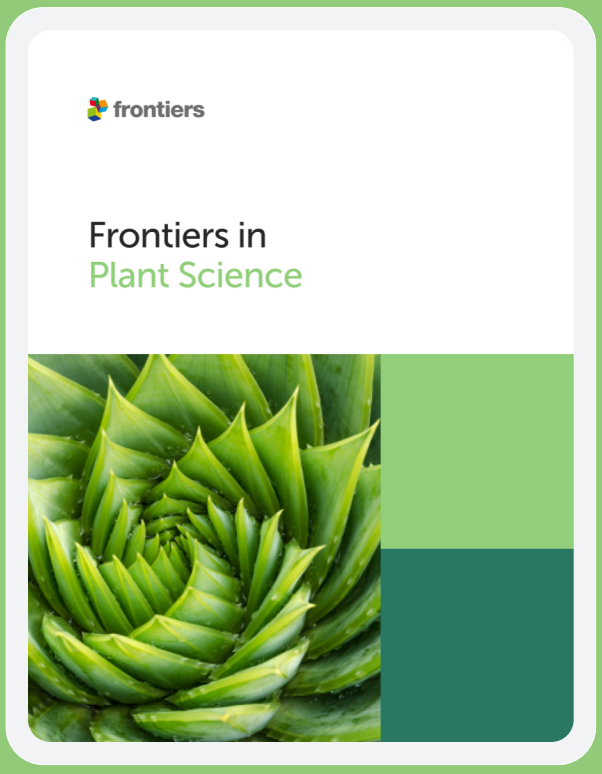

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