

Microbial-mediated induced resistance: interactive effects for improving crop health

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Microbial-mediated induced resistance: interactive effects for improving crop health

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Table of contents

- 05 **Editorial: Microbial-mediated induced resistance: interactive effects for improving crop health**
Debasis Mitra, Anju Rani, Edappayil Janeeshma and Bahman Khoshru
- 08 **Fungal-mediated synthesis of silver nanoparticles: a novel strategy for plant disease management**
Mansoor Ahmad Malik, Abdul Hamid Wani, Mohd Yaqub Bhat, Sazada Siddiqui, Saad A. M. Alamri and Sulaiman A. Alrumman
- 19 **The functional identification and evaluation of endophytic bacteria sourced from the roots of tolerant *Achyranthes bidentata* to overcome monoculture problems of *Rehmannia glutinosa***
Chunli Zeng, Yazhou Liu, Bianhong Zhang, Chenjing Zhang, Niu Li, Leshan Ji, Chaojie Lan, Bin Qin, Yuncheng Yang, Juanying Wang, Ting Chen, Changxun Fang and Wenxiong Lin
- 32 **Next generation plant biostimulants & genome sequencing strategies for sustainable agriculture development**
Shivanshu Garg, Pooja Nain, Ashish Kumar, Samiksha Joshi, Himanshu Punetha, Pradeep Kumar Sharma, Sazada Siddiqui, Mohammed O. Alshaharni, Uthman Balgith Algopishi and Amit Mittal
- 53 **Evaluating the efficacy of microbial antagonists in inducing resistance, promoting growth, and providing biological control against powdery mildew in wheat**
Kariyappa R. Choudaker, Vaibhav Kumar Singh, Abhijeet Shankar Kashyap, Aakash V. Patel, Koshal K. Sameriya, Dhananjay Yadav, Nazia Manzar, Deebea Kamil, Lakshman Prasad and M. S. Saharan
- 70 **Dazomet fumigation modification of the soil microorganism community and promotion of *Panax notoginseng* growth**
Ya-meng Lin, Ming-hua Li, Chun-yan Dai, Yuan Liu, Wen-ping Zhang, Qian Yang, Xiu-ming Cui and Ye Yang
- 82 **The impact of salinization on soil bacterial diversity, yield and quality of *Glycyrrhiza uralensis* Fisch.**
Yangmei Bao, Bin Ma, Neil B. McLaughlin, Ying Niu, Dongqing Wang, Hua Liu, Ming Li and Zhirong Sun
- 95 **Plant growth-promoting rhizobacterium *Bacillus megaterium* modulates the expression of antioxidant-related and drought-responsive genes to protect rice (*Oryza sativa* L.) from drought**
Sanghun Lee, Jung-Ae Kim, Jeongsup Song, Seonbong Choe, Geupil Jang and Yangseon Kim
- 118 **Relative multi-beneficial effect of MOs on plant health of chickpea (*Cicer arietinum* L. var. PG-186)**
Supriya Tomer, Priyanka Khati, Deep Chandra Suyal, Kahkashan Perveen, Faheema Khan and Jayanthi Barasarathi

- 129 **Isolation, identification, and mechanism analysis of plant growth-promoting rhizobacteria in tobacco**
Chuandong Jiang, Fuyu Peng, Li Zhang, Yuqin Zhang, Jie Wang, Junmin Li, Binghui Cui, Changdai Cao, Chengqiang Wang, Yunlei Qin, Ran Wang, Zongpeng Zhao, Jiazhu Jiang, Mingfeng Yang, Mingming Sun, Long Yang and Qiang Zhang
- 147 **Next-generation sequencing-based comparative mapping and culture-based screening of bacterial rhizobiome in *Phytophthora capsici*-resistant and susceptible *Piper* species**
A. Hima Parvathy, R. Santhoshkumar and E. V. Soniya
- 161 **Possible biocontrol of bacterial blight in pomegranate using native endophytic *Bacillus* spp. under field conditions**
N. Manjunatha, Somnath S. Pokhare, Ruchi Agarwal, Nripendra V. Singh, Jyotsana Sharma, Mallikarjun M. Harsur and Rajiv A. Marathe
- 173 **Exploring *Trichoderma* diversity in the Western Ghats of India: phylogenetic analysis, metabolomics insights and biocontrol efficacy against Maydis Leaf Blight disease**
Nazia Manzar, Abhijeet Shankar Kashyap, Manish Roy, Pawan Kumar Sharma and Alok Kumar Srivastava
- 192 **The composite microbial agent controls tomato bacterial wilt by colonizing the root surface and regulating the rhizosphere soil microbial community**
Shuangxi Zhu, Xiaojian Chang, Nana Liu, Yanhui He, Jianwen Wang and Zhansheng Wu



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Editorial: Microbial-mediated induced resistance: interactive effects for improving crop health

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Editorial on the Research Topic

Microbial-mediated induced resistance: interactive effects for
improving crop health

The global population, currently estimated at ~8.06 billion, is anticipated to increase to 9.80 billion by 2050 and 11.20 billion by 2100, as reported by the United Nations Department of Economic and Social Affairs (Graham, 2017; UN-DESA, 2024). To address this demographic expansion, it is imperative to prepare for the heightened demand for food. Nevertheless, the agricultural sector remains heavily dependent on chemical fertilizers, pesticides, and herbicides (Zhou et al., 2024). These practices have significant environmental repercussions, including a reduction in the diversity of soil microorganisms, which can ultimately impair food production. This challenge is exacerbated by climate change, declining soil health, and other stressors. In this context, microbial-mediated induced resistance (MIR) emerges as a promising area of research in agriculture, investigating the potential of microbes to enhance plant resistance to pathogens (Singh et al., 2024). This approach utilizes certain microorganisms, such as bacteria and fungi, to trigger a systemic response in plants, thereby boosting their disease defense mechanisms (Rabari et al., 2023). The effect of MIR on crop health can be substantial, providing sustainable alternatives to traditional chemical-based disease management techniques (Manzoor et al., 2024). Progressing research into the role of microbes in sustainable agriculture will encourage the adoption of innovative methods that enhance soil health, crop yield, and fertility.

Soil microorganisms play a vital role in facilitating plant nutrient absorption, inducing systemic resistance, and mitigating adverse climatic conditions through plant signaling compounds and cross-talk mechanisms (Ruparelia et al., 2022). The interactions between beneficial symbiotic microorganisms and plant roots, along with other soil microbial interactions, enhance nutrient utilization efficiency and activate plant defense mechanisms, thereby contributing to sustainable agricultural production (Alzate Zuluaga et al., 2024). This Research Topic aims to present the latest insights into plant-soil-microbe interactions, which play a crucial role in microbial-induced resistance (MIR). Jiang et al. reported that plant growth, crop yield, and pest & disease control are enhanced by plant

growth-promoting rhizobacteria (PGPR), which are beneficial microorganisms found in close symbiosis with plant roots. In this study, the efficient PGPR strain T1 was isolated and screened from tobacco inter-root soil, and its identity was confirmed through ITS sequencing technology. The soil's physical and chemical characteristics showed significant enhancement, with phosphorus availability rising by 26.26%. Additionally, there was a marked increase in the activity of essential soil enzymes like sucrase, catalase, and urease, reflecting improved soil health and fertility. This research offers valuable insights for the creation of innovative microbial fertilizers and presents strategies for the sustainable advancement of modern agriculture. The study of [Zhu et al.](#) revealed that bacterial wilt, caused by *Ralstonia solanacearum*, significantly hinders the healthy development of tomato seedlings. This study explored the use of biocontrol microbes to combat tomato bacterial wilt, focusing on the behavior of the *Enterobacter hormaechei* Rs-5 and *Bacillus subtilis* SL-44 composite microbial agent (EB) in the rhizosphere soil. The study also evaluated its effects on the soil's microbial community and the growth of tomato plants. EB was found to lower the incidence of tomato bacterial wilt from 77.78 to 22.22% and markedly enhance the biomass, physicochemical properties, and nutrient content of the rhizosphere soil in tomato seedlings. Additionally, there was an increase in the relative abundance of beneficial bacteria such as *Massilia*, *Pseudomonas*, *Bacillus*, and *Enterobacter*, along with an improvement in the diversity of the fungal community. Similarly, [Manzar et al.](#) examined 260 isolates of *Trichoderma* species, identifying the primary ones as *Trichoderma koningiopsis*, *T. asperellum*, *T. caribbaeum* var. *caribbaeum*, *T. lixii*, *T. brevicompactum*, *T. atroviride*, and *T. erinaceum*. Among these, 9% demonstrated significant potential for biocontrol and enhancing crop growth. The use of the effective *Trichoderma* strain TR11 for seed biopriming led to a reduction in the maydis leaf blight (MLB) disease index to 32.92 % and improved growth-promoting characteristics in maize. Additionally, treatments with the TR11 isolate resulted in a 2.5 to 4.2-fold increase in defensive enzyme activities and greater lignification following pathogen inoculation, suggesting bolstered plant defense responses. According to [Manjunatha et al.](#), bacterial blight in pomegranates, caused by *Xanthomonas citri* pv. *punicae* (Xcp), is a highly destructive disease that results in significant financial losses in pomegranate farming. The use of endophytes such as *Bacillus haynesii*, *B. tequilensis*, and *B. subtilis* for controlling this blight led to a 47–68% decrease in the disease index, which is notably more effective than the reduction achieved by the chemical immune modulator (2-bromo-2-nitro-1, 3-propanediol) that is currently recommended for managing the blight. Research conducted by [Hima Parvathy et al.](#) indicates that black pepper (*Piper nigrum* L.) is susceptible to foot rot caused by the soil-borne oomycete pathogen *Phytophthora capsici*. The investigation highlighted compositional variations in the rhizobiome of two *Piper* species, with *P. colubrinum* exhibiting greater diversity and a higher number of differentially abundant genera. Predictive functional profiling of the *P. colubrinum* rhizobiome identified a significant enrichment of functional gene orthologs (KOs), notably chemotaxis proteins, osmoprotectants, and various transport systems that contribute to pathogen resistance. A study by [Tomer](#)

[et al.](#) examined the phosphate solubilizing abilities of *Lysinibacillus macroides* ST-30, *Pseudomonas pelleroniana* N-26, and *Bacillus cereus* ST-6 in relation to chickpea cultivation in the Tarai region of Uttarakhand. The consistent presence of these inoculated P solubilizers throughout the experiment indicates their capability to compete with native microflora and maintain a good shelf life under field conditions, supporting their potential use as commercial fertilizers in the future. [Lee et al.](#) emphasized the role of *Bacillus megaterium* strains CACC109 and CACC119, isolated from a ginseng field, in enhancing drought stress tolerance through plant growth-promoting activities. They explored these mechanisms by assessing the strains' impact on rice growth and stress resilience using *in vitro* assays, pot experiments, and physiological and molecular analyses. The application of CACC109 and CACC119 led to increased expression of genes related to antioxidants and drought response. These strains show promise as biostimulants for boosting plant growth and providing resistance to abiotic stresses in crop production. [Bao et al.](#) found that an increase in soil Actinobacteria and Firmicutes, or a decrease in Gemmatimonadetes and Myxococcota, could create a favorable environment for the sustainable growth of medicinal plant crops in salinized soil ecosystems. [Lin et al.](#) discovered that dazomet, a soil fumigant, effectively controls soil-borne pathogens and boosts levels of total N, P, K, available ammonia nitrogen, P and K in the soil. Its fumigation also increased the relative-abundance of bacteria involved in the biosynthesis of secondary metabolites, while decreasing the relative-abundance of pathogenic fungi and reducing the incidence of soil-borne diseases. [Choudaker et al.](#) highlighted the success of using microbial antagonists, especially the *B. subtilis* DTBS-5, in controlling wheat PM through biocontrol, induced resistance, and improved plant growth, presenting a sustainable alternative to chemical methods. [Garg et al.](#) discussed that plant biostimulants comprise biomolecules like lipids, carbohydrates, proteins, and other secondary metabolites associated with specific nitrogen-containing compounds, terpenes, and benzene ring-conjugated compounds. These secondary metabolites, being crucial precursors, require extensive study for precise calculations of biochemical reactions occurring inside and outside the synthesized living cell. This review underscores sequencing techniques as a basis for generating opportunities in agricultural sustainability. [Zeng et al.](#) study demonstrated a notable rise in the presence of beneficial bacteria in the rhizosphere soil of *Achyranthes bidentata* when subjected to extended monoculture, as indicated by bioinformatics analysis. The functional analysis revealed a variety of plant growth-promoting characteristics among these bacteria, such as the production of indole-3-acetic acid in the range of 68.010–73.250 mg/L, abilities to solubilize P and K, and antagonistic effects against pathogenic fungi (21.540–50.810%). [Malik et al.](#) investigated the effects of biosynthesized silver nanoparticles (AgNPs) derived from *Rhizoctonia solani* and *Cladosporium cladosporioides* through a green synthesis method, assessing their antifungal activity against various pathogenic fungi. A concentration of 15 mg/mL of these AgNPs exhibited strong inhibitory effects on all tested fungal pathogens. Consequently, the findings strongly indicate that silver nanoparticles could play a significant role in managing different plant diseases caused by fungi.

In conclusion, we believe that this Research Topic on “Microbial-mediated induced resistance: interactive effects for improving crop health” will provide significant insights into the recent advancements and benefits of using PGPR and biostimulants to achieve sustainable agricultural production. Additionally, it will highlight the role of microbial inoculants in enhancing crop yields while maintaining soil health.

Author contributions

DM: Conceptualization, Project administration, Supervision, Writing – review & editing, Investigation, Writing – original draft. AR: Writing – original draft, Project administration, Conceptualization, Writing – review & editing. EJ: Project administration, Writing – original draft, Writing – review & editing, Investigation. BK: Project administration, Writing – review & editing, Conceptualization, Supervision, Writing – original draft.

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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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Fungal-mediated synthesis of silver nanoparticles: a novel strategy for plant disease management

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Various traditional management techniques are employed to control plant diseases caused by bacteria and fungi. However, due to their drawbacks and adverse environmental effects, there is a shift toward employing more eco-friendly methods that are less harmful to the environment and human health. The main aim of the study was to biosynthesize silver Nanoparticles (AgNPs) from *Rhizoctonia solani* and *Cladosporium cladosporioides* using a green approach and to test the antimycotic activity of these biosynthesized AgNPs against a variety of pathogenic fungi. The characterization of samples was done by using UV–visible spectroscopy, SEM (scanning electron microscopy), FTIR (fourier transmission infrared spectroscopy), and XRD (X-ray diffractometry). During the study, the presence of strong plasmon absorbance bands at 420 and 450 nm confirmed the AgNPs biosynthesis by the fungi *Rhizoctonia solani* and *Cladosporium cladosporioides*. The biosynthesized AgNPs were 80–100 nm in size, asymmetrical in shape and became spherical to sub-spherical when aggregated. Assessment of the antifungal activity of the silver nanoparticles against various plant pathogenic fungi was carried out by agar well diffusion assay. Different concentration of AgNPs, 5 mg/mL 10 mg/mL and 15 mg/mL were tested to know the inhibitory effect of fungal plant pathogens viz. *Aspergillus flavus*, *Penicillium citrinum*, *Fusarium oxysporum*, *Fusarium metavorans*, and *Aspergillus aflatoxiformans*. However, 15 mg/mL concentration of the AgNPs showed excellent inhibitory activity against all tested fungal pathogens. Thus, the obtained results clearly suggest that silver nanoparticles may have important applications in controlling various plant diseases caused by fungi.

KEYWORDS

antimycotic, antifungal, *Cladosporium cladosporioides*, *Rhizoctonia solani*, silver nanoparticles

Introduction

Nanotechnology exploration has recently sparked significant interest within the domain of material sciences. It stands out as one of the latest breakthroughs imbued with novelty and quintessentially addresses the emerging challenges our world faces. In the pharmaceutical and biomedical industries, nanoparticles exhibit a diverse range of applications, including gene

and drug delivery systems, water disinfection, electronics, biosensors, as well as serving as agents for anticancer, antibacterial, antifungal, and antiprotozoal purposes (Lee and Jun, 2019; Rai et al., 2021; War et al., 2022). In recent years, there has been significant research interest in the biosynthesis and characterization of nanoparticles. This interest is primarily driven by their large surface area, which imparts unique properties and potential applications distinct from their bulk counterparts (Shnoudeh et al., 2019; Sajid and Plotka-Wasyłka, 2020). Up to now, nanoparticles have been synthesized using a variety of chemical and physical techniques. However, biosynthesis approach utilizing biological systems such as plants, fungi, yeast, and bacteria has been explored and adopted globally for nanoparticle biosynthesis due to its environmentally friendly, reproducible, non-toxic, and cost-effective nature (Gudikandula et al., 2017; Hano and Abbasi, 2021). The biosynthesis approach offers many advantages over chemical synthesis (Duan et al., 2015; Roy et al., 2019; Murali et al., 2023). Since biological systems operate as natural reducing, stabilizing, and capping agents, thereby avoiding many processes for the synthesis of nanoparticles, which not only decreases the cost and consumption of chemicals, but also eliminates agglomeration and oxidation of synthesized nanoparticles (Sidhu et al., 2022). Metallic nanoparticles, especially silver nanoparticles (AgNPs) produced through biological sources, have undergone intensive investigation as a potential alternative therapy for a wide range of infections and illnesses (Bhuyan et al., 2017; Al-Ansari et al., 2020; Murali et al., 2021; Nandi et al., 2023).

Silver is widely known to combat certain microorganisms by modifying their cell membrane structure and function (Dakal et al., 2016; Mikhailova, 2020; Das and Dutta, 2021). Silver is employed as a disinfectant in water purification systems due to its ability to destroy bacteria at low concentrations (less than 1–10 m) (Liu et al., 1994; Deshmukh et al., 2019; Bhardwaj et al., 2021; Das and Dutta, 2022). However, silver can be hazardous to animals, freshwater, and marine organisms at greater quantities (Tortella et al., 2020). Interestingly, micromolar quantities of silver are not toxic to humans (Vrček et al., 2016). Therefore, silver has been widely used in the production of various biological and medicinal products. Recent studies revealed that the toxicity of Ag-NPs measured in freshwater depends on the test species (Blinova et al., 2013). For example, Ag-NPs are reported to be toxic for crustaceans at very low concentration ($EC_{50} < 0.1 \text{ mg L}^{-1}$), followed by algae ($EC_{50} = 0.23 \text{ mg L}^{-1}$), but the toxicity to fish is relatively low ($EC_{50} = 7.1 \text{ mg L}^{-1}$, Kahru and Dubourguier, 2010; Asghari et al., 2012). It has been observed that effects of AgNP on the T84 epithelial cells were size- and dose-dependent, with the 10 nm AgNP causing the most significant changes. Changes in permeability of the epithelial cell monolayer, as measured by transepithelial electrical resistance, after exposure to 10 nm AgNP were most dramatic at the highest dose (100 $\mu\text{g/mL}$), but also observed at the lower dose (20 $\mu\text{g/mL}$) (Williams et al., 2016).

Presently, AgNPs are widely utilized across a broad spectrum of applications, ranging from electronic devices to biological tools (Garg et al., 2020; Tufail and Liaqat, 2021; Naganthran et al., 2022). This is likely due to the stability of the particles, which holds significant importance for various applications, particularly in medicine. Additionally, it is crucial that nanoparticles do not agglomerate during their formation, to achieve enhanced stability, maximal yield, and controlled size aggregation of particles, optimization of the

various parameters employed in plant-mediated nanoparticle biosynthesis is essential (Ebrahimezhad et al., 2018; Ahmad et al., 2024). Recent reports have demonstrated the broad-spectrum antibacterial activity of AgNPs against both Gram-positive and Gram-negative bacteria, including multidrug-resistant strains (Liao et al., 2019; Ansari et al., 2021; Yassin et al., 2022). It is significant that AgNPs exhibit multiple modes of inhibitory action against microorganisms, as opposed to the single specific action of antibiotics (Vazquez-Muñoz et al., 2019). Interestingly, AgNPs demonstrate efficacy against various fungi, including *Candida* spp., dermatophytes, and certain phytopathogenic fungi such as *Bipolaris sorokiniana* and *Magnaporthe grisea* (Rajeshkumar, 2019; Mansoor et al., 2021). Conversely, various phytopathogenic fungi remain unexplored, despite their role in causing severe diseases in crucial crop plants, consequently diminishing agricultural yield. Therefore, the present study aimed to address the following questions: (a) to study the preparation and characterization of silver nanoparticles. (b) To study the efficacy of various concentrations of biosynthesized silver nanoparticles against fungal pathogens.

Materials and methods

Fungal culture

In this study, *Rhizoctonia solani* and *Cladosporium cladosporioides* were isolated from soil samples. Pure cultures of these fungi were cultivated on Potato Dextrose Agar and identified based on cultural, morphological, and microscopic characteristics as described by Trappe (1982), Quimio (2001), Boerema et al. (2004).

Preparation of silver nanoparticles

To biosynthesize AgNPs the fungi *Rhizoctonia solani* and *Cladosporium cladosporioides* were cultured separately in 250 mL conical flasks, each containing 100 mL of potato dextrose broth. The flasks were then incubated at $26 \pm 2^\circ\text{C}$ for 72–96 h. Then mycelial mat separation was performed using Whatman filter paper. The medium components were eliminated from the biomass by washing with double-distilled water 3–4 times. Approximately 25 g of fresh biomass was placed in 250 mL conical flasks containing 200 mL of double-distilled water and left for 24–72 h at 25°C . Subsequently, 50 mL of cell filtrate was combined with 50 mL of AgNO_3 silver nitrate solution. Solution and the reaction mixture was kept in orbital shaker at 37°C and 200 rpm for 24 h. The conversion of Ag ions to (AgNPs) was confirmed by color change from yellow to brown. A reaction mixture without AgNO_3 served as a control and was kept alongside the experimental flasks (Sagar and Ashok, 2012; Talie et al., 2020) (Figures 1A–F).

Characterization of silver nanoparticles

Different methods were employed to characterize the biosynthesized AgNPs.



FIGURE 1

(A–F) Cell filtrate of *Rhizoctonia solani* and *Cladosporium cladosporioides* before and after the addition of AgNO_3 solution.

Color change

The reduction of silver ions was routinely monitored visually over a period 24 h. The key presence of a brown color in the reaction mixture is an indicator of the formation of silver nanoparticles. The color change is caused by the activation of surface plasmon vibrations.

UV–visible spectroscopy analysis

Ultraviolet (UV) spectroscopy confirms the formation of silver nanoparticles by reducing silver nitrate. The UV–Vis spectra of the cell filtrate was recorded after 24 h on a UV–Visible absorption spectrophotometer (UV–visible Spectrophotometer 119, SYSTRONICS) with a resolution of 2.0 nm between wavelengths of 350–700 nm possessing a scanning speed of 300 nm/min.

Scanning electron microscopy (SEM)

Scanning electron microscopic analysis was used to measure the size and shape of silver nanoparticles. For SEM, (AgNPs) were biosynthesized using cell filtrate, normally required to be completely dry and the specimen was dried and grounded to a powder.

Fourier transmission infrared spectroscopy (FTIR)

The suspension of AgNPs biosynthesized using *Rhizoctonia solani* and *Cladosporium cladosporioides* was centrifuged at 10,000 rpm for 20 min at room temperature. The resulting residue was washed several

times with sterile distilled water, dried in 40°C and finally the AgNPs were stored in vials. The collected powdered AgNPs were then taken for FTIR analysis in the range of $450\text{--}4,500\text{ cm}^{-1}$.

X-ray diffractometry (XRD)

The AgNPs solution obtained after bio-reduction was purified by centrifugation at 10,000 rpm for 20 min, followed by redispersion of the AgNPs pellet into 1 mL of sterile de-ionized water. X-ray diffraction (XRD) was used to examine the structure and content of the purified AgNPs after they were freeze dried. The dried mixture of AgNPs was collected for X-ray diffractometer analysis of AgNPs production. The colloidal suspensions of AgNPs were analyzed by XRD to validate their crystalline nature in order to verify the results of the UV spectral analyses.

Antifungal efficacy of biosynthesized silver nanoparticles against some selected fungal pathogens

Effect of biosynthesized AgNPs on the mycelial growth of some selected soil pathogenic fungi was analyzed. The agar well diffusion experiment was performed to investigate the antifungal activity of biosynthesized AgNPs against fungal pathogens such as *Aspergillus flavus*, *Penicillium citrinum*, *Fusarium oxysporum*, *Fusarium metavorans*, and *Aspergillus aflatoxiformans*. An aliquot of 0.02 mL of

inoculum of each test fungal pathogen was injected into culture tubes containing 20 mL of molten Sabouraud dextrose agar medium. The culture tubes were homogenized and then emptied into petri plates and allowed to harden under laminar airflow chamber (aseptic conditions). A 5 mm conventional cork borer was used to make wells on the agar plate. Three different concentrations *viz.* 05, 10, and 15 mg/mL of AgNPs were prepared and 50 μ L from each concentration was added to respective wells. Nystatin 50 μ L/disc was utilized (positive control) as a control. The effect of AgNPs on the test fungal infections was analyzed and compared to the reference standard. The antifungal activity was determined using the standard scale of [Norrel and Messley \(1997\)](#).

Results

Plant pathogens such as bacteria and fungi are controlled by different traditional management strategies. However, some of these management measures, such as the use of pesticides, have negative environmental consequences due to their limits. As a result, alternative ways are used that are more environmentally friendly and have fewer negative consequences on human health and the environment. As a result, the antimycotic activity of various AgNPs was tested against fungal pathogens in this work. These AgNPs were prepared by simple technique and used at different concentrations against pathogenic fungi. AgNPs were biosynthesized using *Rhizoctonia solani* and *Cladosporium cladosporioides*. After preparation of these AgNPs they were processed to prepare different concentrations. These different concentrations of AgNPs were screened for their antifungal activity against fungal pathogens.

Characterization of biosynthesized silver nanoparticles using fungi

Different techniques employed for the characterization of biosynthesized AgNPs are given below:

Color change

The Initial indication of AgNPs biosynthesis was confirmed by the color change. The color of *Rhizoctonia solani* and *Cladosporium cladosporioides* fungal filtrates changes from colorless to brown, as seen in the results ([Figures 1A–F](#)). Change in color of the cell free filtrate incubated with silver nitrate solution was visually exhibited after 24 h of incubation which clearly indicates the AgNPs formation.

UV–visible spectroscopy analysis

The reduction of silver nitrate to AgNPs was confirmed by ultraviolet (UV) Spectroscopy. The UV–Visible spectra of *Rhizoctonia solani* and *Cladosporium cladosporioides* cell filtrates showed strong plasmon absorption bands at 425 and 450 nm ([Figures 2A,B](#)).

Scanning electron microscopy (SEM)

The mean particle size and shape of biosynthesized AgNPs were studied using scanning electron microscopy. SEM pictures of silver nanoparticles synthesized from *Rhizoctonia solani* and *Cladosporium cladosporioides* are shown in [Figures 3A,B](#). The aggregated shape of

biosynthesized AgNPs was found to be irregular and spherical. AgNPs range in size from 80 to 100 nanometers.

Fourier transmission infrared spectroscopy (FTIR)

The FTIR spectroscopy was used to examine biosynthesized AgNPs in the range 450–4,500 cm^{-1} . AgNPs made from *Rhizoctonia solani* were found to absorb substantially at various wavelengths (3250.30, 2915.01, 2015.3, 1989.97, 1634.85, 1539.08, 1382.14, 1245.14, and 1047.63 cm^{-1}) respectively. While the absorption bands 2915.01 are related to the -OH of carboxylic acid, the absorption band 3250.30 cm^{-1} is related to the N-H amine stretch. Similar to this, the absorption bands at 1382.14 and 1634.85 cm^{-1} are related to the C-N stretching vibrations of aromatic amines and the unsaturated nitrogen molecules O-NO₂ and nitrate, respectively. Furthermore, the infrared (IR) spectra show bands that reveal the existence of O-H carboxylic acid, N-H amine linkages, C-N aromatic amine linkages, and O-NO₂ unsaturated nitrogen compounds, which may be present in AgNPs as stabilizing caps alongside proteins and amino acid residues. Likewise, the results revealed that biosynthesized AgNPs from fungus *Cladosporium cladosporioides* absorb strongly 3221.4, 2987.03, 2105.4, 1997.8, 1564.12, 1374.10, 1230.0, and 864.5 cm^{-1} , respectively, ([Figures 4A,B](#)).

XRD analysis

During the present study, the crystal structure and particle size of the biosynthesized AgNPs were determined using X-ray diffraction. [Figures 5A,B](#) represents the XRD pattern of AgNPs and biosynthesized AgNPs showed excellent crystal quality. Reference data from ICSD (inorganic crystal structure database) and ICDD (international center for diffraction data) were used and matched with data obtained during the present study using PDXL-2 software. Peaks were seen over the whole spectrum, which ranged from 20 to 90 nm demonstrating the achievement of great purity. The XRD (X-ray diffraction) spectrum demonstrated that the biosynthesized AgNPs were in the form of nano crystals when compared to the reference data.

Antifungal activity of biosynthesized silver nanoparticles on the mycelial growth of some pathogenic fungi employing agar well diffusion method

The findings revealed that AgNPs derived from *Rhizoctonia solani* and *Cladosporium cladosporioides* at various concentrations (05, 10, and 15 mg/mL) inhibited all of the fungal pathogens such as *Aspergillus flavus*, *Penicillium citrinum*, *Fusarium oxysporum*, *Fusarium metavorans*, and *Aspergillus aflatoxiformans*. However, zone of inhibition increased with the increase in concentrations of AgNPs. Furthermore, the lowest concentrations of biosynthesized AgNPs reduce the zone of inhibition against all of the tested fungal strains significantly ([Table 1](#)).

Antifungal efficacy of various concentrations of biosynthesized silver nanoparticles using fungus, *Rhizoctonia solani* on the zone of mycelial growth inhibition of some pathogenic fungi

The results ([Table 2](#) and [Figures 6, 7A–E](#)) revealed that there was found inhibition in all the tested pathogenic fungi at all the concentrations of AgNPs biosynthesized by *Rhizoctonia solani*.

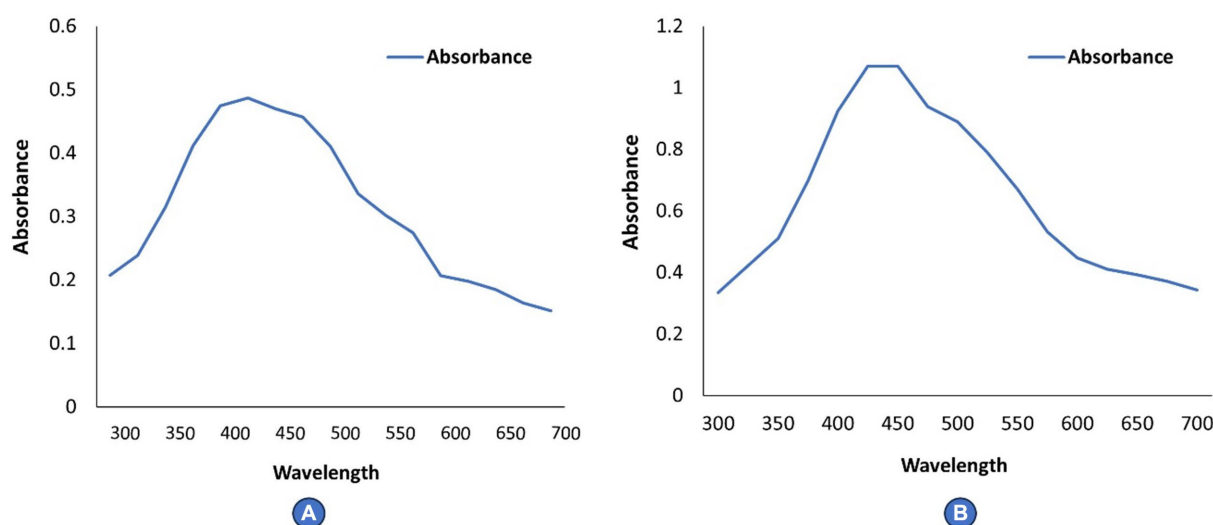


FIGURE 2
(A,B) Depicts UV-Vis spectra of AgNPs.

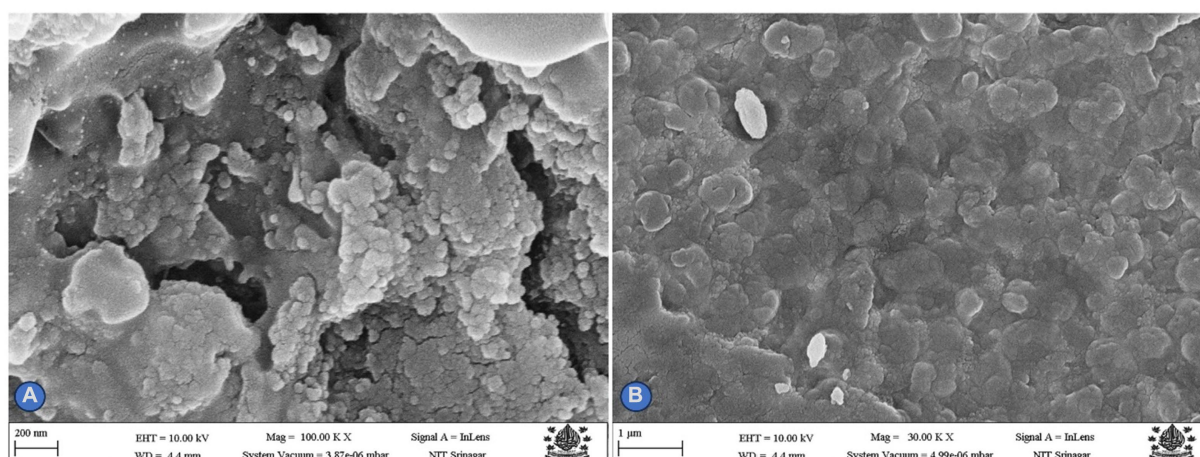


FIGURE 3
(A,B) Depicts SEM micrograph of AgNPs.

However, the maximum zone of inhibition against *Fusarium metavorans* (24.33 ± 0.57) was found at highest concentrations of biosynthesized AgNPs. It was followed by inhibition in mycelial growth of *Aspergillus flavus* (21.00 ± 1.00), *Penicillium citrinum* (17.00 ± 1.00), *Aspergillus aflatoxiformans* (17.00 ± 1.00), and *Fusarium oxysporum* (13.33 ± 0.57) at the same concentrations, respectively. The zone of inhibition in mycelial growth against *Aspergillus flavus* varied from 15.00 to 21.00 mm, and in case of *Penicillium citrinum* varied from 8.00 to 17.00 mm, respectively at different concentrations of AgNPs. Similarly, in case of *Fusarium oxysporum*, the zone of inhibition in mycelial growth ranges from 8.33 to 13.33 mm, for *Fusarium metavorans*, from 15.00 to 24.33 mm, and for *Aspergillus aflatoxiformans*, from 7.66 to 17.00 mm, respectively. The zone of inhibition against all the other tested fungi decreased considerably at the lowest concentrations of biosynthesized AgNPs but to lower extent.

Antifungal efficacy of various concentrations of silver nanoparticles biosynthesized using fungus, *Cladosporium cladosporioides* on the zone of mycelial growth inhibition of some pathogenic fungi

The results (Table 3 and Figures 7F–J, 8) revealed that the AgNPs biosynthesized from *Cladosporium cladosporioides* significantly inhibited development mycelia tested fungi. However, the maximum zone of inhibition against *Aspergillus flavus* (27.00 ± 1.00) was found at the highest concentrations of AgNPs. The highest concentration of AgNPs also caused maximum reduction in the mycelial growth in case of *Fusarium metavorans* (22.00 ± 1.00), *Aspergillus aflatoxiformans* (17.00 ± 1.00), *Penicillium citrinum* (16.00 ± 1.00), and *Fusarium oxysporum* (14.00 ± 1.00) respectively. The zone of inhibition in mycelial growth against *Aspergillus flavus* varied from 21.00 to 27.00 mm, and for *Penicillium citrinum* varied from 7.00 to 16.00 mm,

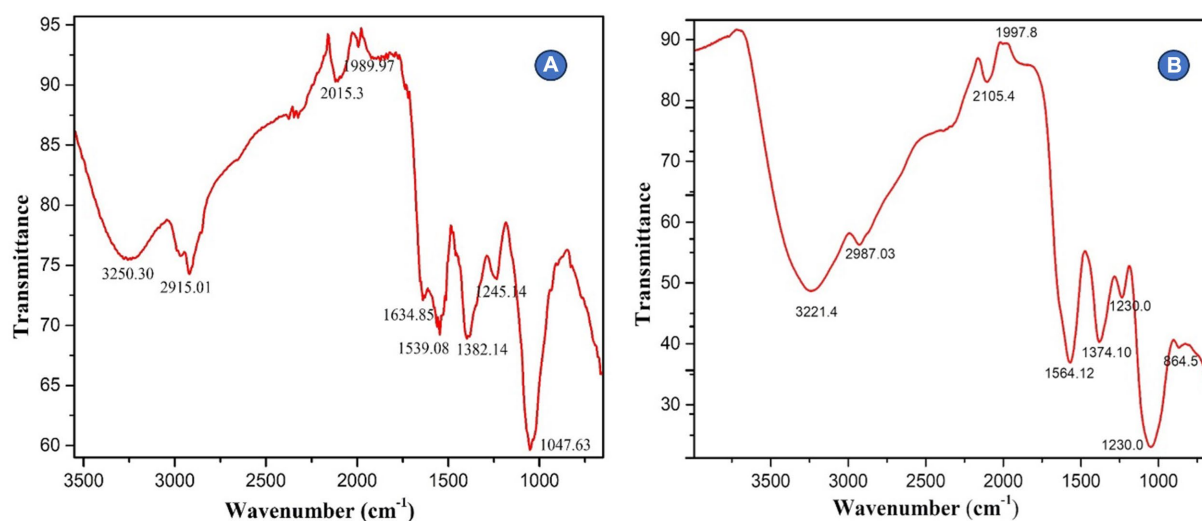


FIGURE 4
(A,B) Depicts FTIR spectroscopy of silver nanoparticles.

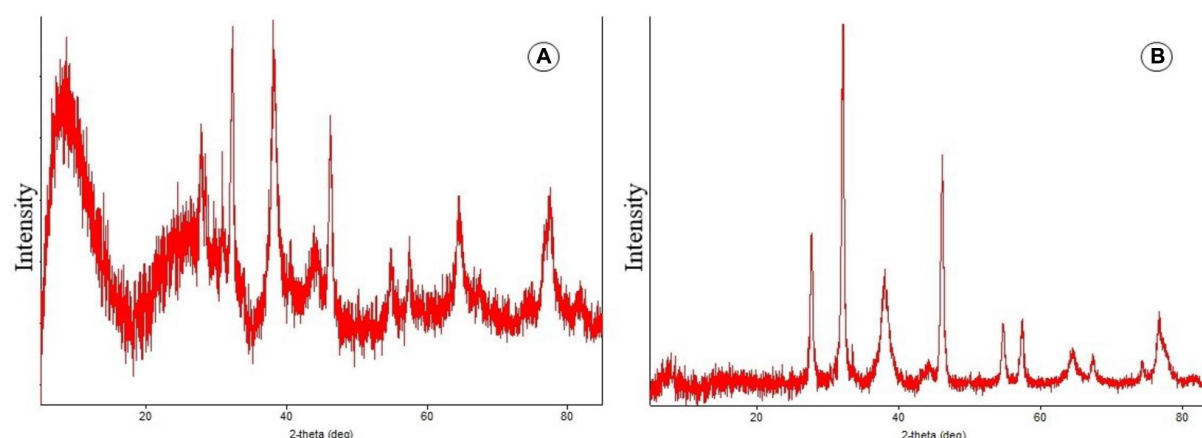


FIGURE 5
(A,B) Depicts X-ray diffraction pattern of AgNPs.

respectively, at different concentrations of AgNPs. Similarly, in case of *Fusarium oxysporum*, the zone of inhibition in mycelial growth ranges from 10.00 to 14.00 mm, in case of *Fusarium metavorans* it varies from 14.00 to 22.00 mm, and for *Aspergillus aflatoxiformans* varied 12.00 to 17.00 mm, respectively. The zone of inhibition against all the other tested fungi also decreases considerably at the lowest concentrations of produced AgNPs.

Discussion

Nano-biotechnology is quickly developing as an important field of modern research, generating the most promising applications in medicine and agriculture in the present climate change scenario (Mariyam et al., 2023). The application of nano-biotechnology in agriculture will help in addressing and solving inherent imperfections and other complex problems in farm production with low input but

with an efficient role due to their unique size (Dimkpa and Bindraban, 2017; Usman et al., 2020). Biosynthesis of AgNPs using green approach provide ecofriendly, clean and effective way out for the biosynthesis of nanoparticles. These nanoparticles differ in shape, size, chemical composition and other properties (Raj et al., 2021).

During the present study, *Rhizoctonia solani* and *Cladosporium cladosporioides* were used to biosynthesize AgNPs, which was proved by the appearance of brown color due to reduction of silver salt into AgNPs by fungal culture filtrates along with the appearance of strong plasmon absorbance bands at 420–450 nm and strong resonance peaks at 440 nm, thus confirming the biosynthesis of AgNPs as has been reported by Khan et al. (2018) and Paul et al. (2023). Many researchers have reported that microorganisms, plant extracts, and fungi can be used to biosynthesize nanoparticles through biological pathways (Koul et al., 2021). Many fungi like *Fusarium oxysporum*, *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium semitectum*, *Penicillium brevicompactum*, and *Cladosporium cladosporioides* have been reported

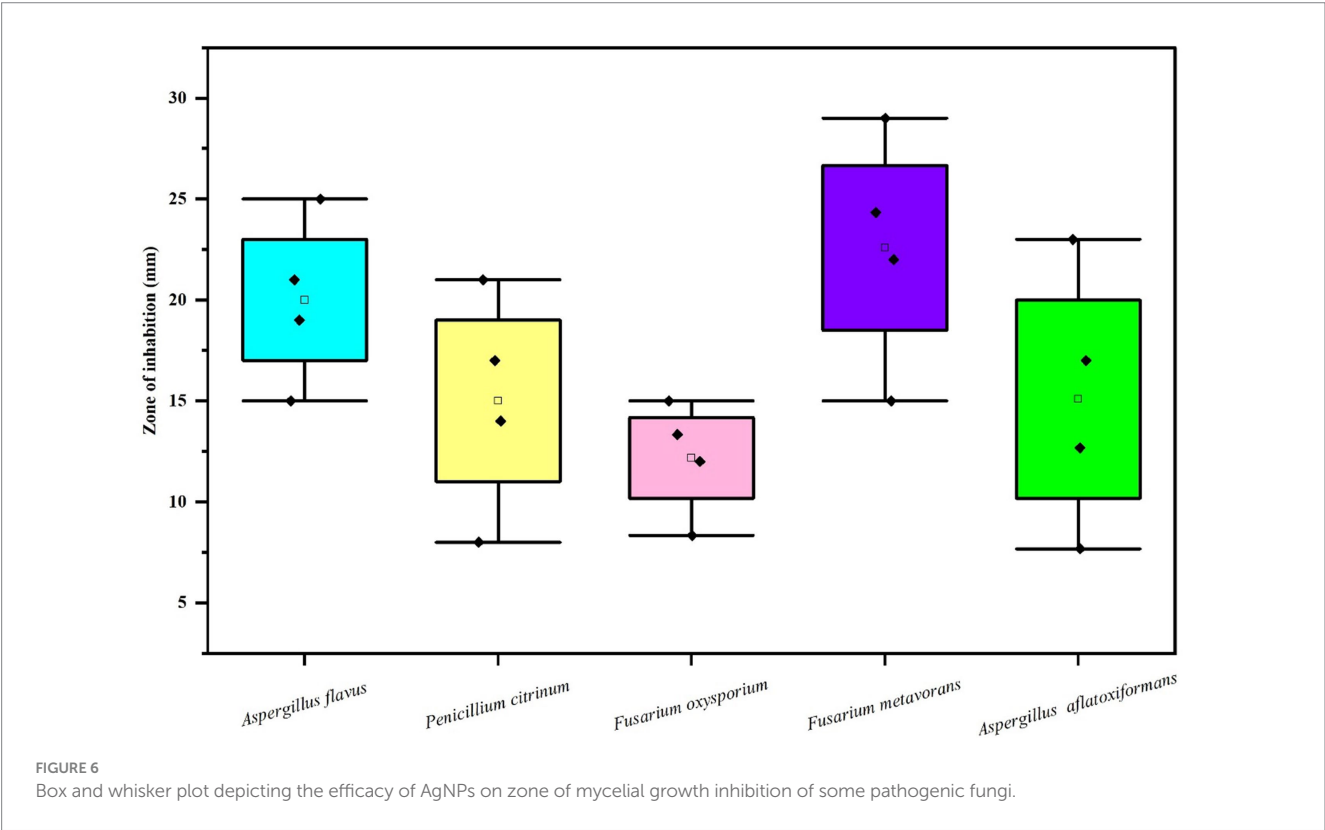
TABLE 1 Geographical location of the sampling sites.

Location	Altitude (asl)	Latitude	Longitude
Gulmarg	2,650 m	34°03'14" N	74°23'88" E
Doodhpathri	2,850 m	33°50'67" N	74°35'15" E
Drang	2,300 m	34°03'32"N	74°25'57"E
Kashmir University Botanical Garden (KUBG)	1,591 m	34°09'66" N	74°50'77"E

TABLE 2 Efficacy of various concentrations of biosynthesized silver nanoparticles on the zone of mycelial growth inhibition of some pathogenic fungi.

Concentration	Zone of inhibition (mm)			
Fungal pathogens	5 mg/mL	10 mg/mL	15 mg/mL	Standard
<i>Aspergillus flavus</i>	15.00 ± 1.00 ^a	19.00 ± 1.00 ^b	21.00 ± 1.00 ^c	25.00 ± 1.00 ^d
<i>Penicillium citrinum</i>	8.00 ± 1.00 ^a	14.00 ± 1.00 ^b	17.00 ± 1.00 ^c	21.00 ± 1.00 ^d
<i>Fusarium oxysporum</i>	8.33 ± 1.52 ^a	12.00 ± 1.00 ^b	13.33 ± 0.57 ^{bc}	15.00 ± 1.00 ^c
<i>Fusarium metavorans</i>	15.00 ± 1.00 ^a	22.00 ± 1.00 ^b	24.33 ± 0.57 ^c	29.00 ± 1.00 ^d
<i>Aspergillus aflatoxiformans</i>	7.66 ± 0.57 ^a	12.66 ± 0.57 ^b	17.00 ± 1.00 ^c	23.00 ± 1.00 ^d

Values are represented as the mean ± SD of three replicates. Duncan's multiple comparison test was used to compare mean values. The numbers that are followed by the similar alphabets do not differ statistically ($p \leq 0.05$).



to be competent enough to extracellularly biosynthesized AgNPs (Rajeshkumar and Sivapriya, 2020; Adebayo et al., 2021) respectively. Our results are in conformity with Verma et al. (2010) and Parveen et al. (2018), who also reported similar results for biosynthesis AgNPs and iron oxide nanoparticles from fungi, respectively. Fungi have many advantages for the production and biosynthesis of nanoparticles in comparison to other types of microorganisms and phyto extracts. This is because the mycelial mesh of fungi is easy to handle and withstands high flow pressure, agitation and many other conditions in bioreactors and other chambers. Scanning electron microscopy revealed that biosynthesized AgNPs were irregular and spherical in aggregate form, with a size ranging from 80 to 100 nm. Similar patterns of biosynthesized AgNPs were observed by Kathiresan et al. (2009) and Jain et al. (2011). Our findings are in accordance with the work of

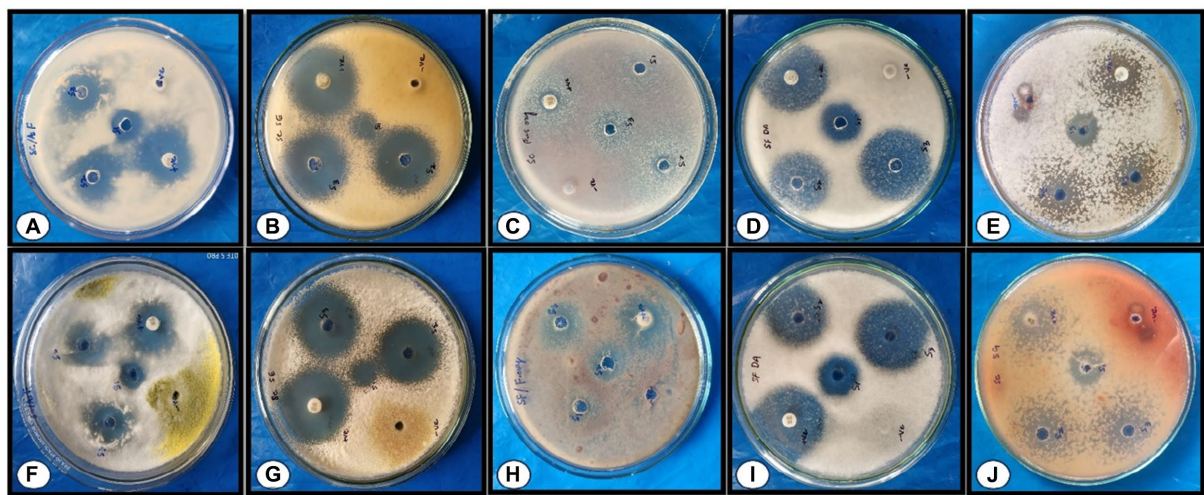


FIGURE 7
Effect of different concentrations of AgNPs on the zone of mycelial growth inhibition of some pathogens. (A–E) depicts the effects of AgNPs biosynthesized from *Rhizoctonia solani* (A) *Aspergillus flavus*, (B) *Penicillium citrinum*, (C) *Fusarium oxysporum*, (D) *Fusarium metavorans*, and (E) *Aspergillus aflatoxiformans*; (F–J) depicts the effect of AgNPs biosynthesized from *Cladosporium cladosporioides* (F) *Aspergillus flavus*, (G) *Penicillium citrinum*, (H) *Fusarium oxysporum*, (I) *Fusarium metavorans*, and (J) *Aspergillus aflatoxiformans*.

TABLE 3 Efficacy of various concentrations of biosynthesized silver nanoparticles on the zone of mycelial growth inhibition of some pathogenic fungi.

Concentration	Zone of inhibition (mm)			
Fungal pathogens	5 mg/mL	10 mg/mL	15 mg/mL	Standard
<i>Aspergillus flavus</i>	21.00 ± 1.00 ^a	25.00 ± 1.00 ^b	27.00 ± 1.00 ^b	31.66 ± 1.52 ^c
<i>Penicillium citrinum</i>	7.00 ± 1.00 ^a	13.00 ± 1.00 ^b	16.00 ± 1.00 ^c	21.00 ± 1.52 ^d
<i>Fusarium oxysporum</i>	10.00 ± 1.00 ^a	12.66 ± 0.57 ^b	14.00 ± 1.00 ^b	16.00 ± 1.00 ^c
<i>Fusarium metavorans</i>	14.00 ± 1.00 ^a	21.00 ± 1.00 ^b	22.00 ± 1.00 ^b	28.33 ± 1.57 ^c
<i>Aspergillus aflatoxiformans</i>	12.00 ± 1.00 ^a	14.00 ± 1.00 ^b	17.00 ± 1.00 ^c	22.00 ± 1.00 ^d

*Values are represented as the mean ± SD of three replicates. Duncan's multiple comparison test was used to compare mean values. The numbers that are followed by the similar alphabets do not differ statistically ($P \leq 0.05$).

Al-Zubaidi et al. (2019) and Talie et al. (2020) who also used different microfungi and macrofungi for biosynthesis of AgNPs. It seems that the pattern aggregation and formation of mycosynthesized silver nanoparticles take place due to the enzymatic reduction of silver metal ions (Rajput et al., 2016). The results from the present study with regard to the bioactivity of biosynthesized AgNPs against phytopathogenic fungi revealed that at different concentrations, biosynthesized AgNPs caused a significant reduction in the fungal mycelial growth in terms of zone of inhibition against all the test fungal pathogens such as *Rhizoctonia solani* and *Cladosporium cladosporioides* indicating their strong antimycotic activity. Similar work was carried out by Abd-Elsalam et al. (2019), Ingle et al. (2020), Padhi and Behera (2021), Sulaiman et al. (2015) and reported antimycotic activity of bi-synthesized AgNPs against fungi, namely *Aspergillus niger*, *Penicillium chrysogenum*, *Fusarium culmorum*, and *Alternaria alternata*. Talie et al. (2020) also reported the potent antifungal activity of biosynthesized AgNPs using *Helvella leucopus* against *Aspergillus niger*, *Penicillium chrysogenum*, *Alternaria alternata* which is in conformity with our work. The results emphasize that these biosynthesized AgNPs will work best as nano-biopesticides, as has been reported by Paramo et al. (2020) and can be incorporated into the integrated disease management module. Antimycotic activity of AgNPs against different species of phytopathogenic fungi of some cereals was

reported by Al-Askar et al. (2013) and *Candida* species by Ishida et al. (2013). Since phytopathogenic fungi are toxic due to the production of mycotoxins which can be easily adsorbed by nanoparticles and impart protection against disease as has been reported by Horky et al. (2018).

Conclusion

This study focused on the biosynthesis of AgNPs from aqueous extract of the fungi, *Rhizoctonia solani* and *Cladosporium cladosporioides*. The biogenic method provides natural agents for reduction, capping and stabilization of AgNPs, which makes the synthesis approach much more cost-effective, non-toxic, reproducible and environmentally friendly, implying that fungi could be a good source of silver nanoparticles. Biosynthesized silver nanoparticles from *Rhizoctonia solani* and *Cladosporium cladosporioides* were crystallite in nature, with an average particle size of 10.100 nm. The study revealed strong antifungal properties of these synthesized AgNPs. A significant zone of mycelial growth inhibition by AgNPs was observed against all the test microorganisms. These silver nanoparticles could be of tremendous use in pharmaceutical industries for various biomedical purposes, as well as in food processing industries for food packaging to reduce contamination and enhance long-term storage and

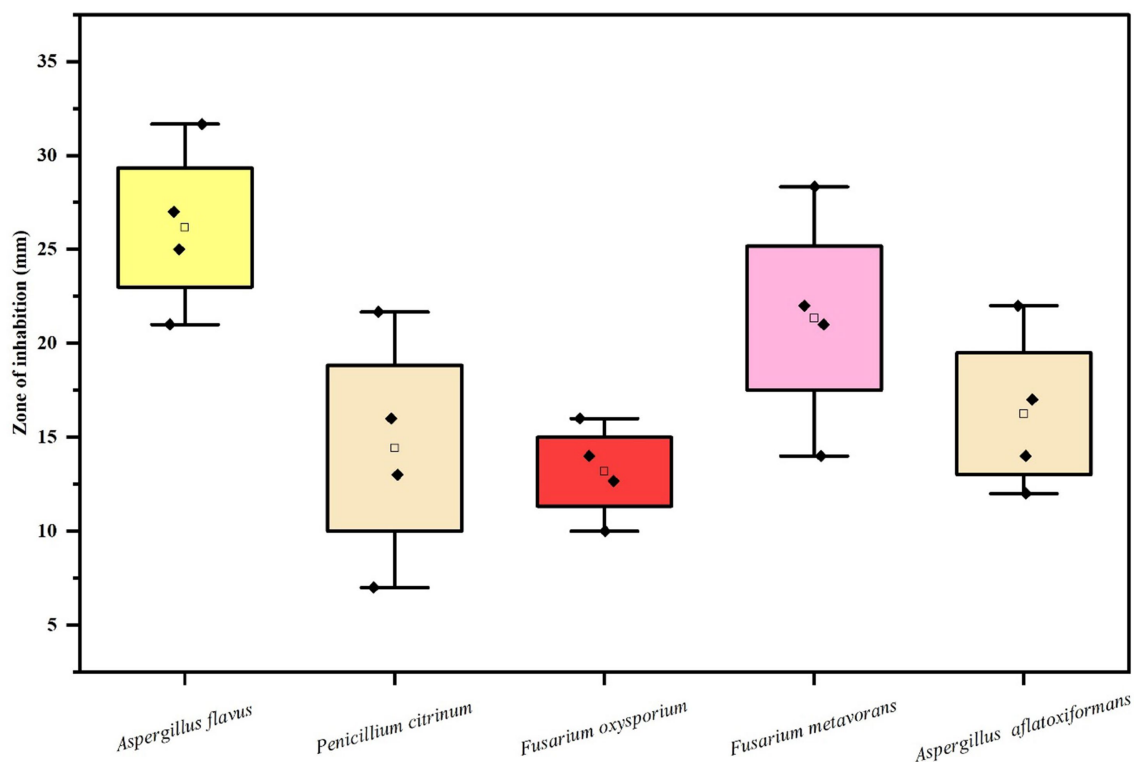


FIGURE 8

Box and whisker plot depicting the Efficacy of AgNPs on zone of mycelial growth inhibition of some pathogenic fungi.

preservation of foods. Metal based nanoparticles such as silver nanoparticles may prove to be very beneficial in the agricultural sector, such as their use as nanopesticides. However, proper investigation into their mechanism of action and evaluating the impact on human health and the environment is required.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. AW: Data curation, Project administration, Resources, Validation, Writing – review & editing. MB: Data curation, Investigation, Project administration, Writing – review & editing. SS: Funding acquisition, Writing – review & editing. SAMA: Writing – review & editing. SAA: Writing – review & editing.

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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 functional identification and evaluation of endophytic bacteria sourced from the roots of tolerant *Achyranthes bidentata* to overcome monoculture problems of *Rehmannia glutinosa*

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The isolation and identification of plant growth-promoting endophytic bacteria (PGPEB) from *Achyranthes bidentata* roots have profound theoretical and practical implications in ecological agriculture, particularly as bio-inoculants to address challenges associated with continuous monoculture. Our research revealed a significant increase in the abundance of these beneficial bacteria in *A. bidentata* rhizosphere soil under prolonged monoculture conditions, as shown by bioinformatics analysis. Subsequently, we isolated 563 strains of endophytic bacteria from *A. bidentata* roots. Functional characterization highlighted diverse plant growth-promoting traits among these bacteria, including the secretion of indole-3-acetic acid (IAA) ranging from 68.01 to 73.25 mg/L, phosphorus and potassium solubilization capacities, and antagonistic activity against pathogenic fungi (21.54%–50.81%). Through 16S rDNA sequencing, we identified nine strains exhibiting biocontrol and growth-promoting potential. Introduction of a synthetic microbial consortium (SMC) in pot experiments significantly increased root biomass by 48.19% in *A. bidentata* and 27.01% in replanted *Rehmannia glutinosa*. These findings provide innovative insights and strategies for addressing continuous cropping challenges, highlighting the practical promise of PGPEB from *A. bidentata* in ecological agriculture to overcome replanting obstacles for non-host plants like *R. glutinosa*, thereby promoting robust growth in medicinal plants.

KEYWORDS

continuous monocultural obstacles, endophytic bacteria, plant growth-promoting, biological control, synthetic microbial consortium

Introduction

In modern agricultural practices, the limitations of arable land resources and the food demands for an increasing human population have driven an increase in intensive cultivation and long-term planting areas of single crops. It has been proved that the problems from consecutive monoculture could inevitably lead to reduced growth and development of crops as well as increased incidence of soil-borne diseases and higher yield penalties, which may entail substantial economic losses and compromise the sustainability of agriculture. Such is the case with Chinese medicinal plants, especially those with tuberous roots, that are continuously grown in the same field for years, often resulting in decreased pH value, soil pathogen enrichment and nutrient sequestration, and consequent reduction in yield and quality of crop in the cropping system. The aforementioned issue significantly hampers the sustainable development of Traditional Chinese Medicine (TCM) in China (Wu and Lin, 2020). Hence, it is imperative to gain a profound understanding of the underlying mechanisms behind continuous cropping obstacles and explore the effective control measures that have garnered substantial research attention.

The well-known traditional Chinese medicine *R. glutinosa*, belonging to the Scrophulariaceae family, is rich in various pharmaceutically active compounds such as iridoids, amino acids, and inorganic ions (Zhang et al., 2008). It is primarily cultivated in its geo-authentic source, Jiaozuo city, Henan Province, located in the central plains of China. However, continuous cropping has resulted in a significant decline in both yield and quality of *R. glutinosa* due to the outbreak of root rot pathogens (Wu et al., 2018; Li Q. et al., 2023). The successful eradication of soil memory resulting from continuous cultivation of *R. glutinosa* necessitates a rotation cycle exceeding 15 years, as well as the excessive use of chemical fertilizers (Yang et al., 2011). Consequently, this relocation process significantly diminishes both the yields and quality of geo-authentic areas for *R. glutinosa* medicinal plants. Exploring sustainable pathways is an effective strategy for alleviating replanting problems in *R. glutinosa* and enhancing crop productivity. Our previous studies have demonstrated that *A. bidentata*, originating from the same geographic source as *R. glutinosa*, exhibits tolerance to continuous cultivation and can grow normally after previous cropping of *R. glutinosa*. Furthermore, intercropping with *A. bidentata* has been found to effectively mitigate the replanting issues associated with *R. glutinosa* (Wang et al., 2019; Liu et al., 2022). The medicinal plant *A. bidentata*, belonging to the Amaranthaceae family, exhibits a unique characteristic of increasing yield and quality over successive years, unlike other medicinal plants that face significant obstacles due to continuous cropping (Li et al., 2010; Wang et al., 2019). Previous studies have demonstrated that *Fusarium oxysporum* derived from three rhizocompartments (rhizosphere, rhizoplane, and endosphere) of *A. bidentata* under a single continuous planting system did not attain dominance.

In fact, the abundance of plant growth-promoting endophytic bacteria (PGPEB) from the root of *A. bidentata* increases with continuous cropping years, particularly *Pseudomonas* and *Bacillus* species (Wang et al., 2021). PGPEB thrive within plants and enhance plant growth under both normal and challenging

conditions by priming plant defenses, improving nutrient uptake, and modulating the synthesis of growth- and stress-related phytohormones (Qin et al., 2018; Borah et al., 2019; Carrión et al., 2019; Lau et al., 2020). Consequently, we hypothesize that the roots of *A. bidentata* could recruit rhizosphere-associated plant growth-promoting bacteria to adapt to continuous cropping environments.

To investigate the potential of PGPEB from *A. bidentata* in promoting its growth and alleviating the continuous cropping problem of *R. glutinosa*, this study aimed to isolate and culture endophytic bacteria from *A. bidentata* roots tolerant to replant disease, screen and identify PGPEB based on their plant growth-promoting and antagonistic traits, and ultimately develop a promising biofertilizer to mitigate replanting obstacles for *R. glutinosa*. This research will provide a technical pathway for exploring ecological approaches to prevent and control monoculture-related challenges, effectively safeguarding local geo-authentic sources while enhancing the yield and quality of TCM.

Materials and methods

Materials

Seeds of the *A. bidentata* variety “Hetaowen” were provided by Cuihong Lu from the Wenxian Institute of Agricultural Sciences, while the *R. glutinosa* variety “Jinjiu” was preserved by the Institute of Agroecology at Fujian Agriculture and Forestry University (FAFU), located in Fujian, China. The soil sample originated from a geo-authentic source in Jiaozuo City, Henan Province, China, with a composition containing 2.16 g/kg total nitrogen, 0.92 g/kg total phosphorus, and 0.99 g/kg total potassium, of which 184.33 mg/kg, 292.55 mg/kg, and 121.86 mg/kg were available, respectively. The pathogenic fungi *F. oxysporum* and *Chromobacterium violaceum* CV026 were preserved by the Institute of Agroecology at FAFU for further study; it is worth noting that CV026 does not possess sensitivity toward N-acyl-homoserine lactones (AHLs) auto-inducers, which are commonly associated with quorum-sensing bacteria (QSB). However, CV026 can be utilized as an AHL biosensor to detect QSB activity (Li et al., 2020).

Methods

Bioinformatic analysis of plant-beneficial traits of endophytic bacteria from *A. bidentata*

In Jiaozuo city (113°22'E, 35°6'N), *A. bidentata* was sown during the final 10 days of June and harvested by mid-November within the same year. Following harvest, wheat cultivation took place from December until June of the subsequent year. Our previous study (Wang et al., 2021) provided a long-term (10 years) dataset comprising four root samples for *A. bidentata* root endophytic bacteria, obtained through continuous cropping: i.e., newly planted (A1Y), three-year consecutive monoculture (A3Y), five-year consecutive monoculture (A5Y), and ten-year consecutive monoculture (A10Y). In this study, we calculated the relative abundance and diversity in each sample by clustering reads from 16S rDNA amplicon sequencing into OTUs at 97% sequence similarities using USEARCH (Edgar, 2013). The resulting OTUs

were aligned against the SILVA database to analyze alpha diversity indices such as Observed OTUs, Chao1, ACE, Shannon, and Simpson index with QIIME2 v1.9.1 according to literature (Bolyen et al., 2019). Community phylogenetic diversity indices including phylogenetic diversity (PD), phylogenetic species variability (PSV), phylogenetic species richness (PSR), and phylogenetic species evenness (PSE) were calculated using the R package picante based on literature (Kembel et al., 2010). Our focus was mainly on new results generated by potential functions of endophytic bacteria of *A. bidentata* based on microbial taxonomy and plant-associated functional traits, such as biocontrol capacity, PGP behavior, and stress resistance, found through the plant-beneficial bacteria (PBB) database (Li P. F. et al., 2023).

Isolation and purification of endophytic bacteria

The root samples of *A. bidentata* were collected from Jiaozuo city in November 2021. The collected root samples were thoroughly rinsed with running tap water to remove soil and subsequently washed with sterile water before processing. Surface sterilization of the samples was conducted as follows: roots were washed twice with 75% ethanol for 5 min each, followed by treatment with 4% sodium hypochlorite for 20 min, and finally washed three times with sterile water. To assess the effectiveness of surface sterilization, 0.1 ml of the last rinse water was plated onto LB agar medium.

The surface-disinfected roots were dissected into small fragments using a sterile scalpel and transferred to a sterile mortar. They were then crushed in 20 mL of sterile phosphate buffer saline (PBS). From this, 1.0 mL of the suspension was sequentially diluted until reaching a dilution factor of 10–3. A 0.1 mL aliquot from the dilution was spread onto LB agar medium, NA medium, and PISA medium (with 50 $\mu\text{g mL}^{-1}$ natamycin) respectively. The plates were subsequently incubated at 37°C for 48 h (Ahmad et al., 2008). After the incubation period, morphologically distinct bacterial colonies on the plates were selectively picked and streaked repeatedly to obtain pure bacterial isolates. All purified bacterial isolates were stored at 4°C.

Evaluate the bacterial endophytes for growth-promoting traits

Phosphate solubilization

Each strain was cultured overnight and transferred onto Pikovskaya's agar medium at 28°C for 5 days to assess their phosphate solubilization ability (Boubekri et al., 2021). The formation of a clear zone around the colony, resulting from tricalcium phosphate utilization, was used as an indicator of phosphate solubilizers. The Phosphate Solubilization Index was calculated by measuring both the colony diameter and halo zone diameter using the following formula: Phosphate Solubilization Index = D/d = Diameter of clearance zone/Diameter of growth (Paul and Sinha, 2017).

Potassium solubilizing

Each strain was cultured overnight and then transferred onto Alexandrov's agar medium at 28°C for 5 days. The presence of a clear zone surrounding the colony was used as an indicator for identifying potassium solubilizers. The Potassium Solubilization

Index (PSI) was calculated by measuring both the diameter of the halo zone and the colony, using the formula $PSI = D/d$ = Diameter of clearance zone/Diameter of growth (Joe et al., 2018).

Siderophore production

The production of siderophores was qualitatively assessed following the method described by Schwyn and Neilands (1987). The strain was cultured overnight and inoculated with 2 μL onto CAS agar medium at a temperature of 28°C for a duration of 3 days. Siderophore production was confirmed by the presence of an orange halo surrounding the bacterial colonies on the medium. The Siderophore Production Index was calculated using the formula Siderophore Production Index = D/d = Diameter of zone of clearance/Diameter of growth (Ghazy and El-Nahrawy, 2021).

Produce IAA

Endophytic bacterial isolates were inoculated into nutrient broth media containing 0.2% (v/v) L-tryptophan and incubated at 28°C for 5 days with vigorous shaking. Controls consisted of 0.2% (v/v) L-tryptophan without bacterial inoculation. At the end of the incubation periods, the bacterial cultures were centrifuged at 12,000 rpm for 10 min at 4°C. 0.1 mL of the supernatant was mixed with one drop of orthophosphoric acid and 0.1 mL of Salkowski's reagent (300 mL concentrated sulfuric acid, 500 mL distilled water, 15 mL 0.5 M FeCl_3). Development of a pink color indicated IAA production after 30 min (Mahgoub et al., 2021). To analyze the amount of IAA produced by each microalgal isolate, absorbance was measured at 535 nm using a spectrophotometer (Rushabh et al., 2020). The concentration of IAA was then calculated based on the standard curve ($y = 0.0029x + 0.0384$, $R^2 = 0.9988$). The standard curve was generated by measuring the absorbance of IAA standards at known concentrations (10 mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L), plotting the absorbance values against the corresponding concentrations, and fitting a linear regression equation to the data.

Quorum sensing

The biosensor strain CV026 was used to streak with 2 μL strain which cultured overnight on the LB plates at 28°C for 48 h. The production of purple pigmentation by CV026 indicated that the co-cultured bacteria could produce QS signal molecules; these were identified as QS bacteria.

In vitro antagonistic activity

The antagonistic activity of bacterial isolates was assessed using the dual culture method on PDA medium *in vitro*. The fungal pathogen was centrifuged (10 min, 6,000 rpm) and its spores were then resuspended in sterile distilled water. Subsequently, the spore suspension was incubated at the center of a fresh PDA medium plate (90 mm in diameter). Approximately 2.5 cm away from the plug, a purified bacterial isolate ($\text{OD}_{600} = 0.6$) was symmetrically inoculated at four sites around the mycelial plug. Control group plates were only inoculated with each fungal phytopathogen. Dual culture plates and control plates were incubated at 28°C for up to 3 days. The percentage of inhibition was calculated using the formula (Swain et al., 2021) Inhibition (%) = $(R1 - R2)/R1 \times 100$, where R1 represents the diameter of control plate and R2 represents the

diameter of dual culture plate. The experiment was repeated three times with three independent replications.

Genotypic characterization and identification

The isolated bacteria were cultured overnight in LB liquid medium. The DNA of these strains was extracted using the E.Z.N.A Bacterial DNA Kit (Omega D3350-01), following the instruction manual for specific operational methods. Proper DNA quality for downstream procedures was assessed by 1% (w/v) agarose gel electrophoresis in TAE buffer. Extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific™).

The extracted DNA samples were subjected to PCR amplification of the 16S rDNA sequence using the universal primers 27F (5'-AGAGTTT-G-ATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') (Dos Santos et al., 2019). The PCR reaction mixture consisted of 25 µL of 2x TransStart® GoldPfu PCR SuperMix, 2 µL Primer 27F (10 µM), 2 µL primer 1492R (10 µM), and 8 ng of DNA template, with a final volume of 50 µL made up with ultra-pure water. The thermal cycling was performed on an Applied Biosystems™ 2720 Thermal Cycler under the following conditions: initial denaturation at 94°C for 5 min, followed by a total of 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension

at 72°C for 1.5 min, and a final extension step at 72°C for 10 min. Aliquots containing 10 µL of each reaction were analyzed on a 1% (w/v) agarose gel in TAE buffer. Subsequently, Tsingke Biotech conducted sequencing analysis on the obtained PCR products. The resulting sequences were assembled, edited, and aligned using DNAMAN and MEGA X software before being compared to those in the GenBank database through Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine their homology with closely related organisms. Finally, in this study, the isolated bacteria were classified to species level based on information from the closest microbes.

In vivo inoculation pot experiments

The pot experiment was conducted in 2022 at the experimental field of the Institute of Agroecology, Fujian Agriculture and Forestry University. *A. bidentata* seeds were sown into pots (25 cm in height, 22 cm in diameter) filled with 4 kg soil sampled from the upper soil layer (5–20 cm) of fields at the Jiaozuo city experiment station. After germination, only one plant was retained per pot. The pot experiment consisted of two treatments and eight replicates: (i) newly planted *A. bidentata* plants inoculated with distilled water only (NPA) and (ii) newly planted *A. bidentata* plants inoculated with a mixed bacterial suspension (NPAB). The

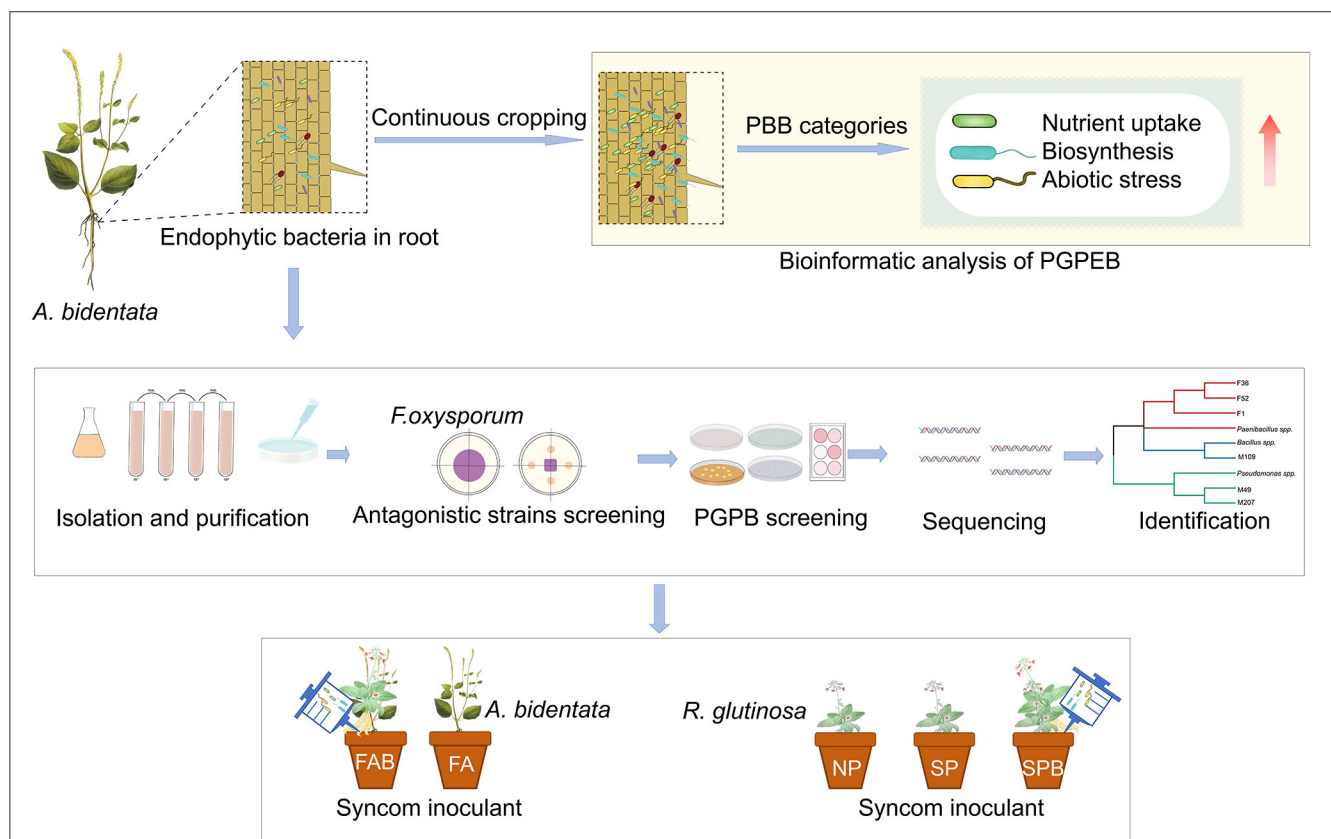


FIGURE 1

Experiment design. SMC: the synthetic microbial consortia; OSMC: the optimizing synthetic microbial consortia; NPA: newly planted *A. bidentata* were inoculated with distilled water only; NPAB: newly planted *A. bidentata* were inoculated with the bacterial suspension mixed; NPR: newly planted *R. glutinosa* were inoculated with distilled water only; CMR: continuous monocultural *R. glutinosa* was inoculated with distilled water only; CMRB: continuous monocultural *R. glutinosa* was inoculated with the bacterial suspension mixed.

experiment commenced in June 2022, and the bacterial suspension was applied to each pot once a week starting on day 30 after thinning out the *A. bidentata* plants, repeated six times in total.

Finally, in November 2022, *A. bidentata* was harvested. Subsequently, an aliquot of 200 μ L from each of the nine selected strains was individually incubated with 100 mL of LB medium and left overnight at a temperature of 30°C under constant agitation at 200 rpm. The bacteria were then collected through centrifugation and resuspended in sterile water. Each strain was adjusted to an OD₆₀₀ value of 0.8 and mixed in equal proportions. For each pot, a total volume of 10 mL from the bacterial suspension mixture was inoculated using the same method for preparing the *R. glutinosa* pot experiment.

The tubers of *R. glutinosa* were surface disinfected in a 0.3% carbendazim solution [N-(benzimidazolyl-2) methyl carbamate] for 5 min, followed by three rinses with distilled water. Subsequently, the tubers were planted into pots (25 cm in height and 22 cm in diameter) filled with soil samples collected from the upper layer (5–20 cm depth) of fields at the Jiaozuo City experiment station. Two types of soils were selected for the experiment: newly planted soil that had never been previously used to grow *R. glutinosa* and 1-year monoculture soil that had been cultivated with *R. glutinosa* for 1 year. The pot experiment involving *R. glutinosa* was designed with three treatments and eight replicates: (i) Newly planted *R. glutinosa* inoculated with distilled water only (NPR); (ii) Continuous monocultural *R. glutinosa* inoculated with distilled water only (CMR); and (iii) Continuous monocultural *R. glutinosa* inoculated with a mixed bacterial suspension (CMRB). In April 2022, two *R. glutinosa* plants were placed in each pot and, after 45 days of planting, they were inoculated once a week with the mixed bacterial suspension for a total of eight repetitions. Finally, in November 2022, *R. glutinosa* was harvested. The fertilization and other field management practices remained consistent across all treatments. The experimental design was shown in Figure 1.

Physicochemical properties of rhizosphere soil

The soil samples were collected using the method described by Wang et al. (2019). Upon harvesting the tuberous roots of *R. glutinosa*, the bulk soil was shaken off and ~2 mm of soil attached to the roots was collected. The samples obtained from each treatment were mixed and then divided into two parts for analysis. One part of the samples was stored at –80°C for DNA extraction, while the other part was used for analyzing the physicochemical properties of the soil. The total nitrogen (TN), phosphorus (TP), and potassium (TK) content were determined by initially digesting the soil using a H₂SO₄-H₂O₂ solution. Subsequently, TN was quantified using the Kjeldahl method, while TP and available phosphorus (AP) extracted with a NaHCO₃ solution were analyzed using a colorimetric method. TK and available potassium (AK), extracted with ammonium acetate, were measured using flame atomic absorption spectrometry. The calculation of available nitrogen (AN) involved converting available nitrogen into ammonia.

Root activity of *R. glutinosa*

The root activity of *R. glutinosa* was assessed using the Triphenyl tetrazolium chloride (TTC) method (Bajracharya,

2004) on the 30th, 60th, 90th, 120th, 150th, and 180th days after planting.

Quantitative PCR of specific microbial taxa in different soils

The total DNA was extracted with BioFast Soil Genomic DNA Extraction Kit (BioFlux, Hangzhou, China) following the instructions. The DNA concentration was measured using a Nanodrop 2000C Spectrophotometer (Thermo Fisher Scientific, United States). A quantitative PCR assay was performed using the CFX96 Real-Time system (Bio-RDA, U.S.A) to determine the abundance of *Pseudomonas* (Ps-F 5'-TTA GCTCCACCTCGCGGC-3'/Ps-R 5'-GGTCTGAGAGGATGATCAGT-3'), *Bacillus* (BacF 5'-GGGAAACCGGGGCTAATACCGGAT-3'/1378 5'-CGGTGTGTACAAGGCCCGGGAACG-3'), and *Fusarium oxysporum* (ITS1F 5'-CTTGATCATTAGAGGAAGTAA-3'/AFP308R 5'-GGAATTAACGCGAGTCCCAA-3'). Each qPCR reaction mixture (20 μ L) consisted of 2 $\times$ Green qPCR MasterMix 10 μ L, forward and reverse primers 0.4 μ L each, sterile water 8.2 μ L, DNA template 1 μ L.

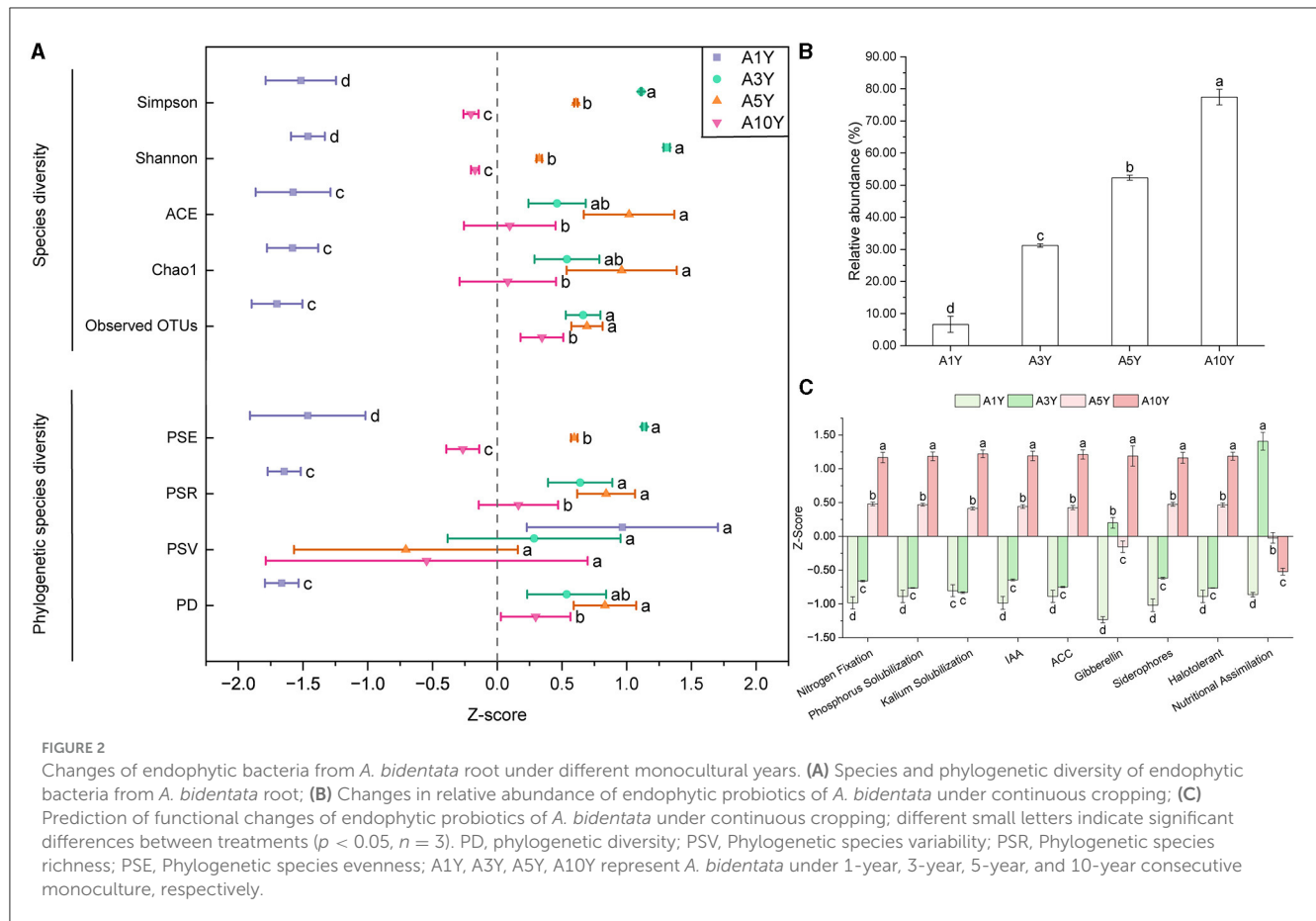
Statistical analysis

Graphs were plotted using Microsoft Excel 2019, Origin 2022b, GraphPad Prism 9.5, R 4.2.9 and SPSS 22.0 statistical software for significance analysis (Duncan's multiple range tests, $\alpha = 0.05$); molecular biology identification was performed using DNAMAN for sequence splicing.

Results

Changes in endophytic bacteria diversity and function potentials of *A. bidentata* under consecutive monoculture

The analysis of Observed OTUs, Chao1, ACE, Shannon, and Simpson indices in the root of *A. bidentata* subjected to long-term monocultivation (A3Y, A5Y and A10Y) revealed that replanted *A. bidentata* exhibited more increased diversity in endophytic bacterial communities compared to newly planted ones (Figure 2A). Moreover, this trend was consistent when considering phylogenetic diversity indices such as PD, PSR, PSV, and PSE (Figure 2A). Based on the PBB database, this study further predicted the potential functions of endophytic bacteria. As depicted in Figure 2B, there was a substantial year-on-year increase in the abundance of beneficial endophytic bacterial community, with a significant rise of 77.44% observed at the tenth year of continuous cropping (A10Y). The classification analysis of beneficial endophytic bacteria (Figure 2C) revealed that successive cropping of *A. bidentata* led to a remarkable enhancement in the abundance of endophytic bacteria exhibiting various functions including nutrient uptake (nitrogen fixation, phosphorus solubilization, potassium solubilization, nutritional assimilation), biosynthesis (IAA, ACC, gibberellin, siderophores),



and response to abiotic stress conditions, particularly evident at the tenth year.

Growth-promoting and biocontrol traits

In consideration of the ultimate objective of this experiment, which aims to address the issue of continuous cropping obstacles in *R. glutinosa* by introducing endophytic bacteria from *A. bidentata*, we have selected the ability to biocontrol *F. oxysporum* as the primary indicator for further strain identification. Through the dual culture method on PDA medium, we isolated 38 strains that exhibited inhibitory effects on the growth of *F. oxysporum* (Figure 3A). The antagonistic activity against pathogenic *F. oxysporum* ranged between 21.54% and 50.81% among these strains (Table 1).

Among them, 30 strains exhibited positive results for phosphorus solubilization, while 10 strains showed positive results for potassium solubilization. The formation of a clear zone on agar containing insoluble phosphate or potassium by these strains indicated their ability to solubilize tricalcium phosphate and potassium, respectively (Figures 3B, C). To screen for the presence of indole-3-acetic acid (IAA), we employed the Salkowski reagent method. Interestingly, we observed that 18 strains exhibited secretion ability, with a more pronounced effect after 24 h (Figure 3D). Additionally, using the widely used CAS assay for

siderophore detection, we identified eight strains as producers based on the development of an orange halo around bacterial colonies in CAS medium (Figure 3E). Furthermore, employing CV026 as a screening tool, we detected a total of 13 strains with quorum-sensing (QS) activity within just 24 h through a significant chromogenic response from the AHL reporter (Figure 3F).

Identification of selected bacteria

Furthermore, the strains were validated by comparing their 16S rDNA nucleotide sequences to the NCBI database using the Basic Local Alignment Search Tool algorithm. Subsequently, these sequences were submitted to GenBank for accession numbers (Table 2). The results confirmed the identification of seven genera, namely *Acinetobacter* sp. (2), *Pantoea* sp. (13), *Raoultella* sp. (9), *Klebsiella* sp. (4), *Paenibacillus* sp. (3), *Bacillus* sp. (2), and *Pseudomonas* sp. (5).

Assessment of potential plant growth-promoting bacteria of *A. bidentata*

We selected nine strains for the application of biofertilizers based on microbial taxonomy and plant-associated functional traits, namely FP1, FP76, FP103, MP171, MB120, MP109, MP59,

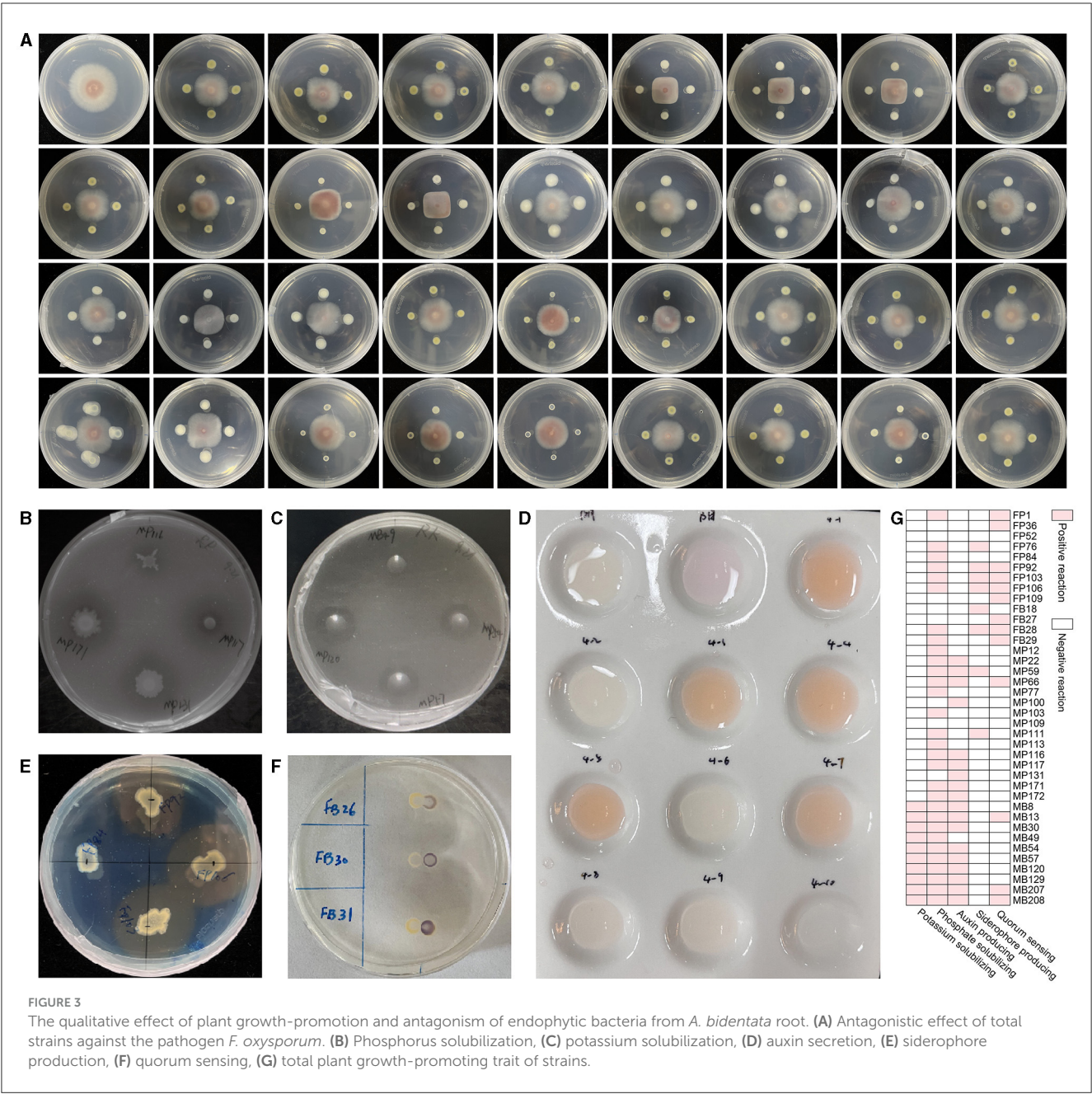


TABLE 1 Inhibition of endophytic bacteria against pathogen.

Strain	Inhibition (%)	Strain	Inhibition (%)	Strain	Inhibition (%)	Strain	Inhibition (%)	Strain	Inhibition (%)
FP1	50.81 ± 0.02 ^a	FP109	47.97 ± 0.01 ^a	MP66	21.54 ± 0.03 ^j	MP117	27.64 ± 0.05 ^{efghij}	MB54	27.64 ± 0.01 ^{efghij}
FP36	48.78 ± 0.00 ^a	FB18	25.02 ± 0.01 ^{hij}	MP77	30.89 ± 0.01 ^{cdefgh}	MP131	27.64 ± 0.01 ^{efghij}	MB57	30.89 ± 0.01 ^{cdefgh}
FP52	49.59 ± 0.01 ^a	FB27	30.89 ± 0.01 ^{cdefgh}	MP100	29.27 ± 0.02 ^{cdefghi}	MP171	35.37 ± 0.01 ^{bcd}	MB120	29.27 ± 0.02 ^{cdefghi}
FP76	32.11 ± 0.06 ^{cdefg}	FB28	34.15 ± 0.03 ^{bcde}	MP103	26.83 ± 0.00 ^{ghij}	MP172	26.02 ± 0.03 ^{ghij}	MB129	26.83 ± 0.03 ^{ghij}
FP84	31.30 ± 0.03 ^{cdefgh}	FB29	27.24 ± 0.05 ^{efghij}	MP109	33.33 ± 0.01 ^{bcdef}	MB8	23.17 ± 0.02 ^{ij}	MB207	33.33 ± 0.06 ^{bcdef}
FP92	31.30 ± 0.03 ^{cdefgh}	MP12	28.86 ± 0.02 ^{defghi}	MP111	27.24 ± 0.04 ^{ghij}	MB13	25.20 ± 0.03 ^{hij}	MB208	23.58 ± 0.06 ^{ij}
FP103	35.77 ± 0.03 ^{bc}	MP22	32.52 ± 0.06 ^{bcdefg}	MP113	33.33 ± 0.01 ^{bcdef}	MB30	21.54 ± 0.04 ^j		
FP106	48.78 ± 0.02 ^a	MP59	39.02 ± 0.04 ^b	MP116	32.93 ± 0.01 ^{bcdef}	MB49	22.76 ± 0.06 ^{ij}		

Different small letters indicate significant differences between treatments ($p < 0.05$, $n = 3$).

TABLE 2 Molecular identification of strains based on 16SrDNA sequence.

Strain	Bacterial name	Accession number	Identity (%) match	Reference organism	Access no. of ref. organism
FP1	<i>Paenibacillus polymyxa</i>	PP449353	100%	<i>Paenibacillus polymyxa</i> strain KF-1	KT962922.2
FP76	<i>Pantoea</i> sp.	PP449354	97.4%	<i>Pantoea</i> sp. strain L-J-70	OP990834.1
FP103	<i>Acinetobacter nosocomialis</i>	PP449355	91.59%	<i>Acinetobacter nosocomialis</i> strain HBU72523	MW365222.1
MP171	<i>Raoultella ornithinolytica</i>	PP449356	99.38%	<i>Raoultella ornithinolytica</i> strain MG	CP017802.2
MB120	<i>Klebsiella</i> sp.	PP449357	99.49%	<i>Klebsiella</i> sp. MTJW-11	KM516019.1
MP109	<i>Bacillus</i> sp.	PP449358	88.54%	<i>Bacillus</i> sp. Pt2	JF900601.1
MP59	<i>Pseudomonas mosselii</i>	PP449359	99.59%	<i>Pseudomonas mosselii</i> strain yy161	MN177227.1
MP131	<i>Pseudomonas koreensis</i>	PP449360	99.58%	<i>Pseudomonas koreensis</i> strain D2	MG269614.1
MB129	<i>Klebsiella pneumoniae</i>	PP449361	99.90%	<i>Klebsiella pneumoniae</i> strain 84	MZ389292.1

MP131, and MB129. The solubilization index and production index indicated that FP76 and FP103 exhibited the strongest phosphorus solubilizing activity among the strains tested. Additionally, MB120 and MB129 demonstrated the highest potassium solubilizing activity while FP76 and FP103 displayed superior siderophore-producing capabilities. Furthermore, using the IAA standard curve to determine concentration levels revealed that MP171 secreted the highest amount of auxin at 73.25 mg/L, followed by MP131 (73.16 mg/L), MB129 (69.64 mg/L), and MB120 (68.01 mg/L) (Table 3). Collectively, a total of nine strains from different genera exhibited a significant positive impact on growth-promoting functions such as phosphate and potassium solubilization, siderophore production, QS activity, IAA secretion, and antifungal activity. Therefore, these nine strains were combined into a synthetic microbial consortia (SMC) without mutual interference and introduced into the pot. The phenotypic growth parameters of *A. bidentata* in the pot are presented in Figure 4. These results demonstrate that inoculation can enhance the root fresh weight by 48.19% and length by 29.35% compared to the uninoculated treatment.

Evaluation of the application of SMC on the growth of *R. glutinosa*

As demonstrated by the aforementioned results, the application of SMC exhibited a growth-promoting effect on *A. bidentata*. However, it remained unclear whether SMC had similar biocontrol and growth-promoting effects on *R. glutinosa*. Therefore, we conducted another inoculation of SMC in the pots containing *R. glutinosa*, following the same procedure as described above. The findings indicated that, compared to replanted *R. glutinosa* without inoculant, the root fresh weight of replanted *R. glutinosa* inoculated with SMC significantly increased by 27.01%, although it did not surpass that of newly planted specimens (Figures 5A–C). Despite the negative impact of continuous planting on plant growth when compared to newly planted specimens, this adverse influence was considerably alleviated to some extent through SMC inoculation. The inoculant facilitated biomass accumulation in *R. glutinosa* and potentially influenced its physiology as well (Figure 5D). It is worth

noting that, throughout all stages of growth and development, CMRB consistently displayed higher root activity than CMR.

Effect and application of SMC on the rhizosphere of *R. glutinosa*

The physicochemical properties of the rhizosphere soil were assessed before and after planting (Table 4). The findings revealed that there were no significant disparities in the physical and chemical characteristics between CMR and CMRB soils, except for higher levels of AK and AP observed in the CMRB soil. Additionally, quantitative PCR results demonstrated a significantly greater abundance of *Bacillus* and *Pseudomonas* genera in CMRB compared to CMR, while *F. oxysporum* exhibited an opposite trend within the rhizosphere (Figure 6).

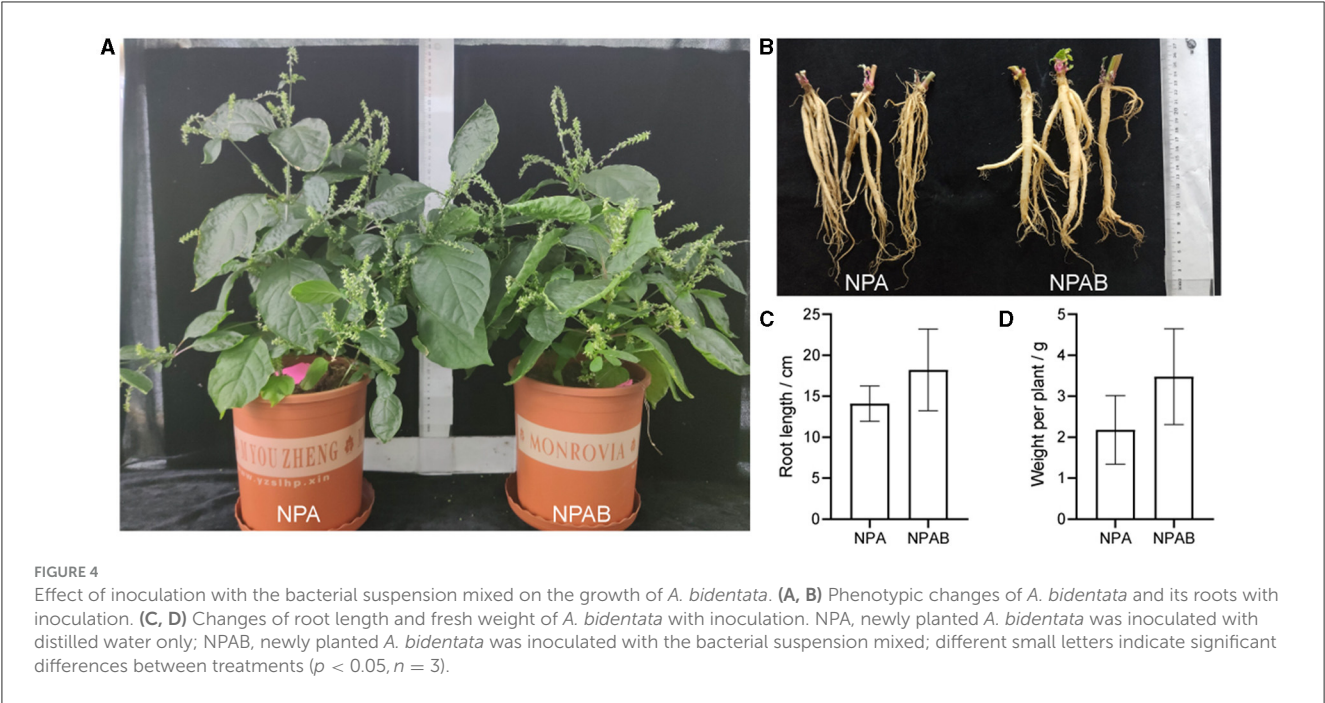
Discussion

Plant-associated microbes play a crucial role in promoting plant growth, development, and stress tolerance (Rodriguez et al., 2019). Harnessing the potential benefits of these microbes offers a sustainable approach to enhancing crop productivity. In this study, we observed a significant increase in α -diversity of root endophytic bacteria from *A. bidentata* under consecutive cropping treatment. Furthermore, the endophytic microbiota exhibited various plant growth-promoting (PGP) traits such as indole-3-acetic acid secretion and phosphate and potassium solubilization, among others. By linking microbial taxonomy with their PGP traits based on PBB database, we propose that these probiotics may enhance host immunity and establish a protective layer during continuous cropping, thereby contributing to the improved yield and medicinal properties of *A. bidentata* after long-term monoculture. Indeed, our analysis demonstrates the PGP traits of these isolates obtained from diverse culturable bacterial endophytes in root samples. These findings provide novel evidence for the presence of culturable root microbiomes in *A. bidentata* and highlight their beneficial traits for plant growth. Overall, our results support the notion that microbiota and hosts co-evolve to mutually

TABLE 3 Plant growth-promoting capacity of nine isolates.

Strain	Quorum sensing	Phosphate solubilization index	Potassium solubilization index	Siderophore production index	Concentration of IAA (mg/L)
FP1	+	1.50 ± 0.46d	-	-	-
FP76	-	3.27 ± 1.10a	-	2.31 ± 0.65	-
FP103	+	3.23 ± 1.09a	-	2.66 ± 0.87	-
MP59	-	-	-	-	48.75 ± 0.67d
MP109	+	1.65 ± 0.42d	-	-	-
MP131	-	2.42 ± 0.70b	-	-	73.16 ± 0.73a
MP171	-	1.95 ± 0.46c	-	-	73.25 ± 0.82a
MB120	-	1.52 ± 0.36d	1.87 ± 0.46	-	68.01 ± 0.76c
MB129	-	2.16 ± 0.71bc	1.94 ± 0.64	-	69.64 ± 0.59b

“+” Represents positive effect; “-” represents negative effect. Different small letters indicate significant differences between treatments ($p < 0.05$, $n = 3$).



support growth and fitness (Dini-Andreote, 2020; Mukherjee et al., 2020; Abdelfattah et al., 2022).

Currently, a wide range of plant species can be colonized by endophytic bacteria, which have been isolated from various plant organs including roots, stems, leaves, seeds, fruits, and tubers (Dwibedi et al., 2022). Our research demonstrated that the root of *A. bidentata* harbored a diverse community of ~100 culturable endophytic bacteria, which played crucial roles in regulating plant growth and developmental processes through quorum sensing (QS), indole-3-acetic acid (IAA) synthesis, phosphate and potassium solubilization, as well as pathogen inhibition. Plate antagonistic experiments combined with DNA sequencing identified the antagonist isolates as *Acinetobacter* sp., *Pantoea* sp., *Raoultella ornithinolytica*, *Klebsiella* sp., *Paenibacillus* sp., *Bacillus* sp., *Pseudomonas mosselii*, *Pseudomonas koreensis*, and *Klebsiella pneumoniae*.

QS plays crucial roles in coordinating the functions of single-celled organisms, such as bacteria, within their family units (Dong et al., 2000). In this study, we discovered that FP1, FP103, and MP109 possess the ability to produce AHL signaling molecules in the QS system. It is well-established that certain gram-negative bacteria can respond to AHL signals and form biofilms (Medina-Martinez et al., 2007). Biofilms represent a communal lifestyle for bacteria, enabling them to exhibit enhanced social cooperation, resource acquisition, and survival capabilities compared to free-living cells (Davies, 2003). For instance, QS regulates both biofilm formation and antifungal activity of *Serratia marcescens* isolated from wheat stems (Liu et al., 2011). Additionally, biofilms aid microorganisms in evading host immunity and effectively colonizing their environment (Pang et al., 2009). Furthermore, endophytic bacteria contribute to crop growth and health by solubilizing potassium and phosphorus while

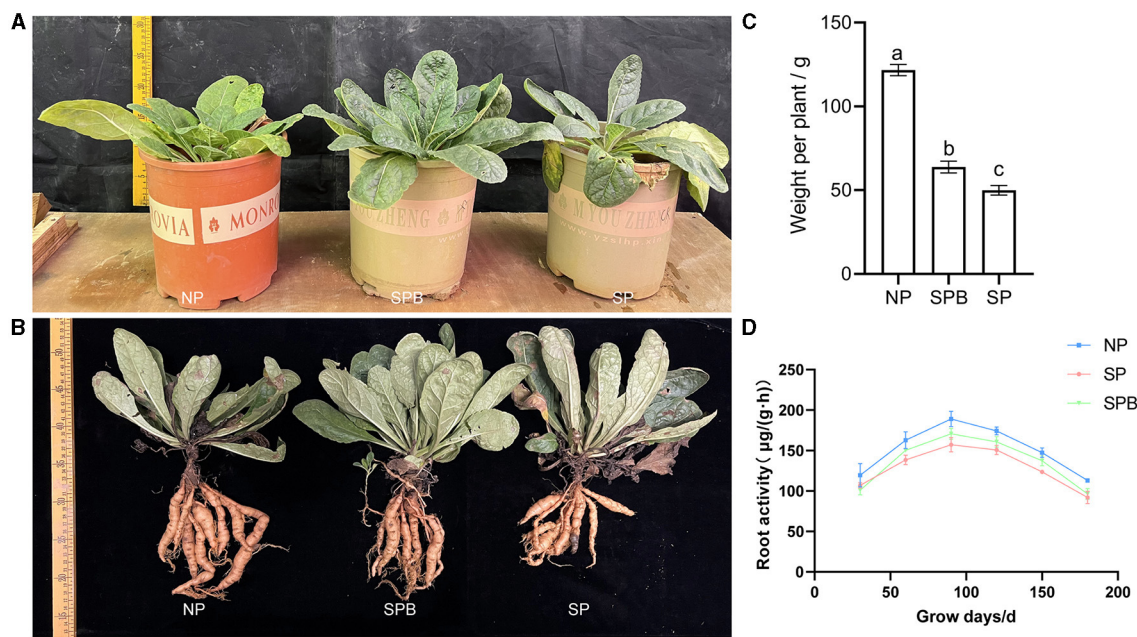


FIGURE 5 Effect of inoculation with SMC on the growth of replanting *R. glutinosa*. (A, B) Phenotypic of *R. glutinosa* and its roots with inoculation. (C) Fresh weight of *R. glutinosa* roots with inoculation. (D) Root activity of *R. glutinosa*. NPR, newly planted *R. glutinosa* was inoculated with distilled water only; CMR, continuously monocultural *R. glutinosa* was inoculated with distilled water only; CMRB, continuously monocultural *R. glutinosa* was inoculated with the bacterial suspension mixed; different small letters indicate significant differences between treatments ($p < 0.05$, $n = 3$).

TABLE 4 Physicochemical properties of rhizosphere soil of *R. glutinosa* under different treatments.

Stage	Treatment	TN (g/kg)	TP (g/kg)	TK (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)
Before planting	NPR	2.16 ± 0.11a	1.00 ± 0.04a	0.99 ± 0.06a	184.33 ± 1.25a	292.55 ± 3.94a	121.86 ± 6.14a
	CMR	2.47 ± 0.11a	1.19 ± 0.06a	0.70 ± 0.12a	265.33 ± 1.70a	257.59 ± 5.71a	166.3 ± 5.40a
Harvest period	NPR	2.14 ± 0.10a	0.98 ± 0.02a	0.97 ± 0.07a	185.00 ± 1.73a	291.5 ± 1.73a	121.43 ± 6.54b
	CMR	2.45 ± 0.21a	1.17 ± 0.05a	0.69 ± 0.18a	265.13 ± 0.58a	256.47 ± 4.65a	165.87 ± 6.92a
	CMRB	2.45 ± 0.15a	1.18 ± 0.07a	0.70 ± 0.10a	265.17 ± 2.52a	257.09 ± 6.74a	166.19 ± 8.14a

TN, the content of total nitrogen; TP, the content of total phosphorus; TK, the content of potassium; AN, the content of available nitrogen; AP, the content of available phosphorus; AK, the content of available potassium; different small letters indicate significant differences between treatments ($p < 0.05$, $n = 3$).

producing siderophores (Dubey et al., 2020). Chen et al. (2015) demonstrated that *Acinetobacter calcoaceticus* Sasm₃ exhibited the ability to enhance the growth of *Brassica napus* L, which aligns with the findings from our study on *Acinetobacter* sp. FP103. Previous studies have reported that *Pantoea* spp. can facilitate nutrient absorption and antagonize pathogens in *Blumeria graminis* (Rahman et al., 2018). Additionally, strains of *Pantoea* sp. were found to be effective against *F. oxysporum*, consistent with observations made in rice (Kouzai and Akimoto-Tomiyama, 2022). Similarly, *Paenibacillus* sp., like FP1, plays a crucial role in pathogen resistance and plant growth promotion through nitrogen fixation, phosphorus increase, and auxin production (Grady et al., 2016). Recent research work on plant growth-promoting bacteria has primarily focused on *Bacillus subtilis* (Errington and van der Aart, 2020). In our study, *Bacillus subtilis* MP109 exhibited significant antagonistic activity against pathogens. *Pseudomonas chlororaphis* (Liu et al., 2022) and *Pseudomonas psychrophile* (Ramírez-Bahena

et al., 2015) are well-recognized as rhizosphere bacteria promoting plant growth.

Pseudomonas mosselii has been demonstrated to be an effective bacterium against the pathogenic fungi *Xanthomonas oryzae* and *Magnaporthe oryzae* (Yang et al., 2023). A previous study revealed that *Raoultella ornithinolytica* can inhibit the growth of *Plutella xylostella* and promote radish seedling growth (Guo, 2023). *Klebsiella* sp. MB120 and MB129 possess the ability to secrete indole acetic acid (IAA), which is consistent with other studies supporting our findings. For instance, *Klebsiella variicola* AY13, a bacteria known for producing IAA, has shown great potential in mitigating flooding stress and improving soybean plant growth (Kim et al., 2017). Additionally, similar to *Raoultella* sp. MP171, a previous study also demonstrated that *Raoultella ornithinolytica* inhibits *Plutella xylostella* growth while promoting radish seedling growth (Guo, 2023). These isolated strains clearly exhibit traits of promoting growth and biocontrol, as previously proven.

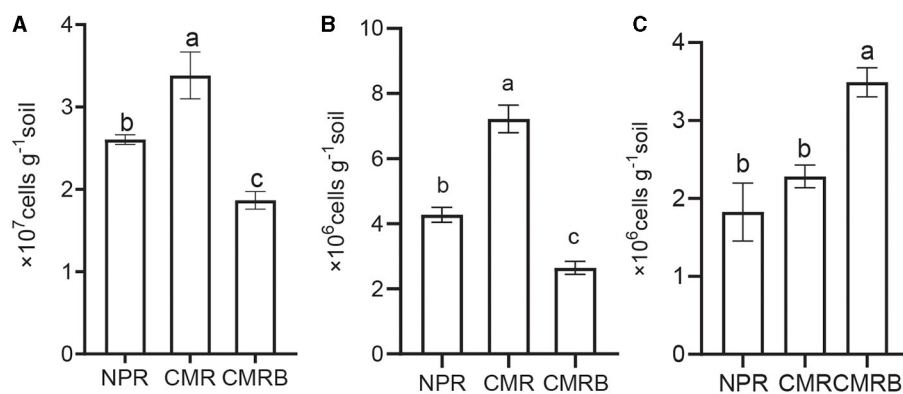


FIGURE 6

Quantitative PCR analysis of specific microbial taxa in different soil samples. (A–C) Quantitative PCR of the genus *Pseudomonas*, *Bacillus*, *F. oxysporum*; NPR, new planted *R. glutinosa* was inoculated with distilled water only; CMR, continuous monocultural *R. glutinosa* was inoculated with distilled water only; CMRB, continuous monocultural *R. glutinosa* was inoculated with the bacterial suspension mixed; different small letters indicate significant differences between treatments ($p < 0.05$, $n = 3$).

Compared to individual organisms, microbial cultures consisting of multiple species exhibit enhanced plant beneficial traits and resistance to environmental perturbations or invasions by other species as part of these microbial communities (Tsoi et al., 2018). The construction of SMC is a crucial approach for studying the function of the microbiome and its interaction with host plants (Vorholt et al., 2017). Our investigation demonstrated that SMC derived from culturable plant growth-promoting endophytic bacteria (PGPEB) isolated from *A. bidentata* have potential applications as biological inoculants for promoting plant growth. Consistent with our findings, reintroduction of isolated endophytes resulted in a significant increase in the length, biomass, and chlorophyll contents of *Cicer arietinum* (Mukherjee et al., 2020). Most of these studies have shown the potential growth promotion effects of endophytes isolated from the same plants (Santoyo et al., 2016).

Meanwhile, other studies have reported the growth-promoting effects of endophytic bacteria on non-host plants (Sessitsch et al., 2005; Ma et al., 2011). Our results from an *in-vivo* inoculation pot experiment, similar to *A. bidentata*, confirmed a reduction in continuous cropping diseases and an enhancement of *R. glutinosa* yield. Remarkably, we demonstrated that the potential for growth promotion by isolated endophytes can similarly exert a significant positive influence on different plants with habitat homogeneity. This research further substantiated that root activity of *R. glutinosa* was enhanced by the inoculant, activating insoluble mineral elements in soil to facilitate nutrient absorption availability. It has been proven that the application of SMC significantly enhances tomato resistance to the pathogen *F. oxysporum* (Zhou et al., 2022). Additionally, qPCR results showed that SMC inoculation increased beneficial bacteria such as *Pseudomonas* and *Bacillus* while decreasing pathogenic *F. oxysporum* amounts in the rhizosphere of *R. glutinosa*, resulting in reduced continuous cropping diseases and improved yield.

Originally, through bioinformatics analysis, we discovered a multitude of endophytic bacteria with PGP traits that were recruited during continuous planting of *A. bidentata*. Subsequently,

we designed an experiment to investigate whether these beneficial endophytes could alleviate the challenges associated with continuous cropping of the non-host plant *R. glutinosa*. Our findings revealed that the isolated flora exhibited a higher tendency to colonize in the rhizosphere of the host plant. Recent studies have also demonstrated that endophytes are primarily sourced from the soil and rhizosphere (Edwards et al., 2015). Given that *A. bidentata* shares similar geo-authentic origins and habitat homogeneity with *R. glutinosa*, it is plausible to assume that they possess comparable rhizosphere microenvironments. Furthermore, we identified PGPEB as a biological inoculant capable of promoting growth in non-host plants while potentially exerting antagonistic effects against pathogens. However, one drawback was encountered: conditional pathogenic bacteria such as *Raoultella ornithinolytica* and *Klebsiella pneumoniae* were unsuitable for use as biofertilizers despite achieving our initial objective. Therefore, it is imperative for us to continuously optimize new and more viable solutions in order to develop effective biofertilizers for field applications. In conclusion, our work provides novel insights into mitigating or eliminating obstacles associated with continuous cropping for sustainable agriculture.

Conclusions

In this study, it was evident that endophytic bacteria isolated from the root of *A. bidentata* exhibited promising potential in promoting plant growth. Furthermore, we demonstrated that these beneficial endophytic bacteria could alleviate the challenges associated with continuous cropping in non-host plant *R. glutinosa*. In other words, the PGPEB isolated from a medicinal plant *A. bidentata*, which possesses tolerance to continuous cropping, displayed biological activity toward *R. glutinosa*, a species facing severe replanting issues. Our future endeavor will focus on continuously exploring more rational approaches for constructing biofertilizer agents suitable for addressing replanting problems in Chinese medicinal plants.

Data availability statement

The datasets presented in this study can be found in online repositories. The accession numbers for NCBI data are found with Table 2 of this article.

Author contributions

CZe: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. BZ: Data curation, Methodology, Software, Supervision, Validation, Writing – original draft. CZh: Investigation, Methodology, Resources, Supervision, Writing – original draft. NL: Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft. LJ: Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft. CL: Data curation, Validation, Writing – original draft. BQ: Data curation, Methodology, Validation, Writing – original draft. YY: Investigation, Supervision, Validation, Writing – original draft. JW: Data curation, Resources, Writing – original draft. TC: Supervision, Validation, Writing – original draft. CF: Supervision, Validation, Writing – original draft. YL: Conceptualization, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. WL: Conceptualization, Investigation, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Supplementary material

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Next generation plant biostimulants & genome sequencing strategies for sustainable agriculture development

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The best environment for plant growth and development contains certain essential metabolites. A broad category of metabolites known as “plant biostimulants” (PBs) includes biomolecules such as proteins, carbohydrates, lipids, and other secondary metabolites related to groups of terpenes, specific nitrogen-containing compounds, and benzene ring-conjugated compounds. The formation of biomolecules depends on both biotic and abiotic factors, such as the release of PB by plants, animals, and microorganisms, or it can result from the control of temperature, humidity, and pressure in the atmosphere, in the case of humic substances (HSs). Understanding the genomic outputs of the concerned organism (may be plants or others than them) becomes crucial for identifying the underlying behaviors that lead to the synthesis of these complex compounds. For the purposes of achieving the objectives of sustainable agriculture, detailed research on PBs is essential because they aid in increasing yield and other growth patterns of agro-economic crops. The regulation of homeostasis in the plant-soil-microbe system for the survival of humans and other animals is mediated by the action of plant biostimulants, as considered essential for the growth of plants. The genomic size and gene operons for functional and regulation control have so far been revealed through technological implementations, but important gene annotations are still lacking, causing a delay in revealing the information. Next-generation sequencing techniques, such as nanopore, nanoball, and Illumina, are essential in troubleshooting the information gaps. These technical advancements have greatly expanded the candidate gene openings. The secondary metabolites being important precursors need to be studied in a much wider scale for accurate calculations of biochemical reactions, taking place inside and outside the synthesized living cell. The present review highlights the sequencing techniques to provide a foundation of opportunity generation for agricultural sustainability.

KEYWORDS

plant biostimulants, biomolecules, next-generation sequencing, metabolites, sustainable

1 Introduction

The plant-based biochemicals, especially secondary metabolites, when released in a local environment influence the growth of other species of plants by combating pathogens and increasing the chances of survival of plants. These compounds providing adequate growth to plant species are termed as plant biostimulants. Agriculture is a complicated discipline concerning the types of biochemicals involved because of the interaction between natural processes. Although the growth of agriculture depends on natural processes such as weather, soil conditions, and fertilizers, human-led effort generates efficient and higher productivity while utilizing minimum agents, which is harmful for the mother nature. This motivates the idea of “sustainable agriculture” as a potential force for human existence and the sustainability of other ecological niches (Divekar et al., 2022).

However, nature does not always support the agriculture sector in a beneficial manner. Pathogens have a wide range of families, specific orders, and overlapping population of species, such as larvae, caterpillars, and moths, which feed on plants only in the form of sap or leaves. The natural selection is followed by organisms according to modification in the set of genes present in their genome that actually develops the ultimate phenotypic effect. However, continuous feeding on plants without any defense mechanism developed by plants will destroy the plant species. This may be further accompanied by pathogenic species of fungi, bacteria, and viruses. If this continues for long, the plants will be extinct, but this is not occurred irrespective of the presence of pathogen and plant feeders because of the biochemicals released by plants in the micro-environment leading to their substantial growth. This leads to development of the concept of plant biostimulants. However, other chemical compounds which deter pathogen's growth and favor plant's growth may also be termed as plant biostimulants. However, chemical interaction is the finest strategy used by living organisms to maintain an overall equilibrium among them. When it comes to the growth of plants, this becomes a plant biostimulant for insects, an insect biostimulant, and fungus, a fungal biostimulant. However, the terms “insect growth retardant” and “fungal growth retardant,” as well as the agents, added to the accurate definition of “plant biostimulant” because they are a part of removing the potential risks to the affected plant. The importance of sustainable agriculture is now brought up in relation to plant biostimulants, whose next generation potential can be used for the opportunities of sustainable agriculture (Nephali et al., 2020).

1.1 Agriculture and plant biostimulants

The agricultural sector has recently been confronted with the simultaneous challenges of maximizing resource utilization, minimizing environmental impact on the ecosystem, and an expanding global population. Chemical fertilizers and pesticides are crucial for agriculture, as they provide cultivators a strong method for

yield and consistent efficiency throughout the seasons in both ideal and sub-standard circumstances. To boost the long-term viability of agricultural production systems, a number of technological advancements have been proposed over the past three decades. These advancements have significantly reduced the use of synthetic agrochemicals such as pesticides and conventional fertilizers. The utilization of regular plant biostimulants (PBs) that have beneficial impacts on the agricultural sector increased the crop efficiency and nutrient use efficiency (NUE), with higher potential of flower blooming, leading to productive fruit development or pod set, plant root development, enhancement in bio-organic product set, and imparting resistance to an extensive variety of biotic and abiotic stressors leading to adequate optimum set of growth conditions for the ecosystem advancement (Colla and Rouphael, 2015). Fertilizers and plant protection products were initially excluded from the definition of PBs. Researchers from Virginia Polytechnic Institute and State University's Department of Crop and Soil Environmental Sciences defined plant biostimulants (PBs) as compounds that stimulate plant growth when applied in small amounts (Zhang and Schmidt, 1997). While fertilizers and soil amendments also promote plant development, they are usually administered in higher quantities. The scientists used the phrase “minute quantities” to distinguish biostimulants from other substances. It was hypothesized that the PBs discussed in this article primarily affect plants. In 2012, the European Commission assigned an *ad hoc* study on PBs to evaluate the materials and substances involved. du Jardin (2015) published this study as: “A Bibliographic Investigation of the Study on Plant Biostimulants.” The accompanying definition was proposed in light of the logical writing (250 logical articles that utilized the expression “biostimulant” in their titles and abstracts). According to the definition, “plant biostimulants refer to substances and materials that, when administered to plants, seeds, or growing media in specific formulations, can alter physiological processes in plants, potentially enhancing growth, development, and/or responses to stress.” The only exceptions to this definition are pesticides and nutrients. According to the research by du Jardin (2015), PB is made up of a wide range of materials, and he identified eight categories of substances that serve as biostimulants. Chemical elements (Al, Co, Na, Se, and Si), inorganic salts such as phosphate and seaweed extracts (brown, red, and green macroalgae), chitin and derivatives (Ghasemi Pirbalouti et al., 2017), anti-transpirants such as kaolin and polyacrylamide, free amino acids and substances that contain nitrogen (peptides, polyamines, and betaines), humic materials, complex organic materials (from sewage) did not have any biostimulants for microorganisms. After 3 years, in the context of “*Biostimulants in Horticulture*” by Colla and Rouphael (2015) and du Jardin (2015) proposed a new definition. Scientific evidence regarding the mode of action, nature, and types of effects that PBs have on agricultural and horticultural crops supported this definition. As indicated by the definition of PBs by du Jardin (2015), “any substance or microorganism that is applied to plants fully intent on further developing sustenance proficiency, abiotic stress resilience,

is a plant biostimulant, no matter what its supplement content.” This definition could be completed by defining PBs as “commercial products containing mixtures of such substances and/or microorganisms.” The definition of PBs has been the subject of intense debate for the past 10 years. Most recently, the following was established by the brand-new Regulation (EU) 2019/1009: An EU compost that revives plant nourishment processes regardless of the supplement content is considered to be plant biostimulant. The purpose is to enhance one or more of the following plant or plant rhizosphere characteristics: (i) the efficiency with which nutrients are utilized, (ii) tolerance to abiotic stress, (iii) the quality characteristics, and (iv) bioavailability of restricted nutrients in the soil or rhizosphere (EU, 2019). To account for broad range, six non-microbial and three microbial PB categories were proposed: (i) humic and fulvic acids (Canellas et al., 2015) (ii) chitosan (Pichyangkura and Chadchawan, 2015) (iii) protein hydrolysates (Gómez-Merino and Cand-Trejo-Téllez, 2015) (iv) phosphites (Varadarajan et al., 2002) (v) seaweed extracts (Battacharyya et al., 2015) (vi) silicon, and (vii) arbuscular mycorrhizal fungi (AMF) (Rouphael et al., 2015), (viii) plant growth-promoting rhizobacteria (PGPR) (Ruzzi and Aroca, 2015; Omidbakhshfard et al., 2019), and (ix) *Trichoderma* spp. (López-Bucio et al., 2015).

Along with all these aspects covered by plant biostimulants, the growth of the agriculture sector clearly relies on biochemical interactions among different species of plants. These interactions proved useful in maintaining the healthy soil conditions, elongated root zone growth, and less pathogenic condition development while minimizing the use of harmful chemical fertilizers, simultaneously achieving the goal of sustainable agriculture.

1.2 The collective data potential

To understand any concept, data generation should be influenced by a set of reasons to determine the concerned research so far across the world. In the case of newly developing field biostimulants, more than 700 scientific studies were published between 2009 and 2019. These studies demonstrate that non-microbial and morpho-anatomical, biochemical, physiological, and molecular plant responses, such as an increase in crop productivity and tolerance to abiotic stresses, can both be induced by PBs (Calvo et al., 2014; Haplern et al., 2015; Nardi et al., 2016; Yakhin et al., 2017; Rouphael et al., 2017a,b,c, 2018a,b; De Pascale et al., 2018).

Information presented here denotes the collective data on 50 scientific studies from highly qualified research studies removing PBs and covering sub-atomic, cellular, and physiological components of fundamental plant biostimulant associations under variable agro-climate zones. In addition, several topics that will be helpful to the scientific community, extension specialists, and business organizations in deciphering the causal/functional mechanism of both microbial and non-microbial biostimulants are discussed. Currently, there is a market trend toward formulations containing both microbial and non-microbial plant biostimulants. However, many of these commercial products lack sufficient scientific evidence to validate their synergistic effects. Therefore, it is imperative to use robust experimental approaches to scientifically characterize the properties of these formulations and their individual impact on crop yield and resistance (Rouphael and Colla, 2020). This future challenge lies in the research, development, and innovation of second-generation PB. The

advantages of PB (Figure 1) reveal the potentials of maintaining the macro-ecosystem (concerned with human influence) and micro-ecosystem (concerned with rhizosphere). The second era of biostimulants in which cooperative energies and corresponding systems can be practically planned should be possible by explaining the rural capability (i.e., to further develop the supplements that used productivity, quality, and resilience to the abiotic stresses) and activity components of PBs.

1.3 Challenges for sustainable agriculture

The major challenges to be encountered for in this direction are biodiversity conservation, protecting the environment from degradation. Wherein habitat expansion that is needed for crops meant to be grown for food supply especially rice, wheat, and maize (de Azevedo et al., 2019), rehabilitation must be ensured (for crops that were grown but not in an adequate amount). Legislation for genetic resources is required, wherein the planned use of genetic materials is carried out through breeding innovations whether classical or modern. On one hand, clearing the forest lands for agricultural production opens the gateways of feeding the over growing population and, on the other hand, overburdens the nature by reducing photosynthetic crops, depleting natural niches and habitats of beneficial animal species, and drastically reducing the natural mineral content of soil. To cope this adequate measures to be taken monitored by government and private organizations and the training and conference programs at national and international levels and self-consciousness among people to protect the forest land. Another major difficulty to forest land is industrialization which depletes cultivated soil and increases pollutants in air and water, thus hampering the agriculture sector in a more drastic way. However, natural calamity such as flood is responsible for soil erosion, but they are not frequent until and unless the forest land is under devastation by human-generated needs. The extension education sector should clearly define the impacts in general body meetings, where the concern for possibility of such mishaps should be clearly described to people ultimately raising the environment-friendly issues.

2 Maneuvering classification data on biostimulants

In view of where the biostimulants are derived from, these biostimulants are divided into two distinct categories. All products with a biological origin, such as those derived from microorganisms or the plant itself, are under one category (biotic biostimulants), and all products without a biological origin, such as those derived from chemicals and physical factors, are under the second category (abiotic biostimulants). In addition, biotic biostimulants may include molecules with a known structure and have a particular composition, or they may be more complex and include multiple molecules with distinct structures. Additionally, biostimulant products can be categorized into two types: microbial, derived from beneficial microbes such as arbuscular mycorrhizal fungi (Giovannini et al., 2020) and plant growth-promoting bacteria, and non-microbial, derived from the extracts of plant microalgae, humic substances, and biopolymers, such as chitosan. This is another way to classify

S. No.	Plant/ Plant tissue	Metabolite	Time	Reference
1.	<i>Hordeum vulgare</i>	a. Glycerophospholipids & Polyketides b. Chlorogenic acid & Hyrdocinnamic acid	a. Seed Germination b. 3-leaf stage	a. Gupta et al 2019 b. Piasecka et al 2017
2.	<i>Oryzae sativa</i>	a. Isoleucine, Phenylalanine & Spermidine b. Lutein & Beta-carotene	a. Flowering b. Maturation stage	a. Roessner et al 2006 b.Zarei et al 2018
3.	<i>Triticum aestivum</i>	a. Ubiquinone, Guanine & Chorismate b. Phenolamides & Polyphenols	a. Mature leaves b. Mature kernels	a. Michaletti et al 2012 b. Matthews et al 2012
3.	<i>Zea mays</i>	a. Asparagine, Glutamic acid & Inositol b. Anthocyanins & Methoxylated flavonoids	a. 8-month old kernel b. Mature kernel	a. Barros et al 2010 b. Jin et al 2017
4.	<i>Sorghum bicolor</i>	Cyanogenic glycosides	4-leaf stage	Mareya et al 2019

FIGURE 1
Cycle of plant biostimulants in nature. This figure represents how the components of nature are regulated by plant biostimulants and the links are established between them.

biostimulant products (Lugtenberg and Kamilova, 2009; Ahemad and Kibret, 2014; Pérez-Montañó et al., 2014; de Vries et al., 2020; Rouphael and Colla, 2020). In particular, there are several ways in which the microbial biostimulants may promote plant growth (Kumari et al., 2022). Biofertilization, root growth stimulation, tolerance to plant stressors, and rhizoremediation are examples of direct effects on plant growth promotion as they involve the interaction between plant roots and biochemicals. On the other hand, plant pathogen control and increased enzymatic activity may indirectly stimulate plant growth (de Vries et al., 2020). Finally, a number of researchers make a distinction between

non-phytohormonal and phytohormonal non-microbial biostimulants, which include compounds containing proteins (Geelen et al., 2020).

2.1 Crucial counterparts of PBs

Certain components that exist naturally within the plant have the potential of acting as a biostimulant, as shown in Table 1. The following section briefly discusses the structure of these crucial counterparts. Moreover, prominent examples are also found

TABLE 1 Primary classification of plant biostimulants.

S. no.	Plant biostimulants	Key points	References
1.	N-containing compounds (amino acids) and protein hydrolysates (PHs)	a) A combination of peptides and amino acids is formulated using animal or plant proteins subjected to chemical, enzymatic, or thermal hydrolysis b) Enhance the physiological and biochemical processes of both primary and secondary plant metabolism c) Are able to mitigate abiotic stress's negative effects	a) Ahmad et al. (2015) and Colla et al. (2015) b) Vioque et al. (2000) and Colla et al. (2014) c) Yakhin et al. Lubyantov (2017)
2.	Humic materials	a) Incorporate humic and fulvic acids, which have distinct characteristics of their own like sub-atomic weight, carbon content, and polymerization degrees b) They might increase the soil's cationic exchange capacity (CEC) by interacting with root membrane transporters (Figure 3)	a) Nardi et al. (2016) b) Canellas et al. (2015)
3.	Extracted seaweeds	a) Brown seaweed extracts, including those from the <i>Ascophyllum</i> , <i>Fucus</i> , and <i>Laminaria</i> genera b) They have a lot of hormone-active compounds, polysaccharides, and polyphenols that help plants grow and develop	a) Khan et al. (2009) b) Craigie (2011) and González et al. (2012)
4.	Biopolymers (chitosans and other polymers)	a) Nematodes, fungi, insects, and crustaceans all naturally contain chitosans that improve plant root growth b) Control plant defense mechanisms that make plants more resistant to biotic and abiotic stressors by controlling the biosynthesis of phytoalexins, degradation and generation of reactive oxygen species (ROS) and pathogenic native proteins	a) Pichyangkura and Chadchawan, 2015 b) Ghasemi Pirbalouti et al. (2017)
5.	<i>Trichoderma</i> , rhizobium, and plant growth-promoting rhizobacteria (PGPR), which are both mycorrhizal and non-mycorrhizal fungi, are biostimulants for microorganisms	a) Symbolic fungi, particularly <i>Glomus</i> -genus arbuscular mycorrhizal fungi (AMF) induces nutrient channeling b) Genus <i>Trichoderma</i> have hyphae that stimulate plant iron uptake c) Beneficial bacteria, also known as PGPBs, that aid in plant growth (<i>Bacillus</i> , <i>Rhizobium</i> and <i>Pseudomonas</i>)	a) Pereira et al. (2019) and Petropoulos et al. (2019) b) López-Bucio et al. (2015) c) Ruzzi and Aroca, 2015
6.	Phosphite (Phi)	a) Phosphate analog (H_2PO_4) are weak acid compounds that affects a variety of plant development and growth processes by regulating water uptake b) Various vegetable crops chelate heavy metal ions and create nutrient channels within plant c) Biostimulatory effects on citrus, avocado, banana, peach, raspberry and strawberry fruits	a) Varadarajan et al. (2002) b) Rickard (2000) , Lobato et al. (2011) , Olivieri et al. (2012) , Tambascio et al. (2014) , and Oyarburo et al. (2015) c) Rickard (2000) , Moor et al. (2009) , and Estrada-Ortiz et al. (2013)
7.	Silicon	Resistant to both environmental stressors (biotic and abiotic) thus eventually participates in plant cell wall formation and provides rigidity to plant. Thus in all maintains overall physiology of plant structure, from roots to shoots	Colla and Rouphael (2015)
8.	Vermicomposts	Vermicompost leachates hormone activity as a result of their high concentration of hormone-like trace elements like cytokinins, indole-acetic acid, eighteen gibberellins and brassinosteroids Vermicompost contains phytohormones belonging to three distinct classes, including auxins gibberellins and cytokinins	a) Aremu et al. (2014) b) Zhang et al. (2015)

outside the plant, where another biological agent such as fungi is involved.

Due to their promising properties, protein hydrolysates, one of the various compounds with biostimulatory activity, are the focus of scientific investigation. In fact, these compounds are made up of soluble peptides and amino acids that are mostly made by heating or enzymatically making proteins from animals or plants (Carillo et al., 2019). The positive impacts of these products are attributed to the stimulation of metabolites associated with plant growth processes and the induction of hormone-like activities, both of which influence plant growth and productivity (Ertani et al., 2009; Colla et al., 2017). These hormones are derived from indole ring such as auxins, which help in encountering salt stress by plants while cytokinins helping the plant during water stress, abscisic acid encountering chilling stress, gibberellic acid helping in ion partitioning, and salicylic acid helping in biomass accumulation (Egamberdieva et al., 2017).

Over the past few decades, seaweed extracts, which are another well-known classes of biostimulants, have gradually become increasingly utilized in farming (Craigie, 2011). These compounds have been used across various crops due to their capability to enhance crop performance, promote tolerance to abiotic stressors, and prolong the shelf life of numerous crop products (Battacharyya et al., 2015). They are generally made up of earthy-colored ocean growth-forming brown algae such as *Ascophyllum nodosum*, *Ecklonia maxima*, and *Macrocystis pyrifera* (do Rosário Rosa et al., 2021), and they contain advancing chemicals or minor components such as iron (Fe), copper (Cu), zinc (Zn), and manganese (Mn) (Sivasankari et al., 2006; Gupta et al., 2011). Other dynamic biomolecules, such as phloroglucinol and eckol, come from the earthy-colored ocean growth-forming brown algae *Ecklonia maxima*, one of the most well-known Kelp species and is utilized as fluid manure (with reduced viscous properties) (Rengasamy et al., 2016). Additionally, the production of carrageenan from *Kappaphycus alvarezii* or other extracts from industrially processed seaweeds (such as carrageenan) may both reduce the carbon footprint of the industrial sector and increase the value of seaweeds (Ghosh et al., 2015).

Trichoderma spp.-derived microbial biostimulants represent another significant category utilized in crop production. These biostimulants enhance plant nutrient levels and resilience to environmental stressors by stimulating root growth and facilitating iron uptake through the activation of ferritin genes (Figure 2). They also promote nutrient absorption and synthesize auxins and secondary metabolites such as peptides and volatile organic compounds (Vinale et al., 2013; Ahmad et al., 2015; López-Bucio et al., 2015; Fiorentino et al., 2018). On the other hand, a number of different fungi have shown biostimulatory activity on crops, boosting plant growth, yield, and oxidative stress response (Drobek et al., 2019). The presence of nitrogen, sulfur, phosphorus, and other essential elements in soil is regulated by various enzymes released by microbes and plants forming a complex system of plant-soil-microbe interaction. The enzymes of this interaction include acid and alkaline phosphatase, sulfur, phosphorus dehydrogenases, and urease activity (Kompala-Bąba et al., 2021).

On the other hand, despite their high biodiversity, very few plant growth-promoting bacteria (PGPB) are utilized in a variety of formulations (Olivares et al., 2017). Their actions may enhance tolerance to biotic stressors by inducing systemic tolerance, synthesizing volatile organic compounds, and leveraging the

presence of PGPB (Kloepper et al., 2004; Glick, 2014; Ruzzi and Aroca, 2015; Bertrand et al., 2021). The primary negative effects that abiotic stressors (such as salinity, drought, and heat) have on plants are alterations in the equilibrium of endogenous hormones (such as the production of ethylene, an increase in abscisic acid, and a decrease in cytokinin levels). As a result, the regulation of plant homeostasis necessitates a reduction in the growth of shoot and root (Yang et al., 2009). Because they encourage the production of indole acetic acid (IAA)—a natural auxin phytohormone—which, in turn, alters the architecture of the roots and causes them to develop, PGPB play a role at this crucial point, which results in a larger root area and more root tips. More meticulously, these extra (exogenous) phytohormones and the endogenous chemicals currently present in plant tissues manage cell multiplication, especially in the roots, working with the uptake of water and minerals from the dirt, which are important for plant development. Roots and shoots can communicate with one another through hormonal signaling by passing endogenous hormones from the xylem to the shoots, which act as hormonal sinks (Wang and Irving, 2011). In fact, the aerial parts of the plant are controlled by the roots. However, a number of additional phytohormones related to the soil microbiome have been discovered in the root-soil environment, in addition to the hormones that the plant may produce internally (Lu et al., 2021). These phytohormones might enter the plant through the xylem flow and manage plant development in light of their equilibrium. The soil microbiome, for instance bacteria and fungi, may also produce phytohormones, which serve as signals for root function (Lu et al., 2022). Plant roots may also produce phytohormones (Rosier et al., 2018). The equilibrium of these *ex-planta* hormones, which also interact with internal plant hormones to govern plant growth and development, is controlled by the synthesis and absorption of the roots, along with the synthesis, absorption, and degradation of hormones by soil microorganisms (Yang et al., 2009; Lu et al., 2021).

Investigation has proposed that substances resembling humic compounds, such as humic and fulvic acids, might exhibit biostimulant characteristics due to their capacity to imitate auxin and cytokinin (Pizzeghello et al., 2013; De Pascale et al., 2018; Puglisi et al., 2018). In general, humic compounds primarily enhance fruit quality and resilience to abiotic stress, promote root growth and morphology, boost nutrient uptake and nitrogen use efficiency (NUE), and enhance overall crop performance (De Hita et al., 2019; Vujinović et al., 2020). The kind of soil and the organic matter present in rhizosphere defines the rate of flow for movement of nutrients and humic acid desired by plants. This creates a synergism between the presence of bioactive compounds and plant growth. Auxins and other precursors are released when humic and fulvic acids are broken down, so these acids may also have hormone-like effects on plant growth (Olaetxea et al., 2018). The release of biomolecules resembling auxin suggested the hormone-like effect of the humic acid on tomato plants, a classical case of biological mimicry, wherein a metabolite having different functional groups represents another metabolite with in-original functional group (Jindo et al., 2012; Pizzeghello et al., 2013; Conselvan et al., 2018; Lucini et al., 2020). Due to the fact that humic-like substances and humic acids in particular can be obtained from a variety of unrefined components, such as regular natural matter, plant tissues, and biowaste, and they have varying effects based on their subatomic weight (Canellas et al., 2015; Scaglia et al., 2017).

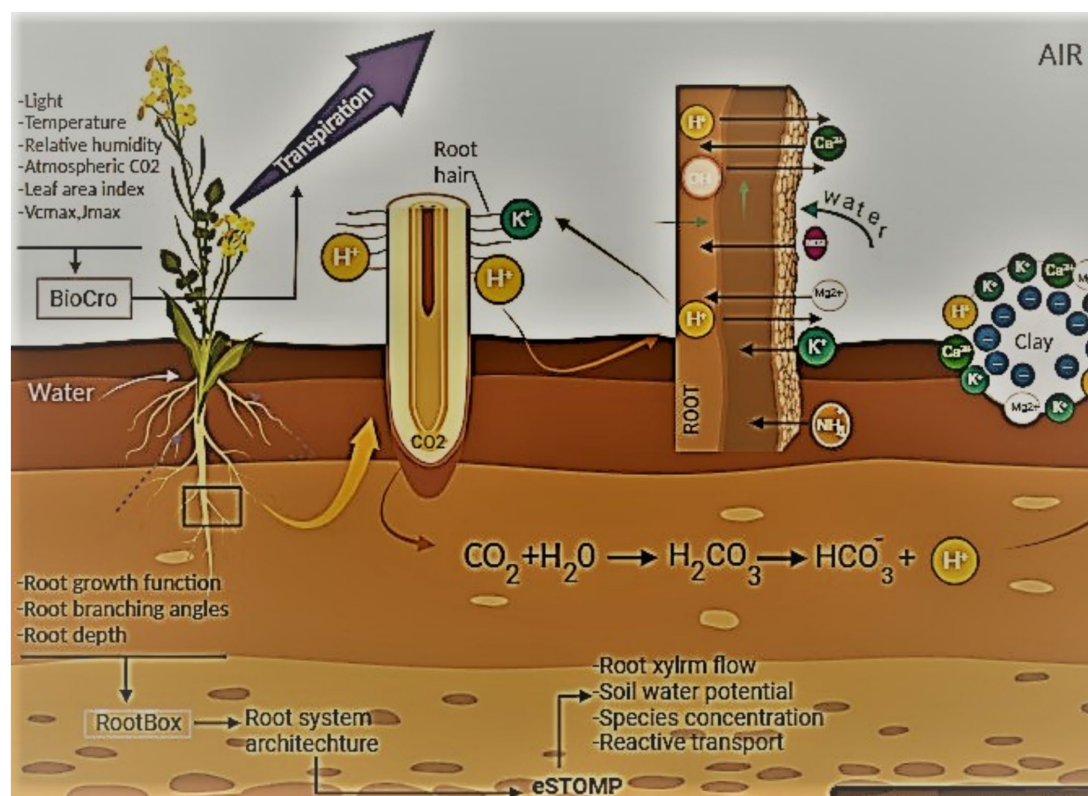


FIGURE 3

Role of humic materials in increasing soil's cation exchange capacity involves interaction between root membrane transporters and minerals and increases soil's cation exchange capacity. This figure illustrates the interactions going on at the level of soil and plant roots, where elements of root biology govern the biochemical interactions with soil's microenvironment.

vary significantly because of composition of chitosan. It is a biopolymer characterized by differing levels of deacetylation and polymerization. As a result, these variations may lead to diverse effects on crops (Malerba and Cerana, 2018). The biostimulant industry is actively exploring waste materials and by-products with significant biological potential, potentially introducing a new category that offers alternative approaches for managing by-products and complementing existing categories (Ugolini et al., 2015; Pane et al., 2016). In this unique circumstance, new biostimulatory items that might further develop plant growth through an auxin-like method of activity have shown promising outcomes on account of the creation of dissolved organic matter (DOM) through anaerobic processing (Messias et al., 2015). Shale water is produced when pyrobituminous shale rock is pyrolyzed. It has been demonstrated that shale water has significant biostimulant effects on horticulture crops and can be utilized as a biofortifying and yield-enhancing agent. Finally, other substances such as vitamins and melatonin have been shown to have biostimulatory effects particularly when subjected to abiotic stress. These substances not only encourage the biosynthesis of secondary metabolites but they also recover the functionality and quality of the final products (Hanson et al., 2016; Teklić et al., 2021).

2.3 Biosafety and ethics

The importance of any compound is defined by parameters such as necessity, employed subjectivity, safe to use, acceptance by people, and positive marketing response. Once a compound is demarcated

crucial and safe, its response of acceptance by public comes from ethical points. Since no animal-derived product is ensured herein yet, the endangered plant supplementary materials are forbidden for the implementation in PBs (see Figure 4).

Biosafety protocols should prioritize Environmental and Human Safety Indices (EHSI) and bioassays over taxonomic classifications (Vilchez et al., 2016; Barros-Rodríguez et al., 2020), especially given the developing nature of the biostimulant industry and the diverse range of substances and organisms that fall under this category. Additionally, careful consideration should be given to unintended consequences that may contribute to adverse effects on reactive nitrogen losses (Souza et al., 2019). One of the primary research topics for the key area of focus on biostimulant characterization of new substances and compounds is the (1) evaluation of the composition of the biostimulant, (2) production procedures that are uniform (3) the description of plant responses particularly when paired with the conditions of environment, (4) determining how biostimulant-containing products affect particular crops, and (5) fine-tuning of application timing and dosage (Toscano et al., 2021).

2.4 Biostimulant-generated chemical interactions in the biological system

2.4.1 Humic substances

Once a living counterpart falls off from its roots of existence and falls onto land, it is marked for decomposition as the current biological

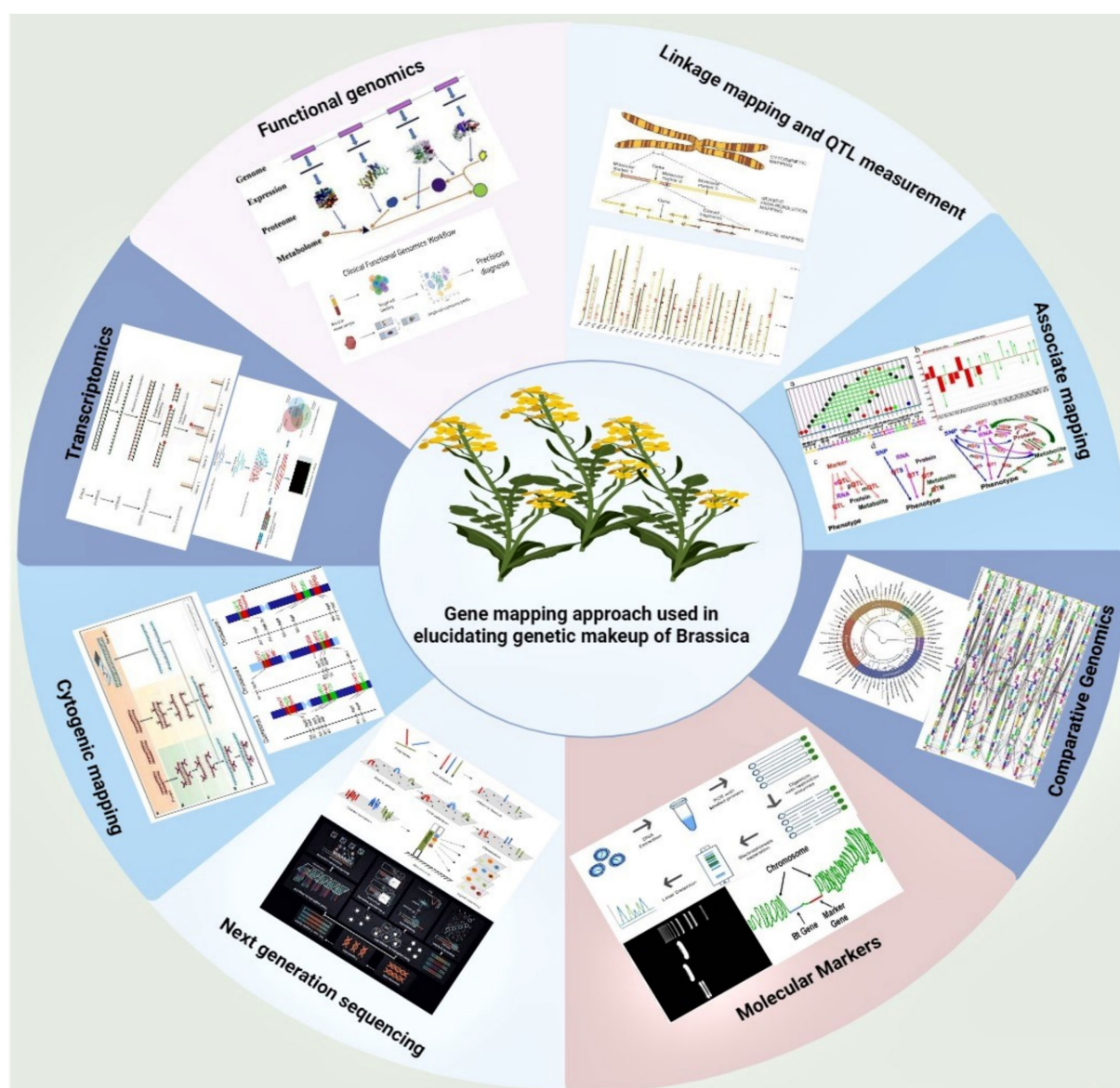


FIGURE 4

Elements of genetic sequence creation: genetic makeup of Brassicaceae deciphered by molecular approaches. This figure depicts the example of brassica and shows how the gene mapping helps in elucidating the complete molecular biology background of any concerned crop.

environment is oxidizing in nature as opposed to reducing one as that was during early evolution. Once the living organism is dead, it heads for decomposition by virtue of process of oxidation under natural environment. Since the oxidation means the loss of electrons, these electrons from decomposed material when released into soil creates an amorphous precipitate which may vary from black to brown in color. These precipitates originated from dead decomposed organic matter are collectively called as humic substances.

Franz Karl Achard noted solid HS formation while mixing a relatively basic material KOH (potassium hydroxide) in peat and extracting it with precipitated decomposed biomass that was naturally formed. This made the pivotal basis of preparing the alkaline extract from the soil. Johns Jakob Berzelius noted the aquatic HS formation from spring water. Moreover, the liquid and solid HS are natural compounds derived from decomposed biomass. However, basic functional groups such as phenol, carboxyl, and ester-related linkages

are common to both types. The nomenclature of HS is defined on the basis of insolubility at low hydrogen ion concentration, whereby HS becomes humic acid. Now, the humic acid is the biological accepted version of HS, governing important nutrient uptake and exchange within soil-plant-water ecosystem. More precisely quoting for humic acids, the microbial degradation and atmospheric oxidation of dead organic matter (in-essential) composed of variable fractions of cellulose (most abundant homopolysaccharide), lignins, and charcoal that give rise to humic acid. Several instances are discussed here which ascertain the enhanced nutrition-based growth of plants (Trevisan et al., 2010; Zanin et al., 2019).

2.4.1.1 Humic acid

- a. Anti-oxidant activity of any metabolite is representative of its extent of its ability to scavenge free radicals. Humic acid promotes the anti-oxidant activity in *Achillea millefolium* L.,

stimulating its content of phenols and flavonols and making it somewhat more tolerant to pathogens. Moreover, it enhances photosynthetic pigment biosynthesis, meaning that plants reside in its basic physiological parameters such as roots, leaves, flowers, and shoots. It is also attributed to fulvic acid (another derivative of HS but soluble at all scales of pH in water) (Bayat et al., 2021).

- b. Genetically, framing the exons by governing the regulation of protein bodies involved for acclimatization of plants induced by heat stress conditions is also another attributed functional scale of action of humic acid and HS. The case of *Arabidopsis thaliana* (Thale cress grass; model organism for plant studies) is peculiar, wherein in response to HS, the concentrations of additional proteins involved in cell trafficking, division, folding, degradation, reactions to heat and inorganic substances, and the cell wall increased, simultaneously reducing centralized distribution of carbohydrates and amino acids (Roomi et al., 2018; Cha et al., 2020).
- c. HS favored the higher yield of *Brassica napus* (a plant from family Brassicaceae with 372 genera), improved plant net photosynthesis, gas exchange rate, electron transport flux, oil quality, and gas exchange rate, and declined in linoleic, erucic corrosive, and solvent sugars (Lotfi et al., 2018; Nasiri et al., 2023). These all physiological parameters are devoid of *in-planta* action, where HS are found absent. Similarly, in another abiotic stress, such as under drought stress, *Capsicum annuum* L. rapidly decreased leaf stomatal conductance and transpiration rates while simultaneously increasing plant biomass and enhancing root development and photosynthesis as a result of an increase in the amount of chlorophyll (Qin and Leskovar, 2018). All these are attributed by the presence of fulvic and humic acids according to the changing value of pH of water.
- d. Proline is the amino acid known to be a marker for all types of biotic and abiotic stresses, and HS stimulate its concentration by activating the enzyme cofactors involved in its biosynthesis. Plant development was observed when studied under dry season pressure by *Echinacea purpurea* L., an expanded centralization of proline. Moreover, an enmarked increase in the content of phenols, flavonoids, total chlorophyll values was observed (Khorasaninejad et al., 2018).
- e. Providing with adequate increase in the amount of photosynthetic machinery, HS stimulate hardiness in the plant growth and yield of *Hordeum vulgare* L. (Abdelaal et al., 2018).
- f. *Rhododendron* spp. is a plant from native Himalayan ranges. A plant growing at this height of >1,200 meters is prone to oxidative damage by the environment. Moreover, chilling and freezing are commonly noticeable in such areas. HS-supplied enrichment is responsible in this plant for an increase in peroxidase genes (POD1) (Elmongy et al., 2020).
- g. Plant biomass and tuber yield are both increased by *Solanum tuberosum* and further created plant advancement, supplement transport, and photosynthetic limits under dry season tension, as attributed to the presence of HS (Man-hong et al., 2020).
- h. *Zea mays* makes nitrogen and water work better as it is a C₄ plant; the upregulation of qualities that are related to nitrate carriers, supplement retention, and the development of water is enhanced due to HS and humic acid (de Azevedo et al., 2019).

2.4.1.2 Fulvic acid

The important difference between humic and fulvic acids is that former is insoluble at low pH in water while the latter is soluble at all pH scales. The beneficial aspects derived from these two are the same except the fact that certain nutrients become bioavailable at a specified pH. Moreover, nature has compromised the nutrient availability to plant roots by creating a pH barrier, beyond which certain nutrients are accessible and non-accessible by plant metabolic molecular machinery. Prominent examples exist where the role of fulvic acid is largely involved in regulation aspects. Some examples are presented here:

- a. Improved germination parameters of *Beta vulgaris* alongside increased quantity of soluble sugar, root size, and yield (Braziene et al., 2021).
- b. By regulating genes related to ascorbate, glutathione, and flavonol biosynthesis, *Camellia sinensis* L. increased the antioxidant defense against water stress and the amount of chlorophyll and water in the leaf and reduced the ROS production in part of the supplementation by fulvic acid (Sun et al., 2020).
- c. Long withstanding agility and ability to harsh climate can overcome by *Hordeum vulgare* due to the presence of essence of fulvic acids (Braziene et al., 2021).
- d. Up-regulation of qualities related to early nodulation flagging, nitrogen digestion, supplement carriers, and hydrolases by *Medicago sativa*, increasing plant yield and biomass (Capstaff et al., 2020).

2.4.2 Protein hydrolysates

Plant biostimulants known as protein hydrolysates (PH) consist of a blend of peptides and amino acids, yielding positive outcomes in plant productivity (Colla et al., 2015). The PH can be classified according to their sources, originating from either animal material or plant material. Their impact is influenced by both their source and the physicochemical or enzymatic processes used to produce them (Colla et al., 2014). Animal protein sources primarily originated from animal by-products, such as epithelial tissue, collagen, feathers, leather, and fish remnants, while plant protein sources are obtained from plant residues such as alfalfa, seeds, legumes, and hay (Schiavon et al., 2008; du Jardin, 2015). PH sourced from animals predominantly contain a blend of free amino acids and peptides (Ertani et al., 2009; Colla et al., 2015). Some examples given below suggest the role PH in nature.

- a. Under dry season stress, *Brassica oleracea* expanded its photosynthetic rate and stomatal conductance and decreased in the negative effects of stress on gas exchange and transpiration rates (Kalużewicz et al., 2017) (as a result of induction from PH).
- b. *Diplotaxis tenuifolia* L. enhances biomass, chlorophyll biosynthesis, and plant yield with an increased photosynthetic rate and antioxidant activity in the leaves and also increased levels of nutrients and organic acids (Caruso et al., 2020) responded in positive sense to PH.
- c. The application of vegetal-derived bioactive compounds promoted the microbiome biodiversity in Lettuce, resulting in

increases in plant biomass and leaf chlorophyll (Luziatelli et al., 2019).

- d. Stomatal conductance and photosynthetic rate were both increased by *Olea europaea*, increased plant growth and biomass, and emphatically affected the sink-to-source proportion (Almadi et al., 2020).
- e. In *Solanum lycopersicon* L., the application of (biostimulant/PH) resulted in enhanced plant growth parameters such as biomass, chlorophyll content, and phenolic content. Additionally, there was an increase in root growth, photosynthetic rate, solvent sugar fixation, antioxidant activity, and nutrient uptake, along with improvements in primary metabolisms. Under drought stress conditions, treated plants exhibited increased biomass, transpiration rates, and stomatal conductance. Furthermore, the treatment helped maintain redox status, protected against oxidative stress caused by reactive oxygen species (ROS), and mitigated the accumulation of cytokines, jasmonic acid, indole-3-acetic acid (IAA), and antioxidants (Ertani et al., 2017; Michaletti et al., 2018; Sestili et al., 2018; Casadesús et al., 2019; Paul et al., 2019; Lucini et al., 2020).

2.4.3 Sea weed extracts

Prominent examples of sea weed extracts (SWE) are given below:

- a. *Ascophyllum nodosum* significantly enhances the ability of *Arabidopsis thaliana* to withstand oxidative stress and drought by reducing the accumulation of reactive oxygen species (ROS) and cellular damage. This is achieved through the downregulation of genes linked to stress-induced growth impairment while simultaneously boosting ROS scavengers, cell cycle, and division processes (Omidbakhshfard et al., 2019; Rasul et al., 2021; Staykov et al., 2021).
- b. Under drought stress, *Glycine max* L. increased chlorophyll content, antioxidant activity, photosynthetic activity, and efficiency and enhanced production and root growth through photo assimilation in response to *Echinochloa maxima* (do Rosário Rosa et al., 2021) under the stimulation of seaweeds.
- c. Multiple seaweed extracts induced an increase in the length of pollen tubes *Solanum melongena* L., as a result of rapidly fertilized ovules, which resulted in improved plant fruiting and flowering (Gaveliené et al., 2021).
- d. Plant biomass, protein, nutrient, and leaf phenolic compound concentrations are all increased in *Spinacia oleracea* L., with reported rate of increase in photosynthesis and production of chlorophyll and enhanced nutritional value (Rouphael et al., 2018a,b).

2.4.4 Microorganisms: plant growth-promoting rhizobacteria

Prominent examples of microorganisms, especially PGPR, are as follows:

- a. The leaves of *Amaranthus hybridus* L. had a higher concentration of nutrients, improved photosynthetic pigments,

nutritional quality, and plant growth under PGPR influence in rhizosphere (Ngoroyemoto et al., 2020).

- b. PGPRs when coexisting with *Brassica napus* contributed to increased seed fatty acid content, augmented plant biomass, improved yield parameters, and overall plant growth (Jiménez et al., 2020).
- c. *Brassica rapa* L. increased plant biomass, number of leaves, root length, and total IAA, P, and N contents (Widawati and Suliasih, 2020) in response to PGPR.
- d. Chlorophyll and NPK contents, plant biomass, and yield were undeniably expanded in *Cicer arietinum* L. in response to PGPR (Rafique et al., 2021).

3 Broad marketing scenario of PBs

As per the latest version drafted by the European Commission, non-microbial organic plant biostimulants encompass natural elements such as humic acid (HA), protein hydrolysates (PH), and seaweed extracts (SWE). The SWE segment controls 37% of the market, while the first two categories control half of the market. Humic substances such as humic and fulvic acids are naturally occurring organic molecules that are the result of the biological and chemical transformations of dead organic matter (Nardi et al., 2016). Most of the times, the humic substances are applied as soil soak but occasionally (fulvic acids) are applied as foliar application. According to du Jardin (2015), the chemical, physical, and biological properties of soils are influenced by humic substances, which have long been considered essential components of soil structure and fertility. The biostimulation effect of HAs on soil nutrient availability and uptake has been linked to a number of mechanisms that affect plant physiology and soil processes: (i) enhancing the structure of the soil, (ii) neutralizing the pH of the soil, (iii) enhancing the phosphorus solubility by interfering with calcium-phosphate precipitation and preventing leaching, (iv) triggering plasma membrane H⁺ ATPase activity, (v) enhancing lateral root induction and hair growth, and (vi) stimulating nitrate assimilation by upregulating the target enzymes (Delgado et al., 2002; García-Mina et al., 2004; Schmidt et al., 2007). According to du Jardin (2015), the biostimulatory action of HAs is significantly influenced by the conditions of soil fertility, with HAs performing better in soils with low organic matter content and low fertility (De Pascale et al., 2018). The wellspring with higher plant execution in light of HAs separated from humidified natural matter (e.g., peat), fertilizers, and vermicomposts instead of those approaching from fossil humus is another factor that contributes to the changeability in the effects of HAs. In addition to their influence on plant metabolism and physiology, both directly and indirectly, numerous studies have indicated the biostimulant properties of humic acids (HAs), particularly their ability to provide stress protection, notably against salinity and drought (García et al., 2012; Petrozza et al., 2014). Salt tolerance and drought tolerance may be mediated by reducing lipid peroxidation and hydrogen peroxide, increasing the amount of proline, and regulating gene expression in different ways, enhancing the chemical, microbiological, and physical properties of the soil and root growth (Calvo et al., 2014).

According to research claims, plant- and animal-based PH, which are composed of a mixture of free amino acids and oligopeptides and

polypeptides that serve as signaling molecules, make up a significant subset of organic non-microbial PBs. PHs are primarily used as foliar sprays, but they can also be used to treat seeds and drench substrates (Colla et al., 2015). In various studies conducted in both greenhouse and open field settings, PHs demonstrated notable effectiveness as plant biostimulants (PBs), triggering physiological and molecular mechanisms that enhance growth and productivity (Colla et al., 2017). The following factors directly affect the biostimulation activity and abiotic stress tolerance of PHs: key proteins engaged with N absorption (NR, NiR, GS, and GOCAT) and C digestion are set off, as are auxin- and gibberellin-like exercises, cancer prevention agent protein movement, shade biosynthesis, auxiliary metabolite creation, citrate synthase, malate, and isocitrate dehydrogenase (Schiavon et al., 2008; Ertani et al., 2009, 2017; Rouphael et al., 2017a, 2018a,b; Sestili et al., 2018). In addition to their direct impact, applying PHs as foliar sprays or substrate drenches also resulted in indirect effects on crop performance and nutritional status (Colla et al., 2014). Indeed, the utilization of PHs has led to enhanced nutrient uptake by expanding the effective soil volume utilized by the root system, notably through the augmentation of root hair diameter, density, and length (Colla et al., 2015).

Further investigation is required to fully understand the mechanisms and modes of action of organic non-microbial plant biostimulants, despite notable advancements in this field. This entails standardizing the raw materials, properties, and extraction techniques and determining the optimal timing, method, and dosage for application under various species and environmental conditions.

4 Advancements in next-generation sequencing

4.1 Components in a genetic sequence development

The genetic material of the plant cell is present inside the nucleus, is accessible by transcriptional and translational machinery of the cell, and is comprised of enzymes and other factors (proteins, carbohydrates, and lipids). Most of the coded information of the cell is displayed by a set of mRNAs (messenger Ribonucleic acid) in genetic elements called codons that ultimately and exclusively give rise to proteins only. However, genes concerned for glycobiology (study of carbohydrates) and lipidome (overall lipid fraction in a cell) are assigned to their function with protein counterparts, whose successive addition and deletion are regulated by a set of regulatory enzymes and genes concerned with the, and this creates a system of cell's function in relation to the environment that it perceives. The coordination among different chemical metabolites as a result of gene expression is displayed as a phenotypic expression. This journey of genotypic to phenotypic expression is called systems biology. Although dynamic in action, the equilibrium is well attained in terms of lipidomics, transcriptomics, proteomics, phenomics and genomics to govern a well-destined living function in terms of systems biology (Breitling, 2010) (Figure 4).

There are several steps in which a way is designed to assign a gene its function, which is scientifically called as annotation. Gene annotation (to assign a gene its function) is a solo feature behind all sophisticated techniques, which utilize the information coded in base

pairs in a stretch of nucleotide. To begin with the isolation of a gene, it has been advanced to a level where nanograms to attograms of DNA (deoxyribose nucleic acid) can be quantified by modern day polymerase chain reaction (PCR). Nonetheless, primers are available at biotechnology product synthesizing industry; the respective amplification is thought to be illuminated in the presence of fluorescent and non-fluorescent markers. However, certain gaps remain like whether PCR amplification is limited to a certain region of the whole gene or certain important regulatory elements are missing from a PCR product that would be the ultimate determinants of gene action and thus functional gene annotation. Gene cloning through vectors has shown us remarkable opportunities to create multiple copies of gene facets and their products. However, to go to the extent of demarcating the single point of gene action, the proper nucleotide base pair number, their relative position, and their length are needed to be met to ascertain specific gene annotation. The true criterion is to count of the number of base pairs and the area that they span the genome through base pair locations. These base pair locations are the sites from where nucleotides are counted in a direction ahead. The allotment of any of four base pairs like one after the other creates a specific sequence in which they are either repeated, non-repeated, overlapped, non-overlapped or may contain a stretch already with defined set of nucleotides. For all the sequencing technologies developed so far, the basic aim is to allocate the sequence of each nucleotide one after the other and generate a sequence, in which it is shown which nucleotide base pair follows the other. This has been achieved with chemical sequencing known as pen-name Maxam and Gilbert sequencing and Sanger sequencing (devised after the name of their inventors). Now, once the locations within a genome have been framed for base pairs, it is not like that a continuous stretch is likely to originate. The random base pairing, overlapping, and deletion (due to technical errors) are the problems commonly encountered while sequencing a fragment of genome. Modern day sequencing or, quite clearly, next-generation sequencing overcomes these limitations, but, to a particular point called sequence depth, segments of base pairs are unknown. The segments initialized for sequencing are aligned by creating a library, called gene library. In this gene library, the overall segments of a gene (intron and exon) from which nucleotides are extracted in base pairs and laid onto a platform of gel runs, followed by automated analyzers, define nucleotide (kind and type) on the basis of its resemblance to the structural output of a gene (Heather and Chain, 2016).

The need of hour is the more sophisticated sequence analyzers which can define sequence depth in an extendable manner. However, evident from current case scenarios is the fact that larger and pure gene library constructs are needed for successful sequencing of gene as the cross-contaminants are hindering agents in successful true-to-sequence generation.

The major focus has been on advancements concerned within and through these sequencing technologies, which have created guiding tools for any biological in-gene action metabolite function and biosynthesis. With biosynthesis, the metabolic pathway which already contains a set/series of enzymatic steps is controlled by in-gene action. Moreover, data are needed to be generated for successful gene annotation for each and every metabolite concerned. Agricultural production is often limited by the presence/absence of crucial metabolites, which ultimately define the quality parameter of a product. Glucosinolates and phytic acid in mustard are important

examples whose elevated and lowered concentrations due to in-gene action actually define the type and palatability of different genotypes and varieties of mustard (Das et al., 2022).

4.2 Sequencing strategies

The time period during which a particular sequencing technology has been invented is given a generation factor. Old sequencing technologies are tended to be summarized in first generation. Quite-a-new sequencing technologies are included in second generation. Relatively-new sequencing technologies are included in third generation and latest sequencing technologies are being put together in fourth generation sequencing technologies. The complete technical details of sequencing techniques concerned are far beyond the scope of present context, but an overall scale of technical implications has been included in the text. Starting from first generation, second generation, third, and fourth generation sequencing techniques, technical implications are being included here. Maxam–Gilbert and Sanger sequencing (also called di-deoxychain termination method) which are being used from 1980s onward are basic techniques with which sequencing progressed into a varsity of version.

4.2.1 Techniques concerned for second generation sequencing

4.2.1.1 454 sequencing or pyrosequencing

Pyrosequencing was first described in 1993 by Nyrén et al. (1993) who used streptavidin-coated magnetic beads, recombinant DNA polymerase devoid of 3' to 5' exonuclease activity (for proofreading), and luminescence detection with the firefly luciferase enzyme. To sequence single-stranded DNA, a mixture of three enzymes (DNA polymerase, ATP sulfurylase, and firefly derived luciferase) and a nucleotide (dNTP) is added. The incorporation of the nucleotide is controlled by measuring the light that is emitted. The incorporation factor for nucleotides, which indicates the number of complementary nucleotides on the template strand, is determined by the intensity of the light. Before the subsequent nucleotide mixture is added, the previous nucleotide mixture is withdrawn. This procedure is meant to be repeated with each of the four nucleotides until the DNA sequence of the single-stranded template is determined.

4.2.1.2 Illumina (Solexa): HiSeq and MiSeq sequencing

In 2006, Solexa Genome Analyzer was purchased and was available for sale in 2007 (Shendure et al., 2005). Before, it is observed as the best sequencing system ensuring >70% strength of the market, particularly with the HiSeq and MiSeq stages. With removable fluorescently labeled chain-terminating nucleotides, the Illumina sequencer, in contrast to Roche 454, produces a more cost-effective output than Roche 454 (Margulies et al., 2005; Metzker, 2010). By PCR span enhancement, called group age, into small provinces known as colonies, the clonally improved layout DNA for sequencing is made (Shendure and Ji, 2008). Sequencing data produce more data per run than other systems (600 Gb, Gigabites), more limited read lengths (approximately 100 bp; base pair), lower expenses, and run times that are essentially longer (3 to 10 days) (Liu et al., 2012). The Next Seq 500, HiSeq series 2500, 3000, and 4000, and HiSeq X series 5 and 10 with mid-to-high output (120–1,500 Gb), as well as the MiSeq, a small

laboratory sequencer with outputs ranging from 0.3 to 15 Gb and turnover rates suitable for targeted clinical and small laboratory applications, are a few of the sequencing machines that Illumina offers.¹ While the top of the line machines utilize the equivalent sequencing and Polony innovation, the MiSeq can deliver sequencing, which brings approximately 1 to 2 days (Liu et al., 2012). The most recent strategy of Illumina to manufacture long per-use application made the TruSeq nano resolving complex retro-transposably evident (McCoy et al., 2014).

4.2.1.3 SOLiD sequencing

Sequencing ligation is used in the Applied Biosystems SOLiD technology, which is now a Life Technologies brand. This method labels a pool of all possible oligonucleotides of a particular length in accordance with the position of the sequence. Annealing and ligation are conducted to oligonucleotides. Whenever the matching sequences are found, the DNA ligase enzyme performs ligation, and a signal indicating the nucleotide at that position is produced. Before the DNA is sequenced, amplification is performed with emulsion PCR. As a result, beads with one copy of the same DNA molecule are placed on a glass slide (Valouev et al., 2008). Amounts and lengths of the subsequent successions are similar to Illumina sequencing. It has been reported that sequencing palindromic sequences by ligation presents some challenges (Huang et al., 2012).

4.2.1.4 Polony sequencing

Polony, the first high-throughput sequencing system, was created at the Harvard George M. Church Laboratory. It was used to sequence the entire *E. coli* genome in 2005. It sequenced an *E. coli* genome utilizing a computerized magnifying lens, ligation-based sequencing science, an *in vitro* matched label library, emulsion PCR, and ligation-based sequencing science at an expense roughly one-tenth that of Sanger sequencing (Shendure et al., 2004). The advancement was approved to Agencourt Biosciences, which was appropriately transformed into Agencourt Individual Genomics and, in the end, included in the Applied Biosystems Strong Stage. Life Advancements, which is currently a division of Thermo Fisher Scientific, joined the project after Applied Biosystems.

4.2.1.5 Massively parallel signature sequencing

In the 1990s, Sydney Brenner and Sam Eletr's Lynx Therapeutics company developed massively parallel signature sequencing (or MPSS), a novel approach to high-throughput sequencing technologies. Bead-based MPSS reads the sequence in four-nucleotide increments using a complicated strategy of adapter ligation and adapter decoding. It was therefore vulnerable to sequence-specific bias or the loss of particular sequences. Lynx Therapeutics only performed MPSS "in-house" due to the complexity of the technology, and no DNA sequencing machines were sold to other laboratories. A partnership was formed in 2004 between Lynx Therapeutics and Solexa, which was later acquired by Illumina. This resulted in the creation of sequencing-by-synthesis, a more straightforward approach that was acquired from Manteia Predictive Medicine and rendered MPSS obsolete. The fundamental features of the MPSS output, which included hundreds

¹ <http://www.illumina.com/systems/sequencing.html>

of thousands of brief DNA sequences, were typical of subsequent high-throughput data types. These were utilized to the arrangement of cDNA (reciprocal DNA) for estimating levels of quality articulation in the MPSS study (Brenner et al., 2000).

4.2.2 Techniques concerned for third generation sequencing

4.2.2.1 Single molecule real time sequencing biosciences

The PacBio RS II sequencer and the SMRT ongoing sequencing framework are exchanged at Pacific Biosciences (Schadt et al., 2010). SMRT cells require 150,000 ultra-microwells at a zeptoliter to perform SMRT sequencing on a 10–21 scale, where a DNA polymerase enzyme-embedded particle is immobilized at the bottom of each well using a biotin-streptavidin framework in zero-mode waveguide (ZMW) nanostructures (biotin-streptavidin interactions are strongest non-covalent biochemical interactions). Nucleotides are added to the growing strand after fluorescently labeled dNTP analogs are incorporated into the single-stranded DNA template by immobilized DNA polymerase. Charged coupled device (CCD) cameras continuously detect the 150,000 ZMWs as a collection of distinct pulses that are transformed into the template sequences. Because all four nucleotides are added simultaneously and measured in real time, the speed of sequencing is significantly faster than that of technologies, in which individual nucleotides are flushed sequentially. Additionally, the read lengths were approximately 900 bp, and the initial accuracy was 99.31% (Metzker, 2010). Longer reads with an accuracy of more than 99.99% were obtained by employing SMRT bell templates, which circularized and sequenced the template multiple times (Travers et al., 2010; Koren et al., 2013). Once sequencing begins, the computational blade center of the system makes use of real-time signal processing, base calling, and quality assessment. The primary analysis software sends the data directly to secondary analysis software, SMRT Analysis, which can process sequencing data in real time. Read-length, distribution, polymerase speed, and quality measurement are all included in this data. Similarly, the optional examination instruments contain a full set-up of devices to investigate single-particle sequencing information for different applications.

4.2.2.2 Heliscope sequencing

The Helicos sequencing system, which Helicos Biosciences sold, was the first commercial implementation of single-molecule fluorescent sequencing (Shendure and Ji, 2008). The sequencing provider SeqLL currently sequences genomic DNA and RNA using the Helicos sequencing system and the Heliscope single-molecule sequencers. Cleavage of DNA is attained, followed by the addition of synthetic poly A sequences (in this case called tailing, as poly A sequences are attached at 3' ends) and finally hybridization to the surface of flow cell, achieving massive (billions of molecules) parallel sequencing. The oligo-dT50 bound on the outer layer of expendable glass stream cells is straightforwardly joined to the poly A-followed sections of DNA particles. When a picture of one nucleotide for every DNA grouping has been caught, the recurrent cycle is come by the joining of fluorescent nucleotides with an ending nucleotide. The procedure is then repeated until the fragments are fully sequenced (Eid et al., 2009; Travers et al., 2010). The sequencing system combines DNA polymerase-based synthesis sequencing with hybridization sequencing (Fuller et al., 2009).

The G + C (Guanine + Cytosine) content and size biases that have been observed in other technologies are virtually eliminated because sample preparation does not involve ligation or PCR amplification. The Heliscope sequencing reads have a mean length of 35 bases and range from 25 to more than 60 bases. Sequenced RNA creates quantitative transcriptomes of cell and tissues (Hart et al., 2010).

4.2.3 Techniques concerned for fourth generation sequencing

4.2.3.1 DNA nanoball sequencing

High-throughput DNA nanoball sequencing was used to help determine an organism's complete genomic sequence. The organization named Complete Genomics used this method to grouping tests presented by various scientists. Using rolling circle replication, small fragments of genomic DNA were amplified into DNA nanoballs. As a result, ligation-unchained sequencing was used to determine the nucleotide sequence (Drmanac et al., 2010). This method of DNA sequencing, as stated by Porreca et al. (2006), enables the sequencing of a large number of DNA Nano balls per run and is more cost-effective than other high-throughput sequencing platforms. Although each DNA nanoball only produces short DNA sequences, mapping the short reads to a reference genome is challenging. Numerous genome sequencing projects have used this innovation, and extra purposes are expected (Xu et al., 2019).

4.2.3.2 Nanopore DNA sequencing

DNA is transformed into an ion current by the method as it moves through the nanopore. This change is caused by the shape, size, and length of the DNA sequence. Each type of nucleotide prevents particles from moving through the pore for a different amount of time. Modified nucleotides are not required because the procedure is carried out in real time. Nanopore sequencing is also known as "long-read" or "third-generation" sequencing, just like SMRT sequencing. In the beginning, this method was based on exonuclease sequencing, in which electrical signals from nucleotides passing through alpha-hemolysin pores were covalently bound with cyclodextrin (Clarke et al., 2009). The current focus areas of nanopore sequencing are protein-based nanopore sequencing and solid state nanopore sequencing. Film protein structures such as hemolysin and MspA (*Mycobacterium smegmatis* Porin A) are used in protein nanopore sequencing, and their ability to distinguish between individuals and groups of nucleotides is extremely promising (Dela-Torre et al., 2012). In contrast, synthetic materials such as silicon nitride and aluminum oxide are used in solid-state nanopore sequencing due to their superior mechanical strength and chemical and thermal stability (Pathak et al., 2012). For this version of sequencing, a fabrication method is required because the nanopore array can contain hundreds of pores with diameters of below 8 µm.

The above described methods of sequencing the DNA are obliged to sophisticated instrumentation and require protective experienced handling. Table 2 shows the forums associated with next-generation sequencing technologies. Although most of the steps are automated, isolation of DNA, creation of library, constructs, and sample injection are key tasks to be performed before any sequencing analysis. The real-time analysis of PCR amplified products along with concomitant gene expression reveals the fact that all important metabolites are biosynthesized not all the times (Table 3). Their expression level varies

TABLE 2 Next-generation sequencing forums and platforms.

S. No.	Next generation sequencing forum	Platform	Platform characters/reads	Generation
1.	Ligation sequencing	SOLiD	>50 bp; <80 bp	2nd
2.	Synthesis sequencing	a) 454 Pyro-Sequencing b) Illumina Sequencing c) Ion Torrent	a) <1,000 bp b) <100 kbp c) <400 bp	2nd
3.	Single molecule long read	a) Bioscience (Pacific) b) Oxford Nanopore	a) <200 kbp b) Precise range (8–20 kbp)	4th

TABLE 3 Tissue time-specific expression of genes in plant/plant tissue in relation to metabolite production.

S. No.	Plant/plant tissue	Metabolite	Time	Reference
1.	<i>Hordeum vulgare</i>	a) Glycerophospholipids & polyketides b) Chlorogenic acid & hydrocinnamic acid	a) Seed germination b) 3-leaf stage	a) Gupta et al. (2019) b) Piasecka et al. (2017)
2.	<i>Oryza sativa</i>	a) Isoleucine, phenylalanine & spermidine b) Lutein & beta-carotene	a) Flowering b) Maturation stage	a) Roessner et al. (2006) and b) Zarei et al. (2018)
3.	<i>Triticum aestivum</i>	a) Ubiquinone, guanine & chorismate b) Phenolamides & polyphenols	a) Mature leaves b) Mature kernels	a) Matthews et al. (2012) and Michaletti et al. (2018)
3.	<i>Zea mays</i>	a) Asparagine, glutamic acid & inositol b) Anthocyanins & methoxylated flavonoids	a) 8-month old kernel b) Mature kernel	a) Barros et al. (2010) b) Jin et al. (2017)
4.	<i>Sorghum bicolor</i>	Cyanogenic glycosides	4-leaf stage	Mareya et al. (2019)

with time according to environmental setup assigned to the cell by nature. Moreover, to quantify these types of metabolites, the important responsible genes (called candidate genes) are monitored throughout the isolation and sequencing steps for plant biostimulatory studies.

To target the candidate genes, at precise gene locus (the position where a particular known segment of gene lies within the DNA segment), CRISPR/Cas9 technology has paved the fruitful results by paving the way for *de novo* addition of gene within the precise functional boundaries of gene promoters and other operational elements of genetic segment. The scientific community discusses different scenarios arising by incorporating the utilization of CRISPR/Cas9 enzymatic system (Cas9 is a kind of caspase) or using the jumping genes action (whereby genes switch locations within a genome; may be inverted segmentation) that may prove devastating in terms of unknown and unexpected gene transfer. However, research has shown very few negligible chances of such a genetic recombination event (due to the presence of spacer in-between *de novo* segments) but cannot be ignored. The benefits of CRISPR/Cas9 are diverse such as short breeding cycle attainment within the same range of atmospheric conditions and higher yield (Ma et al., 2014).

4.3 The potential of next-generation sequencing

The dynamism of agriculture sector is relied also on the crop improvement programs. These include breeding better crops, bio-fortifying crops, understanding the complete genetic make-up, and functional regulation of genes involved. Of all these, it is the functional regulation of genes whose deeper understanding is required in order to fill in the gaps between different sectors of system biology of the concerned agriculturally economic important product.

In case of the role of next-generation sequencing technologies, the inputs provide ascertain fresh gene annotations, thereby focusing the paradigm of information toward the metabolite; so the production may reveal the crucial points of biochemical interactions. Similarly, nickel (Ni^{2+}) is the element that binds phytate molecules in crystalloid bodies of mustard seed and enhances the action of phytic acid in lowering the bioavailability of proteins present when included in human diet. Moreover, a deeper understanding of phytic acid genetic elements becomes crucial and, for that gene sequence, must be validated out. That validation is supported by next generation sequencing methodologies, whose tissue-specific gene expression is highly favored in that area of seed. Moreover, the regulation of other enzymes concerned with Ni^{2+} (divalent in this case) can be linked to switching ON/OFF expression of those genes that provide enzymatic machinery for phytic acid biosynthetic pathway. This establishes a picture of *in vivo* functioning molecular machinery in *in silico* mode. However, to reach that point, one has to start with next-generation sequencing technologies, whose revilement of genetic sequences makes other bioinformatic steps possible (Liu et al., 2023).

The word “next-generation sequencing” depicts the sequencing techniques apart only from the first generation sequencing methodology (Qin, 2019).

5 Revealing the pioneering molecular mechanisms for the production of biostimulants by sequencing strategies

The technique of isotopic labeling provides much tricky shortcut revealing about the presence of biochemicals, especially secondary metabolites as major biostimulants with the first one drafted in late 1950

(Bentley, 1999). However, how gene products are synthesized within the cellular locations is a mystery until researchers utilize gene expression models. Thereby, it becomes an important milestone to cover for, and the story began with landmark groundbreaking articles gifted to the scientific world by logistic scientific minds from 1984 to 1991 (Malpartida and Hopwood, 1984; Cortes et al., 1990; Donadio et al., 1991). By this time, it became clear with the advent of rise in molecular biology gene annotation tools viz., nucleotide cloning and sequencing strategies, which were searched in stretch of DNA obtained from Actinomycetes bacterial species that are responsible for the production of secondary metabolites serving as biostimulants. Actinomycetes are naturally resistant to environmental extreme conditions such as extreme temperature and pH, and it is the production of secondary metabolites that make them tough in response to adverse environmental conditions. This clears the picture of the importance of secondary metabolites. On one hand, secondary metabolites fulfill the role of bacterial survival, and on the other hand, they serve as biostimulants. These molecular revealing are made through high-throughput genome sequencing strategies. Thereby adopting the next-generation sequencing strategies, the genes were found to be organized in gene clusters, the very first one deciphered was that of a fungus, *Penicillium chrysogenum*. The clusters identified were associated with the synthesis of penicillin (a broad range antibiotic, acting as biostimulants by killing heaps of pathogenic bacteria) and an enzyme named L- δ -(α -amino adipoyl)-L-cysteinyl-D-valine (responsible for large-scale peptide synthesis by a nano-meter scale fungi cell, acting as biostimulants by sustaining the growth of such fungal species by producing the enzyme) (Díez et al., 1990). Similar molecules serving as biostimulants were found to be mycotoxins (metabolites produced by fungi responsible for eliminating pathogens of plants). Interestingly, these molecules falls under the class of terpenes and terpenoid-related compounds, of which the special mention is trichothecenes (Hohn et al., 1993). Like penicillin acting as a broad-range antibiotic, the aflatoxin, broad-range anti-fungal compound synthesis, is perceived by *Aspergillus* sp. (Yu et al., 2004).

The cosmid libraries were first used before pre-genomic era, where knocked-out mutants were screened in fungi (Mayorga and Timberlake, 1990; Hendrickson et al., 1999). This was followed by an insertional mutagenesis approach using plasmid recovery (Yang et al., 1996; Chung et al., 2003). However, these are the cDNA expression library, reverse transcriptase polymerase chain reaction (RT-PCR), and subtractive hybrid PCR with suppression analysis, which changed the secondary metabolite screening approach upto the gene level and took it to the accurate prediction of the products at the gene cluster level (Beck et al., 1990; Linnemannstöns et al., 2002; O'Callaghan et al., 2003). These represented a trait of the organism marked by a particular gene or gene cluster level. However, what has limited the screening strategy here is the large genome size which requires high throughput methods. Moreover, the partial gene clusters were selected to deal with candidate genes like that of polyketide production, which naturally influenced the rhizosphere to be a biostimulant in root zone. The development of the PCR methods such as degenerated primers at both flanking ends of DNA revealed the conserved ketosynthase enzymatic domain with many functions to play for with a slight change in the secondary structure of protein domains and motifs and represented by a single nucleotide polymorphism. This opened the gateways for further cosmid library screening, and finally, large dataset gene clusters were screened to a single change in nucleotide stretch (Chooi and Tang, 2012). These

methods revealed the production of fumotoxins (a type of mycotoxin inhibiting pathogenic bacteria growth) and aflatoxins with various classes and sub-classes, thereby making it easy to understand the functional dynamism of gene clusters. To contribute to phylogenomic analysis, the pioneering steps were taken, which resulted in polyketide gene synthesis with eight genome lineages widely spread in fungal kingdom. The use of degenerated primers and cosmid library screening was helpful as they identified partial gene clusters. All these steps were taken prior to whole genome sequencing, as the overlapping fragments may represent the false-positive target hits. Some examples of the fungal biostimulants expressed by candidate genes that were screened are tromin, tryptoquivaline, echinocandinin, and prenyl atedxanthones, which were obtained from *Dothistroma septosporum*, *A. clavatus*, *Emericella rugulosa*, and *A. nidulans*, respectively (Gao et al., 2011; Cacho et al., 2012). To add to the complexity of biostimulants, there are some overlapping genes which tend to produce a different product but with efficient biostimulant capability. Some examples are fumagillin, pseurotin, and fumitremorgin (Wiemann et al., 2013). The illustrative pioneering studies were performed in genomes of the genus *Neurospora*, *Botrytis*, and *Cochliobolus* (Kroken et al., 2003).

To add to the knowledge of screenings of biostimulants produced by animals and plants, there is a need to identify secondary metabolites involved and the gene clusters, if present for such isolates and exudates (Vioque et al., 2000). Since the bio-degradation potential and sub-fractionation are more commonly associated with plants and animal-led biostimulants, the approaches must target individual gene expression patterns in relation to real-time PCR analysis. These are some of the reasons why the mechanisms behind biostimulant-led secondary metabolite action are not much known in plants and animals, as they are by-products of secondary metabolite or the bio-degradation byproduct of secondary metabolites, which are not specified due to a diverse array of biochemicals involved. In case of the role of next-generation sequencing technologies, the connecting links could be established between gene, gene product, by-product enhanced in nature, and, finally, the biostimulant action.

6 Challenges, future prospects, and conclusion

By focusing on the atomic systems that underpin the observed exercises, ranchers will also find it easier to separate the physiological and plant digesting routes involved in this cycle. In addition to being a promising practice that is good for the environment, the use of biostimulants may decrease the usage of agrochemicals (such as mineral fertilizers and chemicals for controlling pests and pathogens) and raise the efficiency with which natural resources are utilized. Additionally, it has the potential to increase the amount of food that is available to feed the world's expanding population and the sustainability of agricultural and horticultural production systems. The most recent information on the categorization of bio-stimulatory agents, their main mechanism of action, and their practical uses in agricultural productivity are presented in this article. Despite the fact that there are many situations in which the administration of a biostimulant benefits the plant and production, more research is needed for the application techniques because it seems necessary to address product and crop specificities.

The variable effects that have been described in the literature are a result of uncertainties in application times, procedures, and doses for biostimulants, which are natural matrices that contain a variety of chemicals from different classes and activities. Last but not least, the crop factor is also important because the genotype greatly influences how people react to biostimulant products, particularly in situations when reactions are unknown and the conditions necessary for the same are present. Taking into consideration the past, new biostimulant products are necessary since the creation and use of biostimulant is a developing relationship. However, this should be addressed utilizing a different approach that emphasizes the synergistic benefits of many biostimulatory specialists rather than administering a single item. By focusing on the atomic systems that underpin the observed exercises, ranchers will also find it easier to separate the physiological and plant digesting routes involved in this cycle. In addition to being a promising practice that is good for the environment, the use of biostimulants may decrease the usage of agrochemicals (such as mineral fertilizers and chemicals for controlling pests and pathogens) and raise the efficiency with which natural resources are utilized. Additionally, it has the potential to increase the amount of food that is available to feed the world's expanding population and the sustainability of agricultural and horticultural production systems.

The various secondary metabolites secreted by plants are of immense importance to humans as they are utilized as drugs against potent diseases, eradicate pathogenic microorganism from the soil which prevents the stimulatory growth of plant, may be used to cure viral infections within the plant-soil system, and regulate the moisture levels in maintaining the humidity. Apart from the above mentioned biological significance imparted by PB, their importance extends to the level of pest control as insecticidal, nematicidal, and fungicidal agents present in nature, which is regulated by nature and produced by plants.

Innovative technologies are continued to work on showing the entire genomic sequence in order to expand the screening of PBs. The availability of the metabolite is ultimately determined by quantification once it has been genetically ruled out and its expression yield has been determined molecularly. The possibilities for investigating PBs are virtually limitless, starting with chromatographic techniques such as Ultra High Performance Liquid Chromatography (UHPLC) by Corporation and continuing through the analysis between nuclear magnetic resonance (NMR) and X-ray crystallography.

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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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Evaluating the efficacy of microbial antagonists in inducing resistance, promoting growth, and providing biological control against powdery mildew in wheat

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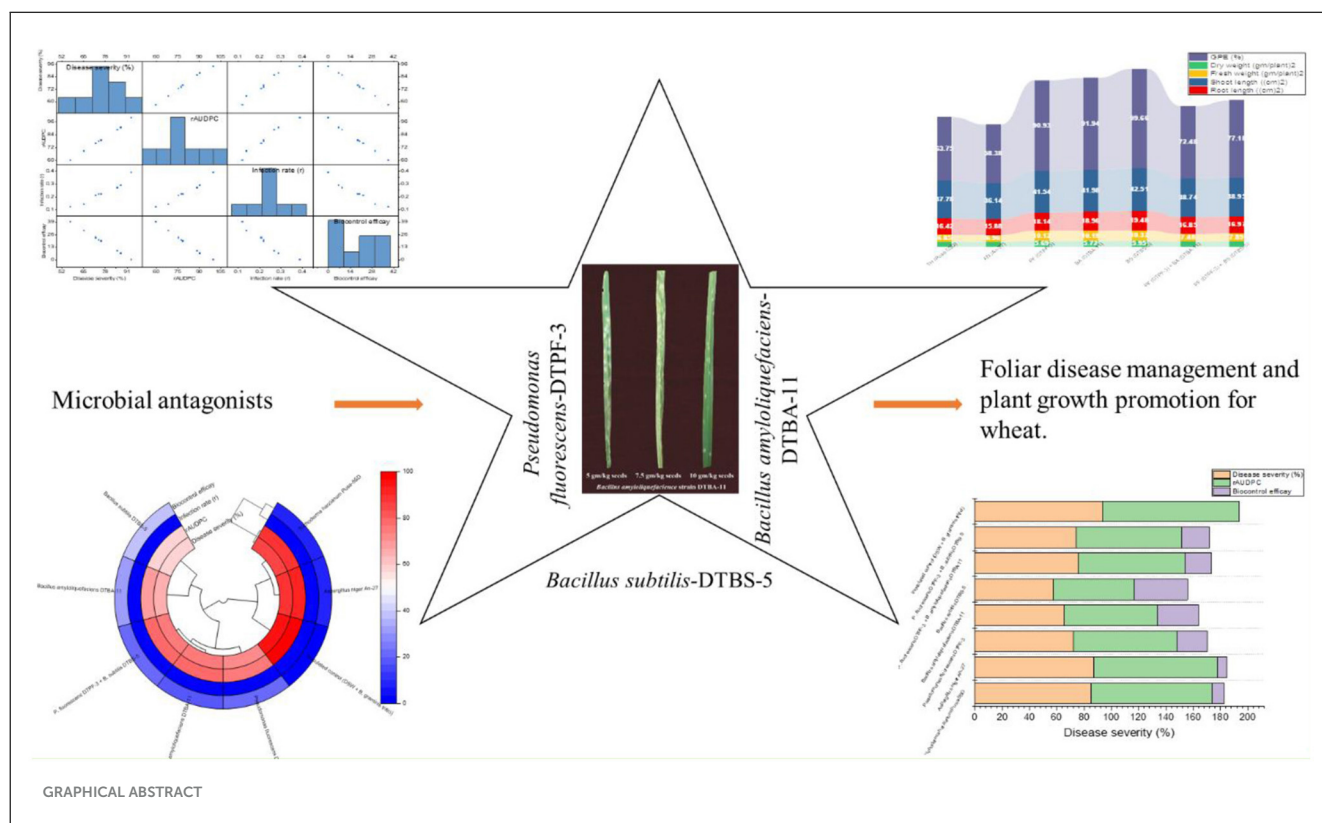
This study evaluates the biocontrol efficacy of three bacterial strains (*Pseudomonas fluorescens* DTPF-3, *Bacillus amyloliquefaciens* DTBA-11, and *Bacillus subtilis* DTBS-5) and two fungal strains (*Trichoderma harzianum* Pusa-5SD and *Aspergillus niger* An-27) antagonists, along with their combinations at varying doses (5.0, 7.5, and 10.0 g/kg of seeds), against wheat powdery mildew. The most effective dose (10 g/kg seeds) was further analyzed for its impact on induced resistance and plant growth promotion under greenhouse conditions. The study measured defense enzyme activities, biochemical changes, and post-infection plant growth metrics. All tested microbial antagonists at 10 g/kg significantly reduced PM severity, with *B. subtilis* strain DTBS-5 outperforming others in reducing PM severity and achieving the highest biocontrol efficacy. It was followed by *B. amyloliquefaciens* strain DTBA-11 and *P. fluorescens* strain DTPF-3, with the fungal antagonists showing no significant effect. Wheat crops treated with *B. subtilis* strain DTBS-5 exhibited substantial increases in defense-related enzyme activities and biochemicals, suggesting an induced resistance mechanism. The study found a 45% increase in peroxidase (POD) activity, a 50% increase in catalase (CAT) activity, a 30% increase in phenolic content, and a 25% increase in soluble protein content in the wheat plants treated with microbial antagonists. The study highlights the effectiveness of microbial antagonists, particularly *B. subtilis* strain DTBS-5, in managing wheat PM through biocontrol, induced resistance, and enhanced plant growth, offering a sustainable alternative to chemical treatments.

KEYWORDS

wheat, microbial antagonist, biocontrol, induce resistance, growth promotion, polyphenol oxidase (PPO), *Pseudomonas fluorescens*, *Bacillus amyloliquefaciens*

Introduction

Wheat (*Triticum* species) is considered a major food source for millions of people worldwide. The increasing global population necessitates additional food production, especially in India, which urgently needs to transform its strategies to boost wheat production and productivity. The sustainable productivity of wheat is of utmost importance due to numerous biotic constraints that affect



grain quality and limit production and profitability. Powdery mildew (PM), caused by *Blumeria graminis* f.sp. *tritici* (), is a very serious disease that follows the three rusts in importance and can limit yield and cause significant economic losses in wheat globally (up to 25%) (Mwale et al., 2014; Basandrai and Basandrai, 2017). Common methods for reducing PM disease include the development of host resistance and the use of fungicides. However, because pathogen virulence and host resistance evolve simultaneously, qualitative resistance quickly loses its efficacy (McDonald and Linde, 2002). Additionally, there are reports of the emergence of fungicide-resistant strains in the pathogen population (Xia et al., 2002; Yang et al., 2011). The widespread and injudicious use of fungicides can also lead to environmental contamination and health risks. Thus, the need to search for non-chemical alternative methods that provide eco-friendly and economical control of wheat PM has become important. As a cost-efficient and environmentally acceptable solution for PM management, biological control agents (BCAs) reduce the likelihood of fungicide-resistant strains emerging. BCAs are becoming increasingly popular as a successful alternative control method. They provide an additional strategy that may be included in integrated management programs for wheat PM disease to increase protection while maintaining profitability and production. To date, numerous microbial antagonists have been reported to be effective in biocontrolling several PM diseases (Elad et al., 1996; Kiss, 2003; Romero et al., 2004), but very few are currently available on the market as bioformulations/commercial products in several countries (Shishkoff and McGrath, 2002; Punja and Utkhed, 2003). However, the majority of the studies and

uses of BCAs have largely been focused on the PM disease of greenhouse vegetables and ornamental crops (Paulitz and Belanger, 2001; Giotis et al., 2012; Medeiros et al., 2012), with very little information available on the uses of microbial antagonists in the biocontrol of wheat PM pathogens (Gao et al., 2015; Russ et al., 2021). Therefore, studies on the evaluation and identification of effective microbial antagonists for bioprotecting wheat against PM disease have become vital. The current study aims to assess the efficacy of several microbial antagonists in bioprotecting plants by inducing systemic resistance and promoting plant growth against *B. graminis tritici* (Bgt).

Materials and methods

Microbial antagonist collection source

This study used five talcum-based bioformulations of different microbial antagonists (two fungal and three bacterial origins) as bioinoculants. The fungal strains *Trichoderma harzianum* Pusa-5SD (TH-Pusa-5SD) (MTCC No.5371) and *Aspergillus niger* An-27 (AN-An-27) were obtained from the Pulse Pathology Laboratory and three bacterial strains *Pseudomonas fluorescens* DTPF-3 (PF-DTPF-3) (KP017263), *Bacillus amyloliquefaciens* DTBA-11 (BA-DTBA-11) (KF850150), and *Bacillus subtilis* DTBS-5 (BS-DTBS-5) (JQ688022) were obtained from the Plant Bacteriology Laboratory of the Division of Plant Pathology, ICAR-IARI, New Delhi.

Phytopathogen cultures

The pathogenic culture of *Blumeria graminis* f. sp. *tritici* (Bgt) was used as an inoculum. These cultures were a mixture of highly virulent and predominant Bgt cultures. The mixed conidial inoculum of Bgt cultures was obtained from the Rice and Wheat Research Centre, C.S.K. Himachal Pradesh Agricultural University, Malan, Himachal Pradesh, India. The mixed conidial inoculum of Bgt cultures was maintained and increased on the highly susceptible wheat cultivar Agra Local in a temperature-controlled growth chamber, following standard protocols and procedures (Pathania et al., 1996).

Plant material

The pure seeds of the wheat cultivar PBW 343 were obtained from the Punjab Agricultural University's Department of Plant Breeding and Genetics in Ludhiana, Punjab, India.

Biological control efficacy of microbials against *B. graminis tritici*

The selected talcum-based bioformulation of all five microbial antagonists and their consortia at three different doses, 5.0 g, 7.5 g, and 10.0 g/kg of seeds, were preliminarily screened for their potential in bioprotecting wheat against PM disease under temperature-controlled greenhouse conditions at the Division of Plant Pathology, ICAR-IARI, New Delhi, India.

Growing seedlings, seed treatment, and pathogen inoculation

Seed sterilization

Wheat seeds were surface sterilized by immersing them in 0.1% sodium hypochlorite for 2 min. This was followed by three rinses with sterile distilled water to remove any residual sterilant. The seeds were then air-dried.

Soil preparation

Field soil was collected from the experimental farm of the Department of Plant Pathology, ICAR-IARI, New Delhi, India. The collected soil was used as the potting medium for this study. To ensure sterility, the soil was autoclaved twice at 24-h intervals at 121°C and 15 psi for 20 min each time.

Seed coating

The dry, surface-sterilized seeds were coated with 0.2% carboxymethyl cellulose (CMC) to act as an adhesive before sowing. Following the coating, the seeds were treated with talcum-based bioformulation of microbial antagonists at a concentration of 10^8 cfu/g. The bioformulation was applied at three different doses: 5.0 g, 7.5 g, and 10.0 g per kg of seeds. For the coating process, the seeds

were placed in a container, and the bioformulation was sprinkled on them. The seeds were then gently shaken to ensure a uniform coating. The microbial-coated seeds were subsequently scattered on a Petri dish and air-dried overnight at $24 \pm 2^\circ\text{C}$.

Provax coating

The seeds treated with Provax-200 were prepared by mixing the fungicide at a concentration of 0.3% (w/w) with the seeds. The seeds were placed in a container, Provax-200 was added, and the mixture was gently shaken to ensure an even coating.

Sowing and growing conditions

The treated seeds were sown in greenhouse pots (15 cm in diameter) filled with the sterilized soil. Four pots, each containing 10 seeds, were prepared for each microbial antagonist treatment and their consortia and for the control treatments. The experimental design was a randomized complete block design (RCBD), and the experiment was conducted twice. The pots were maintained in a temperature-controlled greenhouse at $22 \pm 2^\circ\text{C}$, 50–70% relative humidity (RH), and a 12-h daylight cycle. The plants were irrigated on alternate days.

Pathogen inoculation

At the tillering and boot leaf stages, the seedlings from each treatment and all replications were challenge-inoculated with a conidial suspension of a mixture of *Blumeria graminis* f. sp. *tritici* (Bgt) cultures. The inoculation was performed using a hand sprayer, following the standard procedure described in Paul et al. (1999).

Control treatments

The seeds treated with sterile distilled water only served as the negative control (inoculated with Bgt) and the positive control (untreated and uninoculated healthy plants). The seeds treated with Provax-200 at a concentration of 0.3% (w/w) were used as a fungicide-treated check.

Disease scoring and quantification

The disease developments on each plant in all the replications were scored from 14 days post-inoculation (dpi) with *B. graminis tritici*, following the 0 to 9 scale described by Saari and Prescott (1975). Observations were taken at four time points: 14 dpi, 21 dpi, 28 dpi, and 35 dpi. Disease severity (%) was calculated using the following formula:

$$\text{Disease severity (\%)} = \frac{\text{Proportion of leaf area infected}}{\text{Total leaf area assessed}} \times 100$$

The area under the disease progress curve (AUDPC) for powdery mildew disease development on each treatment

was calculated using the following formula (Milus and Line, 1986):

$$AUDPC = \frac{(X_1 + X_2)}{2} \times (t_2 - t_1) + \frac{(X_2 + X_3)}{2} \times (t_3 - t_2) + \frac{(X_3 + X_4)}{2} \times (t_4 - t_3)$$

where X_1 , X_2 , X_3 , and X_4 refer to the disease severity recorded at 14, 21, 28, and 35 dpi, respectively.

N_1 is the interval day between X_1 and X_2 , and N_2 is the interval day between X_3 and X_4 .

The relative area under the disease progress curve (rAUDPC) was calculated using the following formula:

$$rAUDPC = \frac{\text{line/genotype AUDPC}}{\text{susceptible AUDPC}} \times 100.$$

The apparent infection rate (r) was estimated using the following formula (Van der Plank, 1963):

$$r = \frac{1}{t_2 - t_1} \left(\log_e \frac{X_2}{1 - X_2} - \log_e \frac{X_1}{1 - X_1} \right),$$

where X_1 is the disease severity recorded at date t_1 , X_2 is the disease severity recorded at date t_2 , and $t_2 - t_1$ is the interval in days between these dates.

Biological control efficacy

The biocontrol efficacy of microbial antagonists was calculated using the following formula (Guo et al., 2004):

$$BCE = \frac{D_C - D_T}{D_C} \times 100,$$

where D_C is the disease severity (%) of the infected control, recorded at 14, 21, and 28 days post-inoculation (dpi).

D_T is the disease severity (%) of the treatment group, recorded at 14, 21, and 28 days post-inoculation (dpi).

Determination of microbial antagonists for induced resistance abilities

The dose of microbial treatments that showed maximum biocontrol efficiency against powdery mildew disease during preliminary tests was further studied for their induced resistance abilities in wheat cv. PBW 343 upon infection with *B. graminis tritici*. The assessment of various defense-related enzymes, including peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT), and chitinase, along with biochemical tests and total phenol content, was conducted on the wheat seeds treated with microbial antagonists (using seed soaking treatment). These assessments were performed upon infection with *B. graminis tritici* using the most effective dose of the microbial antagonists and their consortia (10 g per kg of seeds). The experiments were carried out under potted-plant conditions in a temperature-controlled greenhouse at the

Division of Plant Pathology, ICAR-IARI, New Delhi. Leaves (1 g each) from each treatment in all the replications were taken at different time points, 0, 24, 48, 72, 96, and 120 h post-inoculation (hpi) with *B. graminis tritici*. After homogenizing the leaf samples with 10 ml of phosphate buffer (pH 6.8, 0.1 M), the samples were split into two equal pieces, each holding 5 ml. One part was centrifuged for 15 min at 4°C at 12,000 rpm, and the clear supernatant was used as a source of enzyme. The other 5-ml portion was taken for biochemical activity estimation and analysis.

Estimation of peroxidase activity

Peroxidase (POD) activity was quantified using Kashyap et al. (2021)'s protocols. The enzymatic source material was obtained from the assay's supernatant. The composition of the reaction mixture included 1.5 mL of 0.05 M pyrogallol, 0.5 mL of extracted enzyme solution, and 0.5 mL of 1% H_2O_2 , which was subjected to incubation at a controlled temperature setting of 28°C, allowing a fluctuation range of $\pm 2^\circ C$. Initial optical density readings of the reaction mixture were standardized to a baseline of zero at 420 nm wavelength using a spectrophotometric device, with subsequent measurements of optical density alterations occurring at 20-s intervals for a duration of 3 min. A heat-inactivated enzyme preparation served as the negative control within this experimental framework. POD activity levels were deduced from the rate of optical density variation within the reaction mixture, denominated as the alteration in optical density per minute per gram of protein present in the fresh tissue sample.

Estimation of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was quantified following the methodology of Mayer et al. (1965). The assay's reaction mixture comprised 1.5 mL of 0.1 M sodium phosphate buffer at a pH of 6.5 and 200 μL of enzyme extract. The initiation of the enzymatic reaction was marked by the addition of 200 μL of 0.01 M catechol. The reaction mixture was incubated at ambient temperature, and the absorbance was measured at a wavelength of 495 nm to determine PPO activity. Absorbance variations were systematically recorded at 30-s intervals over a span of 2 min. The measure of PPO activity was articulated as the rate of absorbance change per minute per gram of protein in fresh tissue samples.

Estimation of catalase activity

Catalase (CAT) activity assessment was performed following Chandlee and Scandalios (1984)'s protocols, incorporating minor adjustments. The supernatant served as the source for the enzyme assay. The enzymatic breakdown of H_2O_2 was monitored by reducing the absorbance at 240 nm. The activity of the enzyme was quantified as the rate of decrease in absorbance per minute per gram of protein in fresh tissue samples.

Estimation of chitinase activity

Chitinase activity was assessed colorimetrically by quantifying the N-acetyl-D-glucosamine (NAG) liberated from colloidal chitin. The preparation of colloidal chitin was executed in adherence to the

methodology of [Berger and Reynolds \(1958\)](#). Chitinase activity was determined by comparing the measured values to a standard curve of NAG.

Estimation of phenol content

Total phenolic content was quantified using the Folin-Ciocalteu reagent, as described by [Singleton et al. \(1999\)](#) and [Lee et al. \(2004\)](#). Samples weighing 2 g each were homogenized in 80% ethanol at ambient temperature (25°C), followed by centrifugation at 10,000 rpm for 15 min at a low temperature (4°C), with the supernatant retained. The residue underwent two additional extractions with 80% ethanol, and the solvents were evaporated using a rotary evaporator at 40°C for 1 h. The dried residue was redissolved in 5 mL of distilled water. A 100-μL aliquot of this solution was diluted to 3 mL with water, and 0.5 mL of Folin-Ciocalteu reagent was added. Following a 3-min interval, 2 mL of 20% sodium carbonate solution was introduced, and the mixture was thoroughly mixed. The development of color occurred, and the absorbance was measured at 650 nm using a Bausch and Lomb spectronic-21 UVD spectrometer 60 min post-mixture, with catechol serving as the reference standard. The results were presented as mg catechol/100 g of fresh weight material.

Estimation of soluble protein content

The soluble protein content of the enzyme extracts was calculated using the bovine serum albumin Bradford methodology ([Bradford, 1976](#)).

Effect of microbial antagonists on plant growth promoting traits

The pot experiment with the most effective dose of microbial antagonists and their consortia (10 gm/kg seeds) was also conducted to assess plant growth promotion abilities in wheat cv. PBW 343 under greenhouse conditions. This study includes the inoculum concentration of the talc-based bioformulations (10⁸ cfu/gm) and the Bgt (10⁵ conidia/mL). Thirty-five days post-seed treatment (dpt) with microbial antagonists alone and 35 days post-inoculation (dpi) with the *B. graminis tritici* pathogen, whole plants along with roots were uprooted from each treatment in all the replications. The root and shoot of each plant cut from the crown region were measured for plant length (cm). The fresh weight of both root and shoot parts was determined through direct weighing, followed by oven-drying at 60°C for 72 h, after which the dry weight was recorded. Plant length and dry weight parameters were utilized to assess the growth-promoting effects of bacterial antagonists. Evaluations were carried out for the application of microbial antagonists alone and in conjunction with *B. graminis tritici* to determine the relative impact of these antagonists on plant biomass. Comparisons were made between an infected control (plants inoculated with a conidial suspension of *B. graminis tritici*) and a non-treated control (healthy plants without treatment). The calculation of the relative growth promotion efficacy (GPE) attributed to the microbial antagonists was based on the dry weight

of the plant (incorporating both root and shoot dry weights), following the methodology described in [Singh et al. \(2012\)](#), [Manzar et al. \(2021\)](#), [Mahawer et al. \(2023\)](#).

Statistical analysis

The experiments were conducted using a randomized complete block design (RCBD). The presented data are from the illustrative experiments conducted twice. Statistical analysis was conducted, and means were calculated using the Duncan Multiple Range Test (DMRT) to identify significant differences between treatments at the level of p of ≤ 0.05 . SPSS software (version 16.0) was employed for data analysis.

Results

Biocontrol potential of microbial antagonists against *B. graminis tritici*

The objective of the preliminary potted-plant experiments was to ascertain the effects on disease development, rAUDPC, apparent infection rate (r), and biological control efficacy (BCE) in wheat against the powdery mildew pathogen, *B. graminis tritici*, under temperature-controlled greenhouse conditions. All five talcum-based bioformulations of microbials and their consortia were tested at three different doses (5.0 g, 7.5 g, and 10.0 g per kg of seeds).

Disease severity

The severity of powdery mildew disease was assessed at weekly intervals, from 14 days post inoculation (dpi) to 35 dpi, across various treatments and replications. The inoculum concentration of the talc-based bioformulations was 10⁸ cfu/gm, and the Bgt was 10⁵ conidia/mL. The results indicated that microbial antagonists and their consortia, applied at a dose of 10 g/kg seeds, significantly reduced disease severity compared to other doses [5.0 g ([Supplementary Figure 1](#)) and 7.5 g ([Supplementary Figure 2](#)) per kg of seeds]. The infected control exhibited substantially higher mean disease severity (93.56%) than the treated groups. The seeds treated with *Bacillus subtilis* DTBS-5 (BS-DTBS-5) at 10 g/kg demonstrated superior efficacy, with the lowest mean disease severity (8.24%) and maximum biocontrol efficacy (91.19%). BA-DTBA-11 and PF-DTPF-3 were also used at the same dose, exhibiting mean disease severities of 11.44% and 17.14% and mean biocontrol efficacies of 87.77% and 81.68%, respectively. Consortia treatments PF-DTPF-3 + BS-DTBS-5 and PF-DTPF-3 + BA-DTBA-11 at 10 g/kg seeds were moderately effective, with mean disease severities of 27.41% and 32.45% and mean biocontrol efficacies of 70.70% and 65.31%, respectively. However, the fungal antagonists TH-Pusa-5SD and AN-An-27 at each dose showed limited efficacy against powdery mildew. The scattered plot matrix appears to have a strong negative correlation between disease severity and biocontrol efficacy, as higher efficacy leads to lower disease severity ([Figure 1](#)). BS-DTBS-5, BA-DTBA-11, and PF-DTPF-3 at 10 g/kg seeds exhibited

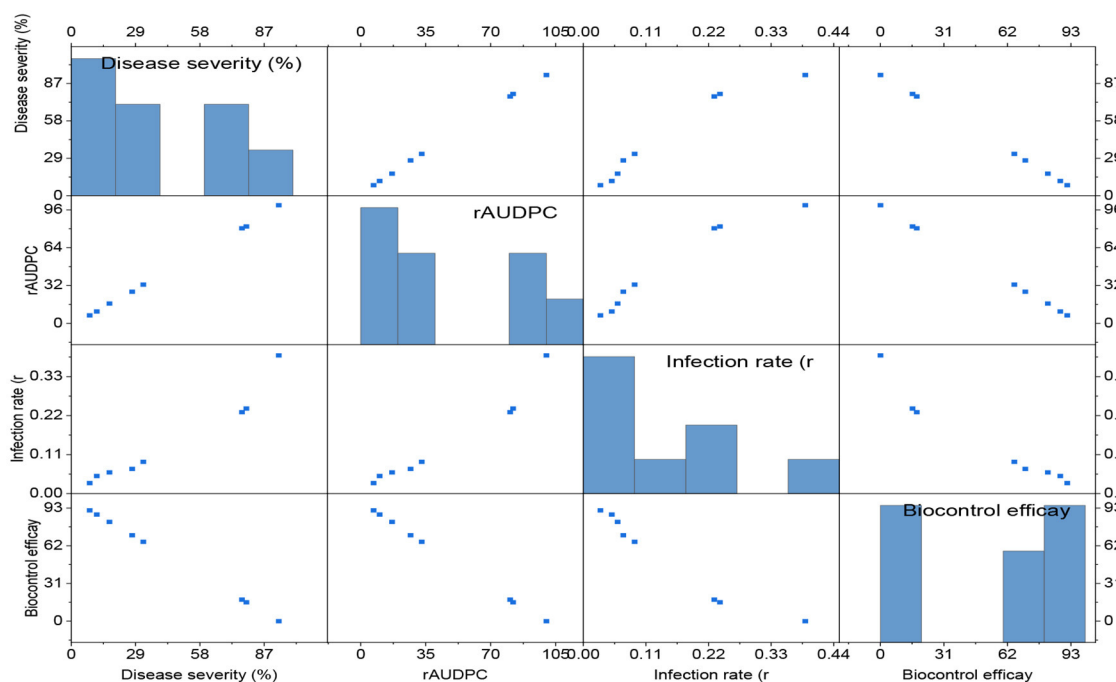


FIGURE 1

The comparative efficacy of biological control agents (treatment dose of 10 g/kg seeds) against powdery mildew disease parameters. Disease severity (%), rAUDPC, and infection rate (r). The data are based on disease observations made four times weekly and a mean of four replicates with 10 plants.

superior biocontrol efficacy, making them promising candidates for controlling powdery mildew in wheat.

Progression of disease

The disease progress curves (DPCs) were determined by plotting mean disease severity against time (Figures 2–4). The DPCs obtained for the infected control followed a typical sigmoid curve. The seeds treated with the antagonist *BS-DTBS-5* at 10 gm/kg showed slight disease progression, followed by *BA-DTBA-11* and *PF-DTPF-3* at the same dose, indicating higher effectiveness against the powdery mildew pathogen in wheat. In control pots, we observed a prolonged log phase (rise in disease severity) after 14 dpi. The seeds treated with consortia of *PF-DTPF-3* + *BS-DTBS-5* and *PF-DTPF-3* + *BA-DTBA-11* showed a prolonged lag phase (low amount of disease) up to 21 dpi, which could be attributed to the moderate effectiveness of antagonists. The seeds treated with fungal antagonists, *T. harzianum* Pusa-5SD and *A. niger* An-27, exhibited continued log phase (disease progression) after 14–21 dpi, which indicated their ineffectiveness in controlling powdery mildew development on wheat.

Relative Area under the disease progress curve

The infected control exhibited the highest mean relative AUDPC (rAUDPC) value (100) compared to the treated groups (Supplementary Table 1). Among the treatments, the lowest mean rAUDPC value (6.88) was observed with the application of the antagonist *BS-DTBS-5* at a rate of 10 g/kg seeds, followed by

BA-DTBA-11 (10.15) and *PF-DTPF-3* (16.82), indicating effective disease control by these antagonistic treatments. Additionally, mean rAUDPC values of 26.78 and 32.82 were recorded with the combinations of antagonists *PF-DTPF-3* + *BS-DTBS-5* and *PF-DTPF-3* + *BA-DTBA-11* at 10 g/kg seeds, respectively, showing moderate effectiveness against the disease (Figures 5, 6). The fungal antagonists, viz., *TH-Pusa-5SD* and *AN-An-27*, treated seeds at all three doses and recorded higher rAUDPC values (82.05–90.65) than other treatments, including the infected control. Due to the ineffectiveness of these fungal antagonistic treatments, the higher rAUDPC value showed higher disease severity, which is positively associated with the percent disease severity (Milus and Line, 1986).

Apparent infection rate (r)

The apparent infection rate (r) of all tested microbial antagonists and their combinations was lower than that of the infected control, with a mean r -value of 0.39 (Supplementary Table 1). Specifically, *BS-DTBS-5*, followed by *BA-DTBA-11* and *PF-DTPF-3* at 10 g/kg seeds, consistently exhibited reduced disease severity, resulting in minimal disease progression over time, with mean r -values of 0.03, 0.06, and 0.07, respectively. Similarly, the combinations of *PF-DTPF-3* + *BS-DTBS-5* and *PF-DTPF-3* + *BA-DTBA-11* at 10 g/kg seeds displayed moderately reduced disease severity, with mean r -values of 0.08 and 0.09, respectively. The fungal antagonists, such as *TH-5SD* and *AN-An-27*, treated at all three doses, exhibited the highest mean r -values (0.23–0.29), indicating ineffective control against the disease based on disease severity

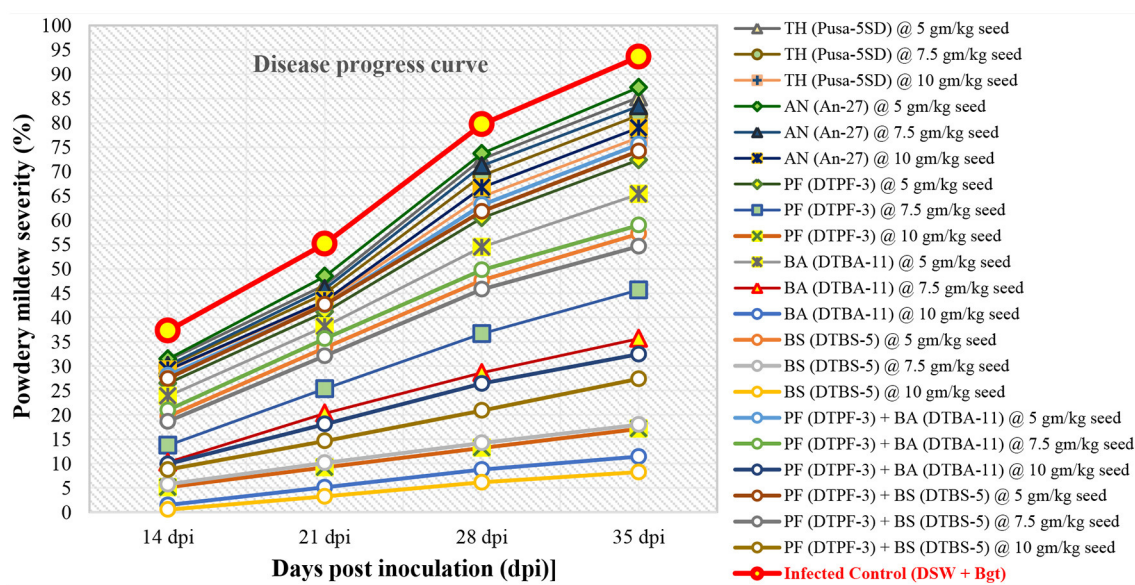


FIGURE 2
Development of powdery mildew caused by *B. graminis tritici* on wheat cv. PBW 343 treated with microbials and their consortia under greenhouse conditions [TH (Pusa-5SD): *Trichoderma harzianum* strain Pusa-5SD; AN (An-27): *Aspergillus niger* strain An-27; PF (DTPF-3): *Pseudomonas fluorescens* strain DTPF-3; BA (DTBA-11): *Bacillus amyloliquefaciens* strain DTBA-11; and BS (DTBS-5): *Bacillus subtilis* strain DTBS-5]. DSW: sterile distilled water; Bgt: *B. graminis* f. sp. *tritici*. The data are based on disease observations made four times weekly and a mean of four replicates with 10 plants.

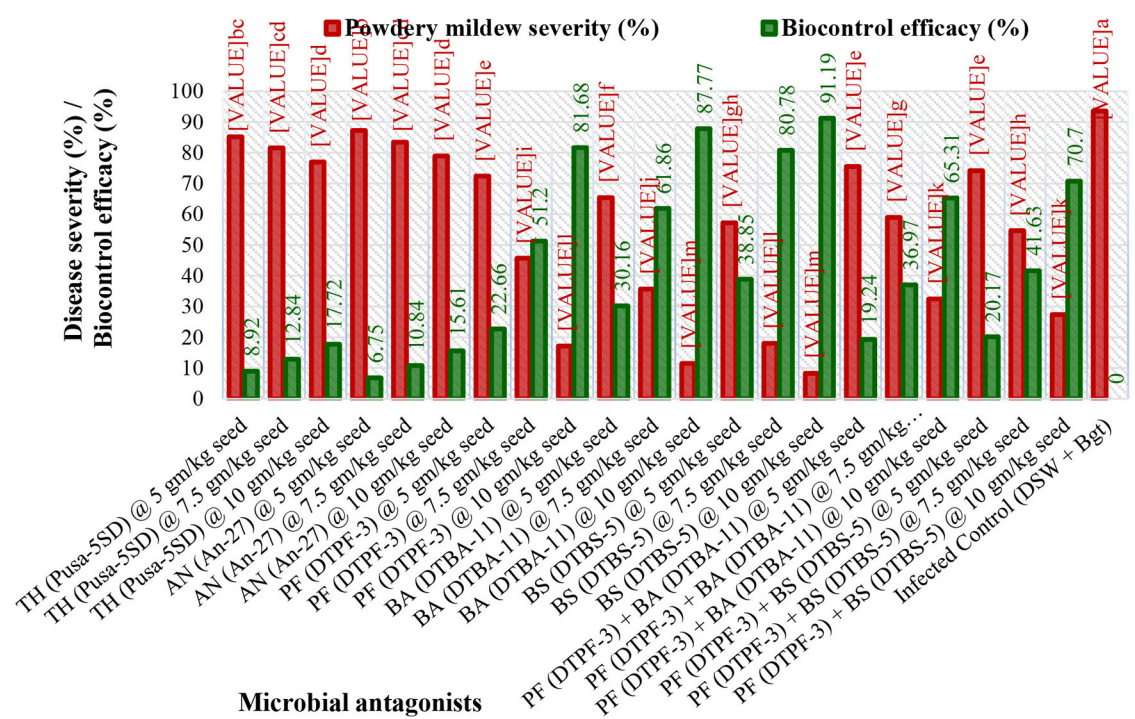
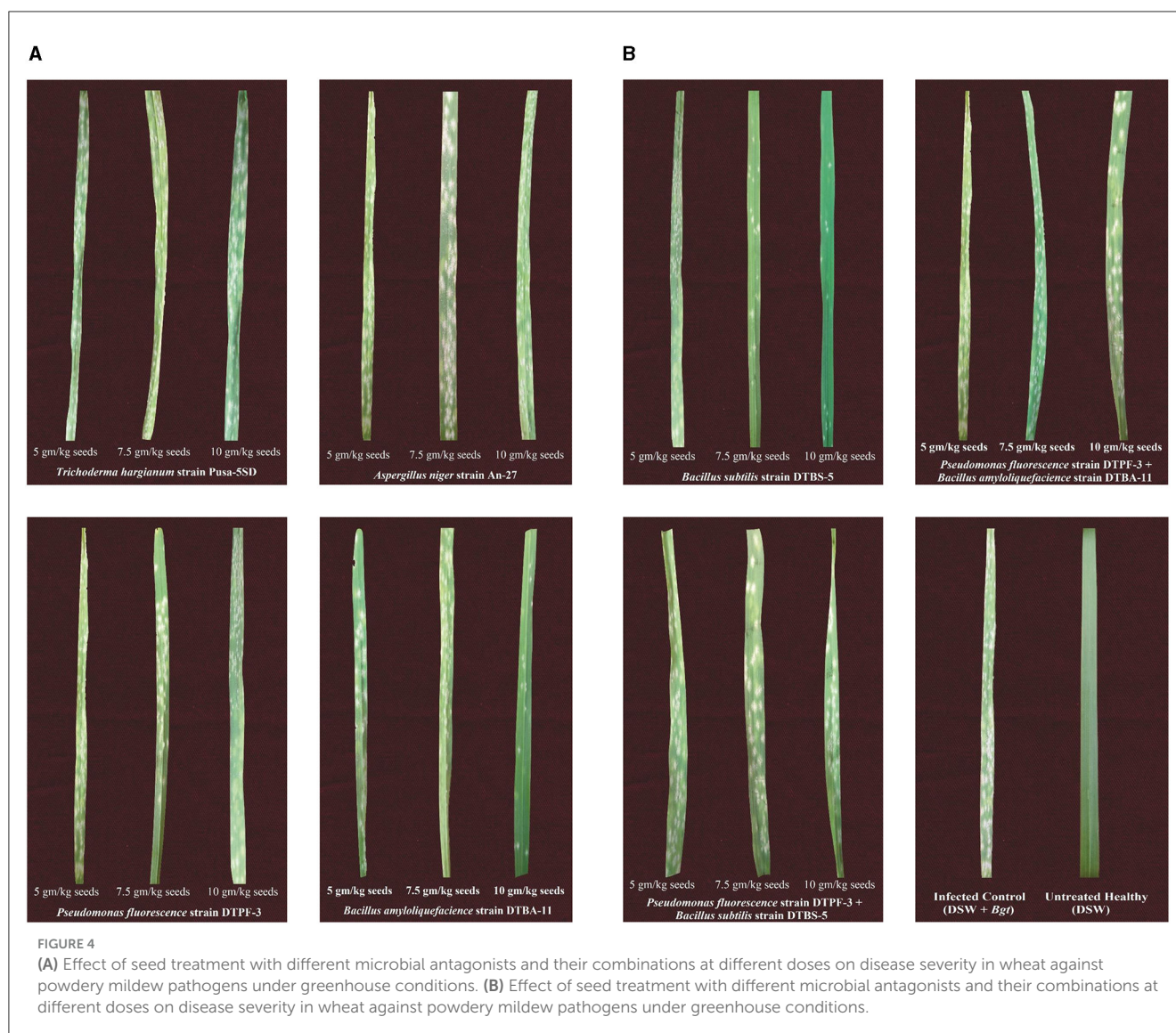


FIGURE 3
Effect of seed treatment with microbial antagonists and their combinations at different doses on disease severity and biological control efficacy in wheat against powdery mildew pathogens under greenhouse conditions. [TH (Pusa-5SD): *Trichoderma harzianum* strain Pusa-5SD; AN (An-27): *Aspergillus niger* strain An-27; PF (DTPF-3): *Pseudomonas fluorescens* strain DTPF-3; BA (DTBA-11): *Bacillus amyloliquefaciens* strain DTBA-11; and BS (DTBS-5): *Bacillus subtilis* strain DTBS-5]. DSW: sterile distilled water; Bgt: *Blumeria graminis* f. sp. *tritici*. The data are based on the mean of four replicates with 10 plants. The means followed by the same letters on the bar diagram are not significantly different, as determined by Duncan's Multiple Range Test at the 5% level.

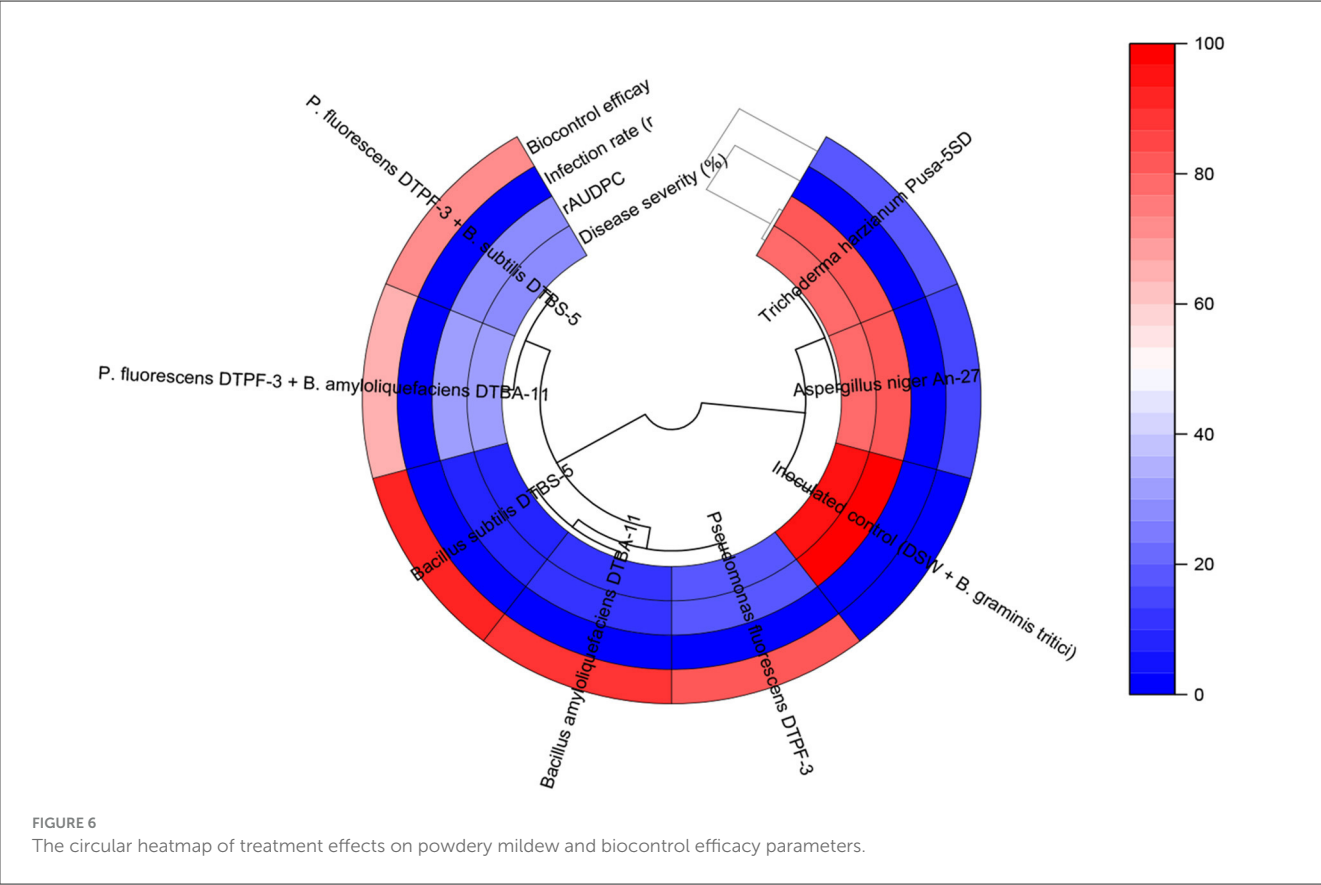
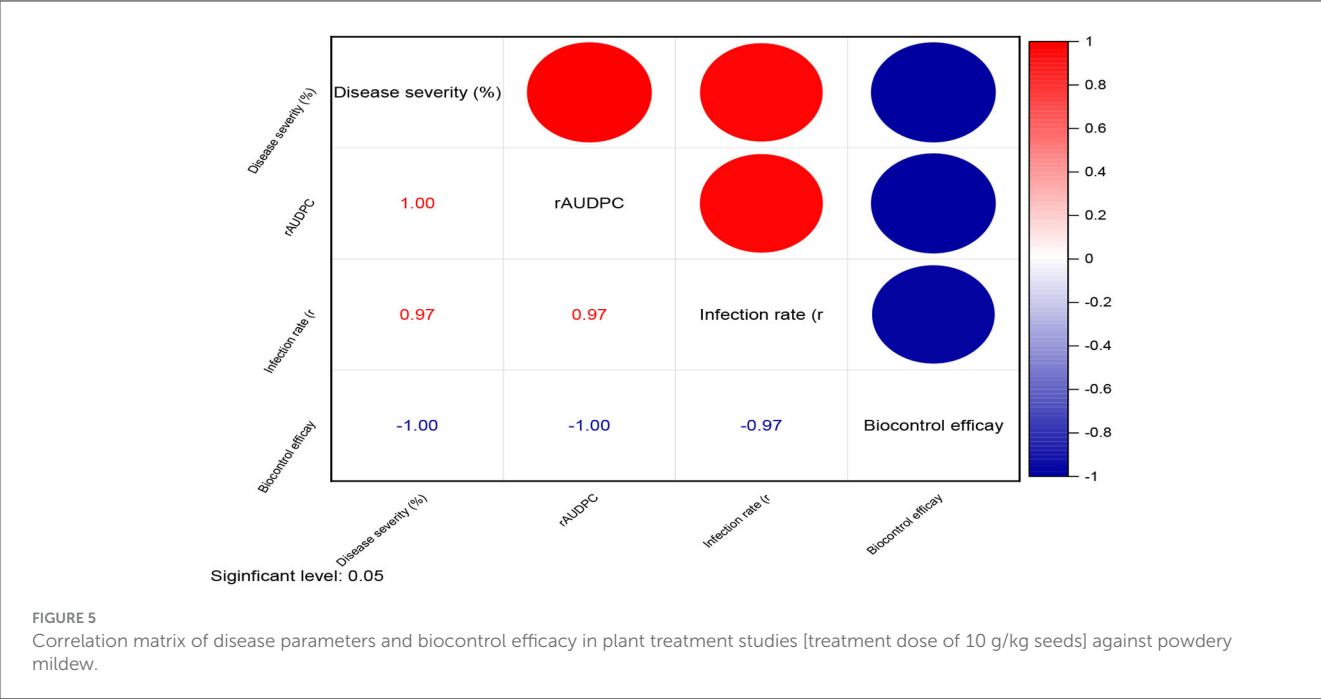


and rAUDPC values. In this study, treatments classified as highly effective biocontrol agents demonstrated infection rates of <0.06 , while those categorized as moderately effective displayed infection rates ranging from 0.07 to 0.09 concerning other disease parameters such as disease severity and rAUDPC (Figure 7).

Expression of defense-related enzymes and biochemical activity changes

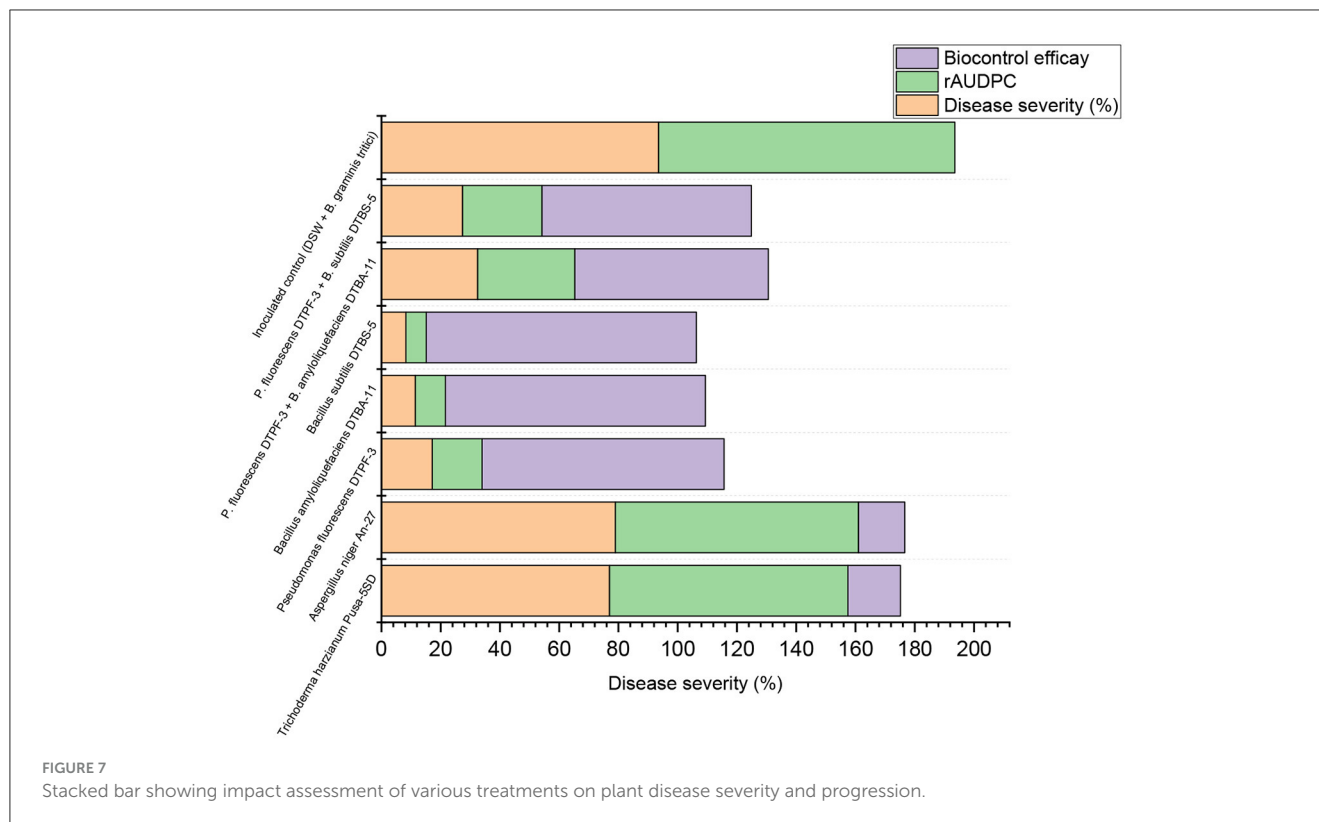
The different defense-related enzymes and biochemical tests were assessed spectrophotometrically in the antagonist-treated wheat tissues after infection with *B. graminis tritici* (*Bgt*) (Figures 8, 9). The peroxidase enzyme activity was found to be highest in the wheat treated with the antagonists *BS-DTBS-5* and *Bgt* after 120 h of infection (0.276 specific activity/min/mg protein), followed by *BA-DTBA-11* (0.189 specific activity/min/mg

protein) and *PF-DTPF-3* (0.151 specific activity/min/mg protein). Comparatively, a moderate increase in POD activity was also noticed in the wheat treated with combinations of antagonists *PF-DTPF-3* + *BS-DTBS-5* (0.092 specific activity/min/mg protein) and *PF-DTPF-3* + *BA-DTBA-11* (0.084 specific activity/min/mg protein) and *Bgt* after 120 hpi. Apart from the untreated healthy and the infected control, no significant increase in POD activity was detected in the wheat treated with the fungal antagonists *TH-Pusa-5SD* (0.072 specific activity/min/mg protein) and *AN-An-27* (0.071 specific activity/min/mg protein) and *Bgt* after 120 hpi. Polyphenol oxidase (PPO) activity in wheat was found to be comparatively higher (0.249 specific activity/min/mg protein) in the plants treated with bioagent *BS-DTBS-5*, followed by *BA-DTBA-11* (0.175 specific activity/min/mg protein) and *PF-DTPF-3* (0.135 specific activity/min/mg protein) and *Bgt* after 120 h of infection (Figure 8). A slight increase in PPO activity was also noted in the wheat treated with mixtures of *PF-DTPF-3* + *BS-DTBS-5* (0.082 specific activity/min/mg protein) and *PF-DTPF-3* + *BA-DTBA-11* (0.081 specific activity/min/mg protein) and



Bgt after 120 hpi. Catalase (CAT) activity in the wheat treated with *BS-DTBS-5* was the highest and remained at high levels (7.918 specific activity/min/mg protein) up to 120 h after *Bgt* infection, followed by *BA-DTBA-11* (7.241 specific activity/min/mg protein) and *PF-DTPF-3* (6.982 specific activity/min/mg protein).

Chitinase activity in the wheat seeds treated with *BS-DTBS-5* showed peak activity of chitinase (2.9 units) at 48 hpi and maintained high levels (6.9 units) up to 120 hpi with *Bgt*, followed by *BA-DTBA-11* (5.7 units) and *PF-DTPF-3* (5.2 units). A reasonable increase in chitinase activity was also observed



in the wheat treated with combinations of antagonists *PF*-DTPF-3 + *BS*-DTBS-5 (4.8 units) and *PF*-DTPF-3 + *BA*-DTBA-11 (4.6 units) up to 120 hpi with *Bgt* pathogen. Total phenol content in the wheat treated with *BS*-DTBS-5 increased (13.5 mg catechol/g F.W.) just after 24 h of pathogen infection and maintained at high levels (29.5 mg catechol/g F.W.) up to 120 hpi with *Bgt*, followed by *BA*-DTBA-11 (26.4 mg catechol/g F.W.) and *PF*-DTPF-3 (23.8 mg catechol/g F.W.) (Figure 9). Phenol content was also found to be moderately increased in the wheat treated with combinations of antagonists *PF*-DTPF-3 + *BS*-DTBS-5 (21.8 mg catechol/g F.W.) and *PF*-DTPF-3 + *BA*-DTBA-11 (20.9 mg catechol/g F.W.) up to 120 hpi with *Bgt* pathogen.

Soluble protein content in wheat was also found to be comparatively higher (28.5 mg g⁻¹ F.W.) in the plants treated with bioagent *BS*-DTBS-5, followed by *BA*-DTBA-11 (21.4 mg g⁻¹ F.W.) and *PF*-DTPF-3 (19.4 mg g⁻¹ F.W.) and *Bgt* after 120 h of infection. However, soluble protein content was also noted to increase moderately in the wheat treated with blends of *PF*-DTPF-3 + *BS*-DTBS-5 (15.5 mg g⁻¹ F.W.) and *PF*-DTPF-3 + *BA*-DTBA-11 (14.2 mg g⁻¹ F.W.) and *Bgt* after 120 hpi. In this study, significantly higher levels of defense-related enzymes (POD, PPO, catalase, and chitinase) and biochemicals (total phenolic content and soluble protein) activity changes were noticed in the wheat seeds treated with the antagonist *BS*-DTBS-5, followed by *BA*-DTBA-11 and *PF*-DTPF-3 at 10 g/kg seeds. Antagonistic combinations were also found to moderately induce resistance, while no significant results were noticed with the seed treatment with fungal antagonists after *Bgt* infection. The observed increase in defense enzyme activities such as

POD, PPO, CAT, and chitinase, along with higher phenolic and soluble protein contents, suggests that microbial antagonists play a crucial role in priming the plant's innate immune response. Specifically, the 45% increase in POD activity and the 50% increase in CAT activity highlight the effectiveness of *Bacillus subtilis* strain DTBS-5 and *Bacillus amyloliquefaciens* strain DTBA-11, respectively, in managing the plant's oxidative stress. The 30% increase in phenolic content and the 25% increase in soluble protein content further support the role of these biocontrol agents in enhancing the biochemical defense of wheat plants against *B. graminis tritici*.

Plant growth promotion attributes

The plant growth promotion abilities were evaluated in the wheat seeds bioprimered with microbial antagonists, both alone and co-infected with *Bgt*, under greenhouse conditions. The results revealed significant variations in the wheat seeds treated with the tested microbial antagonists alone and in conjunction with *Bgt* for various vegetative traits, including plant length, fresh weight, dry weight, and growth promotion efficacy (Figures 10A, B, Supplementary Table 2). In the seeds treated solely with microbial antagonists, the highest mean root length of 19.48 cm and shoot length of 42.51 cm were observed with *BS*-DTBS-5, followed by *BA*-DTBA-11 (18.98 cm and 41.98 cm) and *PF*-DTPF-3 (18.14 cm and 41.54 cm). In the seeds treated with combinations of antagonists *PF*-DTPF-3 + *BS*-DTBS-5 and *PF*-DTPF-3 + *BA*-DTBA-11, the mean root lengths were 16.97 cm and 16.85 cm and mean shoot lengths were 38.95 cm and 38.74 cm, respectively. The

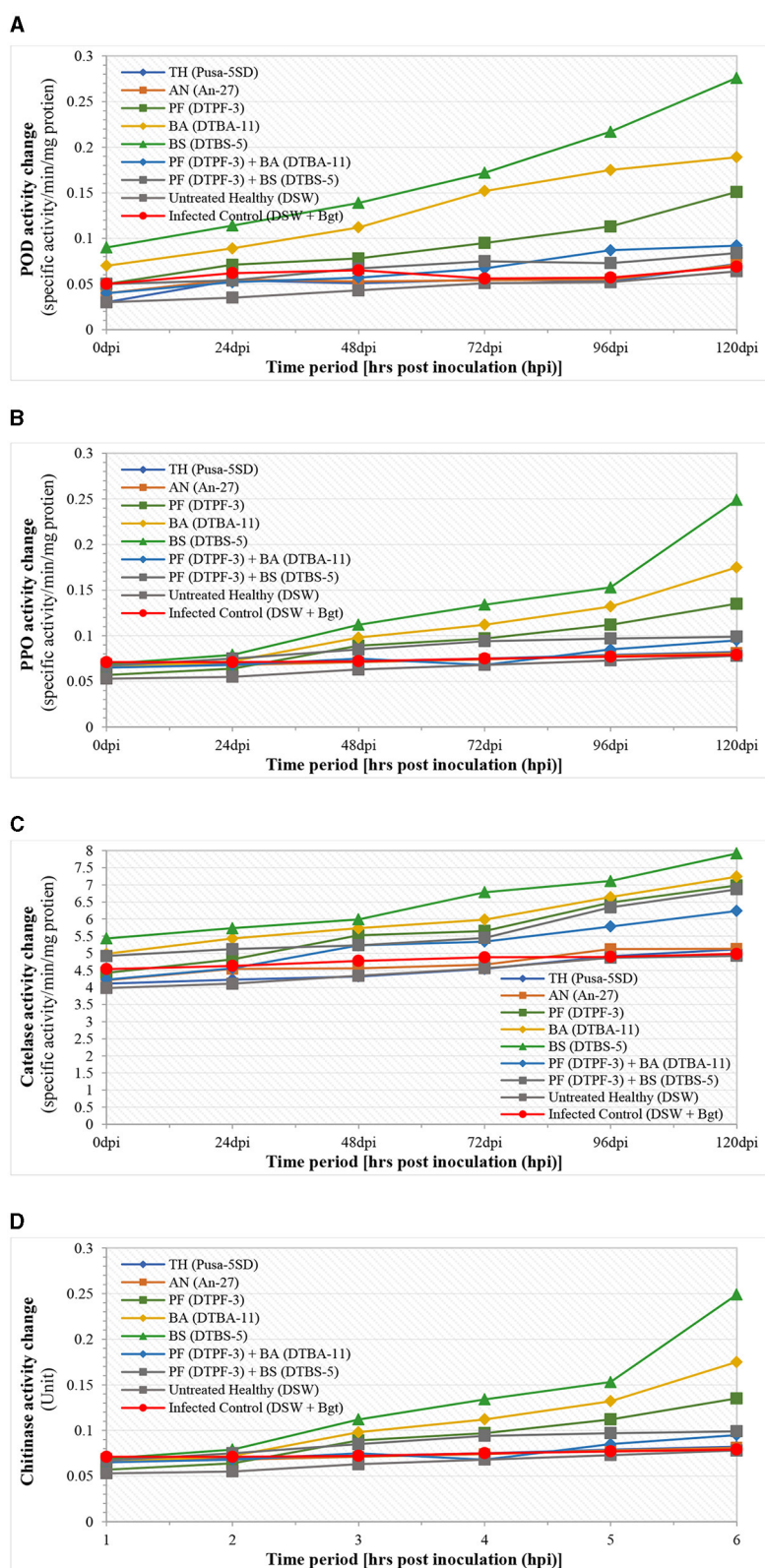


FIGURE 8

(A) Peroxidase (POD), (B) polyphenol oxidase (PPO), (C) catalase (CAT), and (D) chitinase activities in microbial antagonist-treated wheat tissues after infection with *B. graminis tritici* (Bgt). [TH (Pusa-5SD): *Trichoderma harzianum* strain Pusa-5SD; AN (An-27): *Aspergillus niger* strain An-27; PF (DTPF-3): *Pseudomonas fluorescens* strain DTPF-3; BA (DTBA-11): BA-DTBA-11; and BS (DTBS-5): BS-DTBS-5]. DSW: sterile distilled water.

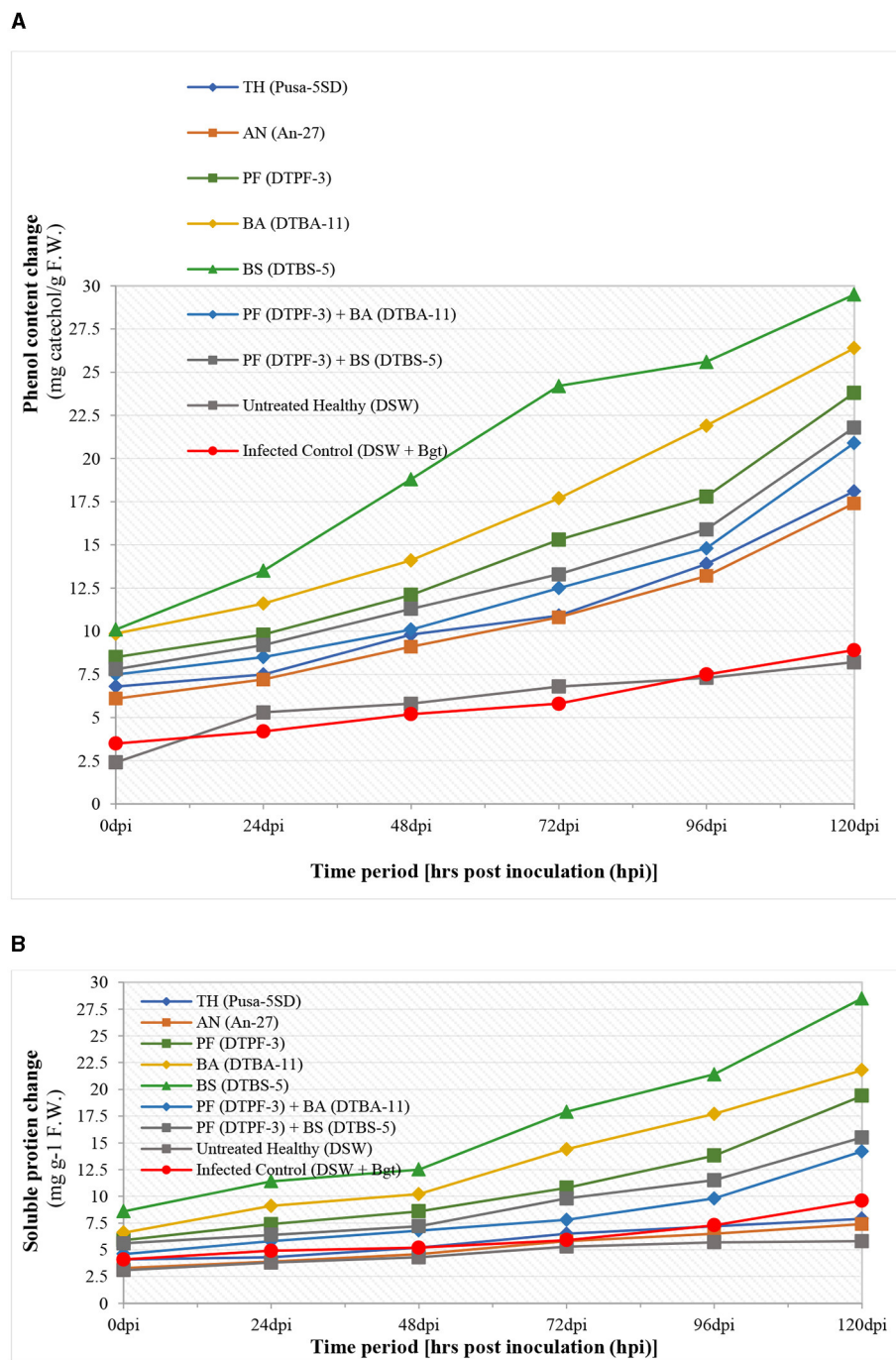


FIGURE 9

Biochemicals [total phenolic content (A) and soluble protein (B)] activity changes in microbial antagonist-treated wheat tissues after infection with *B. graminis tritici* (Bgt). [TH (Pusa-5SD): *Trichoderma harzianum* strain Pusa-5SD; AN (An-27): *Aspergillus niger* strain An-27; PF (DTPF-3): *Pseudomonas fluorescens* strain DTPF-3; BA (DTBA-11): *Bacillus amyloliquefaciens* strain DTBA-11; and BS (DTBS-5): *Bacillus subtilis* strain DTBS-5]. DSW: sterile distilled water.

maximum mean plant dry weight (5.95 gm) was recorded with BS-DTBS-5, followed by BA-DTBA-11 (5.72 gm) and PF-DTPF-3 (5.69 gm). The antagonistic combinations of PF-DTPF-3 + BS-DTBS-5 and PF-DTPF-3 + BA-DTBA-11 also exhibited mean plant dry weights of 5.28g and 5.14g, respectively. The growth promotion efficacy (GPE) was significantly higher (99.66%) with BS-DTBS-5, followed by BA-DTBA-11 (91.94%) and PF-DTPF-3

(90.93%). The antagonistic combinations of PF-DTPF-3 + BS-DTBS-5 and PF-DTPF-3 + BA-DTBA-11 also exhibited GPEs of 77.18% and 72.48%, respectively. In the seed treatment with microbial antagonists and infected with *B. graminis tritici*, the maximum mean root length of 18.89 cm and shoot length of 41.95 cm were noticed in BS-DTBS-5, trailed by BA-DTBA-11 (17.01 cm and 40.45 cm) and PF-DTPF-3 (17.75 cm and 40.98 cm).

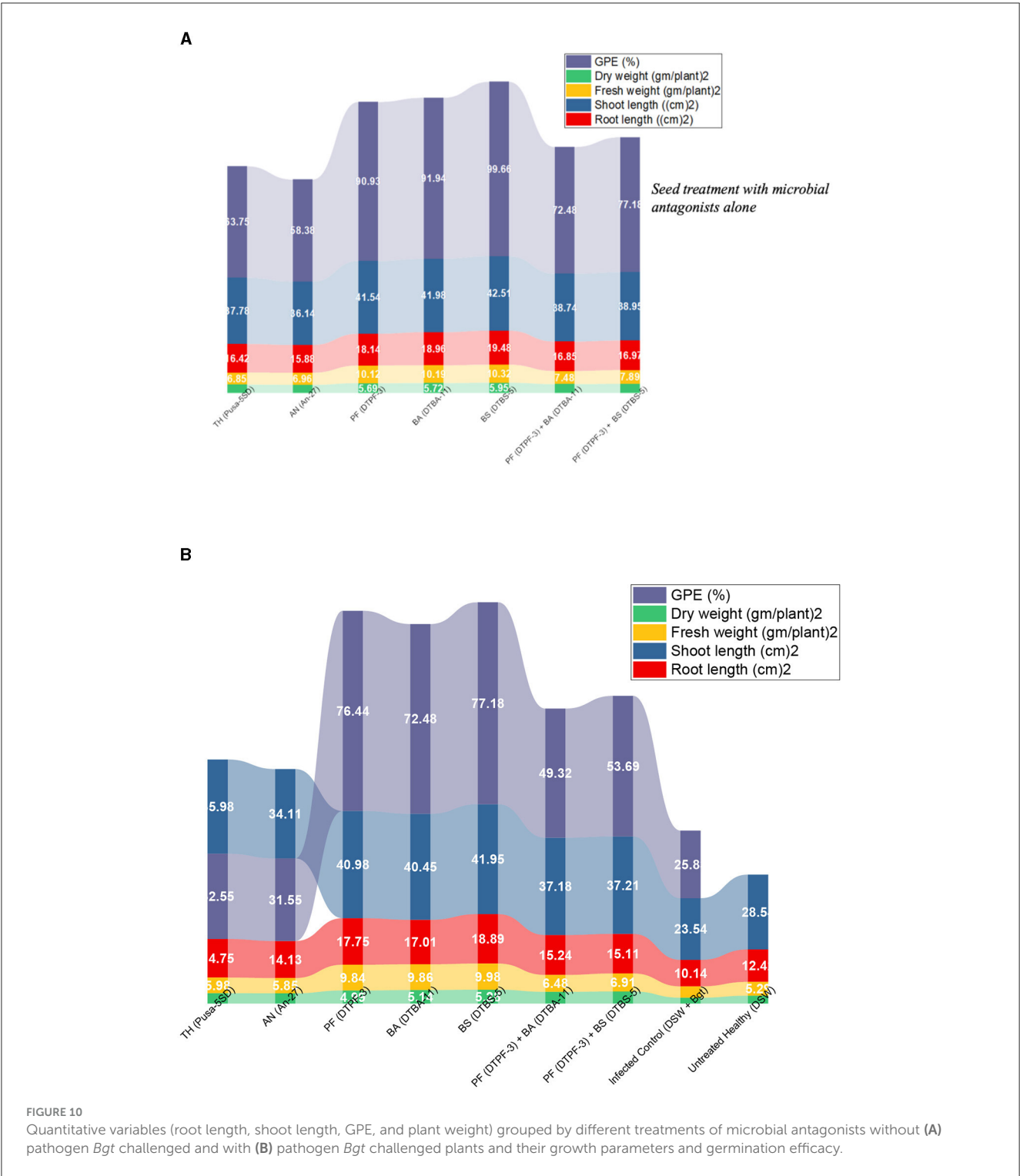


FIGURE 10 Quantitative variables (root length, shoot length, GPE, and plant weight) grouped by different treatments of microbial antagonists without (A) pathogen *Bgt* challenged and with (B) pathogen *Bgt* challenged plants and their growth parameters and germination efficacy.

The combinations of *PF*-*DTPF*-3 + *BS*-*DTBS*-5 and *PF*-*DTPF*-3 + *BA*-*DTBA*-11 gave mean root lengths of 15.11 cm and 15.24 cm, and mean shoot lengths of 37.21 cm and 37.18 cm, respectively. The maximum mean plant dry weight (5.28 gm) was recorded in *BS*-*DTBS*-5, followed by *BA*-*DTBA*-11 (5.14 gm) and *PF*-*DTPF*-3 (4.99 gm). The antagonistic mixtures of *PF*-*DTPF*-3 + *BS*-*DTBS*-5 and *PF*-*DTPF*-3 + *BA*-*DTBA*-11 were yielded mean plant dry weights of 4.58 g and 4.45 g, respectively. The GPE was higher (77.18%) in *BS*-*DTBS*-5, followed by *BA*-*DTBA*-11 (72.48%) and *PF*-*DTPF*-3 (76.44%). The antagonistic combinations of *PF*-*DTPF*-3 + *BS*-*DTBS*-5 and *PF*-*DTPF*-3 + *BA*-*DTBA*-11 also recorded GPEs of 53.69% and 49.32%, respectively.

In this research, the wheat treated with microbial antagonists alone showed no discernible variation in plant growth promotion activity. On the other hand, the wheat treated with microbial antagonists and infected with the *Bgt* pathogen showed notable differences in PGP characteristics.

Discussion

The microbial antagonists, including PF-DTPF-3, BA-DTBA-11, BS-DTBS-5, TH-Pusa-5SD, and AN-An-27, had not been previously evaluated against the wheat powdery mildew pathogen. However, they were chosen for our investigation based on the biocontrol potential that different researchers have documented in other pathosystems (Mondal et al., 2000; Sheroze et al., 2003; Dubey et al., 2007; Anand and Reddy, 2009; Liu et al., 2009; Singh et al., 2010; Hui et al., 2013; Tan et al., 2013; Meena et al., 2017; Sánchez-Montesinos et al., 2021; Manzar et al., 2022; Kashyap and Manzar, 2023). In this study, BS-DTBS-5 emerged as the most effective biocontrol agent, which aligns with the reports by Singh et al. (2010). The synergistic effects of *B. subtilis* and *B. amyloliquefaciens* have been observed to enhance bioefficacy and decrease the incidence of black rot caused by *Xanthomonas campestris* pv. *campestris*. The biocontrol efficacy and plant growth promotion abilities of bacterial antagonists, specifically BA-DSBA-11 and DSBA-12, were assessed against the bacterial wilt of tomato cv. Pusa Ruby under greenhouse conditions. It was observed that plants treated with BA-DSBA-12 exhibited superior growth-promoting efficacy compared to those treated with *B. pumilus* MTCC-7092 (Singh et al., 2016). Multiple microbial antagonists, such as *Pseudomonas fluorescens* PDS1, *Bacillus subtilis* BDS1, *Bacillus cereus* UK4, *Bacillus amyloliquefaciens* UK2, and *Bacillus subtilis* KA9, have been reported for their antagonistic and growth promotion activity against Chilli Wilt disease caused by *Ralstonia solanacearum* (Kashyap et al., 2021, 2022; Kashyap and Manzar, 2023). Our studies showed that BA-DTBA-11 and PF-DTPF-3 at 10 g/kg seeds significantly reduced PM severity by 11.44% and 17.14%, respectively, with mean biocontrol efficacies of 87.77% and 81.68%. A similar finding was recorded in a case study of rust, where biocontrol agents *P. fluorescens* strain PF-1 (5 gm/L water) and *B. subtilis* strain BS-1 (5 gm/L water) were found to be moderately effective in controlling wheat stripe rust by 41.83% and 39.92%, respectively (Singh et al., 2016). Our findings align with those of Gao et al. (2015), who demonstrated the biocontrol potential of four strains of endophytic bacteria (E1R-j, E1R-h, ECL5, and Em7) against wheat powdery mildew, *Bgt*. Additionally, they reported that spraying E1R-j 24 h before *Bgt* inoculation inhibited conidial germination and appressorial development, with a 42.7% reduction in appressorial development and a 43.3% reduction in conidial germination compared to the water control. *Bacillus* organisms, being gram-positive bacteria, have many advantages that make them a potential candidate for the biological control of plant diseases. *Bacillus* can produce spores that resist UV light and adverse conditions, qualifying them for effective commercial bioformulation (Raaijmakers et al., 2002). Gram-negative bacteria from the genera *Pseudomonas* and *Erwinia* have also gained significant recognition as biological control agents (Cartwright et al., 1995; Braun-Kiewnick et al., 2000; Shoda, 2000; Slininger et al., 2000; Costa et al., 2001). *B. amyloliquefaciens* strain YN201732, a beneficial endophyte isolated from tobacco, was found effective in controlling tobacco powdery mildew and inducing systemic resistance. It was also found to be effective in inducing polyphenol oxidase and chitinase activity changes in tobacco plants infected with powdery mildew (Santoyo et al., 2012; Kannoja et al., 2019). Single nucleotide polymorphisms (SNPs) obtained from

Pseudomonas putida (PpFT1) and *B. subtilis* (BsBN3) at 50, 100, and 150 ppm were found to be most effective in reducing powdery mildew severity by 83.3, 89.7, 84.6, and 91.0%, respectively. SNPs obtained from *T. harzianum* at 150 ppm have been reported to reduce PM severity by 82.0% (Farhat et al., 2018). The wheat crop treated with microbial antagonists *B. subtilis* strain DTBS-5 and pathogen *Bgt* showed greater elicitation of defense-related enzymes (POD, PPO, CAT, and chitinase) and biochemicals (total phenolic and soluble protein content) at 120 hpi, followed by another antagonist *B. amyloliquefaciens* strain DTBA-11 and *P. fluorescens* strain DTPF-3. Similar elicitations of defense-related enzymes and biochemical activities were observed in wheat seed biopriming with a mixture of antagonists, *P. fluorescens* strain DTPF-3 + *B. subtilis* strain DTBS-5 and *P. fluorescens* strain DTPF-3 + *B. amyloliquefaciens* strain DTBA-11 at 120 hpi. The strain DTBS-5 likely produces specific elicitors or signaling molecules that are recognized by the wheat plant's innate immune system. Upon recognition, these signals trigger a cascade of defense responses, including the activation of enzymes such as POD, PPO, CAT, and chitinase, which play crucial roles in strengthening the plant's defense barriers against pathogens. The conclusions drawn from our study align with the research conducted earlier (Manzar et al., 2021; Kashyap et al., 2022), indicating that seed biopriming utilizing *Bacillus* sp. or biocontrol agents leads to the elicitation of defense-related enzymes such as PAL, peroxidase, and polyphenol oxidase within 48 h, thereby priming resistance against *Bgt*. Specifically, our findings are consistent with the activities of polyphenol oxidase and peroxidase in the tomato plants treated with *B. amyloliquefaciens* SQRT3 strain and *R. solanacearum* compared to other treatments, which is consistent with previous reports (Shishkoff and McGrath, 2002; Punja and Utkhede, 2003). Apart from the untreated healthy and the infected control, no significant increase in defense-related enzymes or biochemical activity changes were noticed in the wheat seeds treated with the fungal antagonists *T. harzianum* strain Pusa-5SD and *A. niger* strain An-27. This variation in efficacy across microbial antagonists emphasizes the complexities of plant-microbe interactions and the need to select appropriate biocontrol agents based on their mode of action and compatibility with the host plant's defensive system. The results highlight the potential of using microbial antagonists to enhance crop resilience through induced resistance, offering a sustainable alternative to chemical pesticides (Kashyap et al., 2022). This study observed significantly higher levels of defense-related enzymes and biochemical activity changes in *B. subtilis* strain DTBS-5-treated plants, followed by *B. amyloliquefaciens* strain DTBA-11 and *P. fluorescens* strain DTPF-3. Comparatively, antagonistic mixtures were also found to be moderate in terms of induced resistance and biochemical changes, whereas fungal antagonists showed non-significant results (Singh Vaibhav, 2017; Farhat et al., 2018; Manzar et al., 2021, 2022; Jatoh et al., 2024).

The observed increase in defense enzyme activities such as peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT), and chitinase, along with higher phenolic and soluble protein contents, suggests that microbial antagonists play a crucial role in priming the plant's innate immune response. This priming effect is consistent with the results of previous studies, which have demonstrated that microbial antagonists can induce systemic resistance in plants by activating various defense mechanisms (Van Loon et al., 1998;

Pieterse et al., 2014). The 45% increase in POD activity and the 50% increase in CAT activity highlight the effectiveness of *Bacillus subtilis* strain DTBS-5 and *Bacillus amyloliquefaciens* strain DTBA-11, respectively, in bolstering the plant's oxidative stress. Peroxidase and catalase are key enzymes in the plant's antioxidant defense system, which helps mitigate the damage caused by reactive oxygen species (ROS) generated during pathogen attacks. The enhancement of these enzyme activities by biocontrol agents has been well documented. For instance, Zhang et al. (2007) reported that *Bacillus subtilis* increased POD and CAT activities in cucumbers, leading to improved resistance against Fusarium wilt. The 30% rise in phenolic content and the 25% increase in soluble protein content further support the role of these biocontrol agents in enhancing the biochemical defense arsenal of wheat plants against *Blumeria graminis* f. sp. *tritici* (Bgt). Phenolic compounds are known to contribute to plant defense by strengthening cell walls and acting as antimicrobial agents. Increased phenolic content following treatment with microbial antagonists has been reported in various crops. Aziz et al. (2003) observed elevated phenolic levels in grapevine treated with *T. harzianum*, which correlated with enhanced resistance to *Botrytis cinerea*.

Similarly, soluble proteins, including pathogenesis-related (PR) proteins, play a critical role in plant defense mechanisms. PR proteins, such as chitinases, degrade the cell walls of fungal pathogens, thereby inhibiting their growth and proliferation. The increase in soluble protein content observed in this study is in line with previous findings from the study by Benhamou and Brodeur (1998), who demonstrated that *T. harzianum* induced the accumulation of PR proteins in cucumber, enhancing its resistance to *Pythium ultimum* (Benhamou and Brodeur, 1998; Van Loon et al., 1998; Aziz et al., 2003; Zhang et al., 2007; Pieterse et al., 2014; Singh Vaibhav, 2017).

Conclusion

The study concludes that treating wheat seeds with microbial antagonists at 10 g/kg significantly mitigates the severity of powdery mildew (PM), with *B. subtilis* strain DTBS-5 emerging as the most effective. This strain not only reduced PM severity to the lowest observed level of 8.24% but also achieved the highest biological control efficacy (BCE) at 91.19%. Close behind in effectiveness were *B. amyloliquefaciens* (DTBA-11) and *P. fluorescens* (DTPF-3), demonstrating considerable disease control capabilities. Despite their potential, fungal antagonists such as *T. harzianum* (Pusa-5SD) and *A. niger* (An-27) were ineffective against PM. The study also highlighted the ability of microbial antagonists to induce defense mechanisms in wheat, with notable increases in defense-related enzymes and biochemicals following treatment with the most effective strains. Microbial antagonists promoted plant growth, with *B. subtilis* strain DTBS-5 enhancing root and shoot length and plant dry weight. This research highlights the potential of microbial antagonists as a dual-function tool for controlling wheat PM and promoting plant growth, presenting a viable, eco-friendly alternative to chemical fungicides and bacterial antagonists that outperform fungal antagonists for foliar disease management.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

KC: Writing – original draft, Methodology, Investigation, Conceptualization. VS: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. AK: Writing – review & editing, Visualization, Software, Formal analysis, Data curation. AP: Validation, Writing – review & editing, Methodology. KS: Formal analysis, Writing – review & editing, Methodology. DY: Validation, Methodology, Writing – review & editing, Data curation. NM: Software, Formal analysis, Data curation, Writing – review & editing. DK: Methodology, Investigation, Funding acquisition, Writing – review & editing. LP: Project administration, Data curation, Writing – review & editing. MS: Writing – review & editing, Supervision, Resources, Funding acquisition.

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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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Dazomet fumigation modification of the soil microorganism community and promotion of *Panax notoginseng* growth

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Introduction: *Panax notoginseng*, a medicinal herb in China, is attacked by several pathogens during its cultivation. Dazomet (DZ) is a soil fumigant that is effective in controlling soil-borne pathogens, but its long-term effects on *P. notoginseng* growth and soil properties are unknown.

Methods: We conducted field experiments over two consecutive years to assess the impact of three concentrations of DZ fumigation (35 kg/666.7 m², 40 kg/666.7 m², and 45 kg/666.7 m²) on soil physicochemical properties, microbial diversity, and *P. notoginseng* growth. Correlation analyses were performed between microbial community changes and soil properties, and functional predictions for soil microorganisms were conducted.

Results: DZ fumigation increased total nitrogen, total phosphorus, total potassium, available phosphorus, available potassium, and ammonia nitrogen levels in the soil. DZ fumigation promoted the nutrient accumulation and improvement of agronomic traits of *P. notoginseng*, resulted in a 2.83–3.81X yield increase, with the highest total saponin content increasing by 24.06%. And the 40 kg/666.7 m² treatment had the most favorable impact on *P. notoginseng* growth and saponin accumulation. After DZ fumigation, there was a decrease in the relative abundance of pathogenic fungi such as *Fusarium*, *Plectosphaerella*, and *Ilyonectria*, while beneficial bacteria such as *Ramlibacter*, *Burkholderia*, and *Rhodanobacteria* increased. The effects of fumigation on soil microorganisms and soil physicochemical properties persisted for 18 months post-fumigation. DZ fumigation enhanced the relative abundance of bacteria involved in the biosynthesis of secondary metabolites and arbuscular mycorrhizal fungi, reduced the relative abundance of plant–animal pathogenic fungi, reduced the occurrence of soil-borne diseases.

Conclusion: In conclusion, DZ fumigation enhanced soil physicochemical properties, increased the proportion of beneficial bacteria in the soil, and rebalanced soil microorganism populations, consequently improving the growth environment of *P. notoginseng* and enhancing its growth, yield, and quality. This study offers a theoretical foundation for DZ fumigation as a potential solution to the continuous cropping issue in perennial medicinal plants such as *P. notoginseng*.

KEYWORDS

Dazomet, fumigation, *Panax notoginseng*, saponins, soil microbial diversity, function

1 Introduction

Panax notoginseng (Araliaceae), a traditional Chinese medicinal herb used for blood-stasis dispersal, bleeding cessation, and reduction of pain and swelling, is commonly used to prevent and treat traumatic injuries, cardiovascular, and cerebrovascular diseases. The primary active components of *P. notoginseng* include saponins, polysaccharides, amino acids, and volatile oils (Liu et al., 2020). Saponins exhibit activities such as anti-inflammatory, anticoagulant, and anti-tumor effects (Cheng et al., 2024). Polysaccharides from *P. notoginseng* have pharmacological properties that enhance immunity, lower blood sugar, mitigate radiation effects, reduce inflammation, and provide antioxidant benefits (Xing et al., 2021). Dencichines, found in the amino acids of *P. notoginseng*, has been identified as the key active ingredient for its hemostatic effect (Ji et al., 2023). In 2022, *P. notoginseng* in China was grown on 24,300 hectares and generated a total value of approximately 5.6 billion US dollars. However, continuous cropping poses significant challenges for successful *P. notoginseng* cultivation. For example, planting at intervals of less than 10 years can result in greatly reduced yields. During continuous growth of *P. notoginseng*, the deterioration of soil physicochemical properties and the accumulation of allelochemicals can cause an imbalance in the rhizosphere microbial community. This can result in increased pathogen populations causing *P. notoginseng* disease outbreaks (Ling et al., 2014; Tan et al., 2017).

Soil fumigation is an effective strategy employed to manage soil-borne diseases and address continuous cropping challenges. Dazomet (DZ) is commonly used due to its efficacy and low toxicity. DZ fumigation can rebalance the soil microbial ecosystem by reducing soil pathogens and enhancing populations of beneficial bacteria. Following DZ soil fumigation in apple orchards, biocontrol bacteria like *Pseudomonas*, *Bacillus*, and *Sphingomonas* increased while fungal pathogens such as *Fusarium*, *Pseudallescheria*, and *Kernia* declined. These changes fostered apple seedling growth (Chen et al., 2022). Dazomet is used to prevent soil-borne diseases in fruits, vegetables and Chinese herbal medicinal material, like rape clubroot, watermelon phytophthora blight and cucumber fusarium wilt (Tian et al., 2014; Hwang et al., 2017; Ge et al., 2021). At an application rate of 274.5 kg/hm², DZ prevented chrysanthemum rotavirus damage and wilt disease occurrence (Castello et al., 2022). DZ also reduced American ginseng root rot disease incidence by 69.39% (Peng et al., 2024), inhibited ginger root knot nematodes for up to 12 weeks, and improved ginger yields by 37.37% (Eo and Park, 2014; Wang et al., 2022).

DZ fumigation controls soil-borne diseases and also enhances crop agronomic traits. For example, DZ fumigation increased strawberry plant height, chlorophyll content and fresh weight. This increased soluble solids in fruits by 4.40% and yield by 4.32% (Li et al., 2023), in addition to improving the survival of *Codonopsis pilosula* (Campanulaceae) by 42.4% (Wang et al., 2021); the abundance of morel mycelium was also enhanced, and the number and yield of morel fruiting bodies increased (Chen et al., 2023).

To reduce the continuous cropping challenges of *P. notoginseng* cultivation, we studied the effects of DZ fumigation on soil physicochemical properties, microbiota and *P. notoginseng* growth over two consecutive years. The goal of the present study was to determine optimal DZ fumigation methods for reducing disease problems, particularly for perennial medicinal plants such as *P. notoginseng*.

2 Materials and methods

The experiment was conducted in Bozhu Town, Wenshan Zhuang and Miao Autonomous Prefecture, Yunnan Province (103°55'19"E, 23°27'18"N). Our experiment started in 2020 and continued until it concluded in 2022. The study utilized red soil that had been cultivated with *P. notoginseng* since 2010. One-year-old *P. notoginseng* seedlings were used (Yunnan Qidan Pharmaceutical Co., Ltd.).

2.1 Fumigation experiment

The field trial plots were 2×11 m, incorporating four concentration treatments with three replicates: CK (0 kg/666.7 m²), DZ35 (35 kg/666.7 m²), DZ40 (40 kg/666.7 m²), and DZ45 (45 kg/666.7 m²). Fumigation commenced on November 2, 2020. After plowing the plots to a depth of 20–30 cm, dazomet (DZ, C₅H₁₀N₂S₂, Nantong Shizhuang Chemical Co., Ltd.) was evenly applied to the soil surface, thoroughly mixed using a rotary tiller and immediately covered with 0.06 mm thick plastic film (Shandong Longxing Technology Co., Ltd.). The film was removed on December 7, 2020, after 35 days of fumigation, and the *P. notoginseng* seedlings were transplanted on December 27, 2020, following local agricultural practices.

2.2 Soil and plant sample collection

Soil samples were collected on December 7, 2020 (1 month post-fumigation) and March 26, 2022 (18 months post-fumigation) at a depth of 0–15 cm using a five-point sampling method (Bao, 2000). Samples were labeled CK1, DZ351, DZ401, and DZ451 for the 1 month post-fumigation, and CK2, DZ352, DZ402, and DZ452 for the 18 month post-fumigation. Each sample was divided into two parts: one part was sieved through 2 mm mesh, frozen in liquid nitrogen, and stored at −80°C for microbial analysis. The other part was air-dried naturally for analysis of physicochemical properties.

Samples of *P. notoginseng* plants were collected on September 29, 2021, from each treatment, including 15 randomly selected *P. notoginseng* plants. Parameters such as leaf area, length, width, thickness, and weights of different parts of *P. notoginseng* were measured using appropriate tools. These samples were dried, powdered, sieved, and stored for nutrient element analysis.

2.3 Physicochemical properties of soil

Soil N, P and K contents were measured as described in Soil and Agricultural Chemistry Analysis (Bao, 2000). In brief, total nitrogen (TN) was determined by the semi-micro Kjeldahl method (ZG/KDN-102F). Ammonium nitrogen (NH₄⁺-N) was determined by the 2 mol·L^{−1} KCl extraction - indophenol blue spectrophotometry (UV-2600, 625 nm). Nitrate nitrogen (NO₃[−]-N) was determined by the CaSO₄ and H₂O extraction - Phenol disulfonic acid colorimetric method (UV-2600, 420 nm). Total phosphorus (TP) was determined by HClO₄-H₂SO₄ digestion - molybdenum antimony colorimetric method (UV-2600, 880 nm). Available phosphorus (AP) was determined by the 0.5 mol·L^{−1} NaHCO₃ extraction - molybdenum

antimony colorimetric method (UV-2600, 880 nm). Total potassium (TK) was determined by the NaOH fusion - flame photometric method (FP6410). Available potassium (AK) was determined by the $1.0 \text{ mol} \cdot \text{L}^{-1} \text{ NH}_4\text{OAc}$ extraction-flame photometric method (FP6410).

2.4 Nutrient element determination in plants

Analysis of total nitrogen (N), total phosphorus (P), and total potassium (K) content in plants used the NY/T1017-2011 standard. The plant samples were digested with $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$, and N was determined using the Kjeldahl method (ZG/KDN-102F), P content was determined molybdenum antimony colorimetric method (UV-2600, 880 nm), and K content was determined using flame photometry (FP6410).

2.5 Saponin content determination

The sample solutions were prepared according to the method described in [Chinese Pharmacopoeia \(2020\)](#). A total of 0.3 g of each sample was weighed, 10 ml of 70% methanol was added, and the mixture was extracted by ultrasonication for 30 min and centrifuged at $3,000 \times g$ for 15 min. Then, the supernatant was harvested, the residues were extracted again, and the supernatants of the two extracts were combined. Next, 25 ml of 70% methanol was added, and the sample solution was filtered through a $0.22\text{-}\mu\text{m}$ filter membrane.

HPLC equipment (Agilent 1,200) was equipped with a column (BDS C18, $250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$) and a detector (203 nm) packed with ODS-bonded silica gel ($5 \mu\text{m}$ particle size). The separation system consisted of water (A) and acetonitrile (B). The injection volume was $10 \mu\text{l}$, and the flow rate was 1.0 ml/min . The chromatographic conditions included elution for 0–25 min with 20% B, 25–30 min with 25% B, 30–41 min with 41% B, 41–55 min with 45% B, 55–80 min with 74% B, 80–90 min with 100% B and 90–100 min with 20% B. The concentrations of saponins were obtained according to the standard curve and peak area.

2.6 High-throughput sequencing of soil microbiota

The DNA was extracted with the TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., DP812). The universal primers for 16S are F (AGRGTTTATYNTGGCTCAG), R (TASGGHTACCTGTTASGAGATT), and the universal primers for ITS are F (CTTGGTCATTTAGAGGAAGTAA) and R (TCCTCCGCTTATTGATGATTGC). A quality-controlled library is sequenced using PacBio Sequel. The raw subreads are corrected to obtain Circular Consensus Sequencing (CCS) sequences using SMRT Link version 8.0. Subsequently, the lima software (v1.7.0) is employed to identify CCS sequences of different samples based on barcode sequences. The cutadapt 1.9.1 software is used for primer sequence identification and removal, and length filtering to obtain Clean-CCS sequences devoid of primer sequences. The UCHIME v4.2 software is

then utilized to identify and remove chimeric sequences, resulting in Effective-CCS sequences.

2.7 Data processing and analysis

OTU clustering: The sequences are clustered at a 97% similarity level using USEARCH version 10.0 ([Edgar, 2013](#)), with a default threshold filter of 0.005% of the total sequenced reads for OTUs ([Bokulich et al., 2013](#)). Species annotation: Using the SILVA (16S) and UNITE (ITS) reference databases, the feature sequences are taxonomically annotated using a naive Bayesian classifier combined with alignment methods. This approach provides species classification information for each feature, with a classifier confidence level of 0.7. Subsequently, the community composition of each sample is analyzed at various taxonomic levels (phylum, class, order, family, genus, species) using the QIIME software to generate species abundance tables. Finally, R language tools are utilized to visualize the community structure of each sample at different taxonomic levels. Functional predictions for bacteria and phenotypic predictions for fungi are, respectively, performed using PICRUSt2 (16S) and FUNGuild (ITS). The bioinformatics analysis of this study was performed with the BMK online platform.¹ The data is processed using Microsoft Excel 2021, and graphs are created using GraphPad Prism 9.5 and Origin 2018. Statistical analysis such as one-way ANOVA is conducted using SPSS 23 software.

3 Results

3.1 Effects of DZ fumigation on the growth and development of *Panax notoginseng*

DZ fumigation significantly enhanced the emergence and survival rates of 2-year-old *P. notoginseng* ([Figure 1A](#)). Compared to CK, the emergence rates increased by 30.17–33.32% with DZ fumigation, and there were no significant differences among the DZ concentrations. The survival rates were 35.71% for CK and 81.6, 90.4, and 84% for the DZ35, DZ40, and DZ45 treatments, respectively. This indicated that DZ treatment greatly enhanced the emergence rate and survival rate of 2-year-old *P. notoginseng*, and the DZ40 treatment provided the most favorable outcome.

DZ fumigation promotes the growth of *P. notoginseng* plants, but there was no significant difference between different concentrations of dazomet ([Supplementary Table 1; Supplementary Figure 1](#)). The DZ treatment stimulated the accumulation of both aboveground and underground biomass of *P. notoginseng*. Compared to CK, the dry weight of the main root increased by 67.78, 90.06, and 76.83% under DZ35, DZ40, and DZ45 treatments, respectively ([Figure 1B](#)) and resulted in significant yield increases per acre of 2.83, 3.81 and 3.16X. In the DZ40 treatment, the aboveground dry weight of *P. notoginseng* was 19.32 and 4.247% higher than the DZ35 and DZ45 treatments, respectively, while the underground dry weight increased by 14.32 and 7.73%,

¹ <https://international.biocloud.net/>

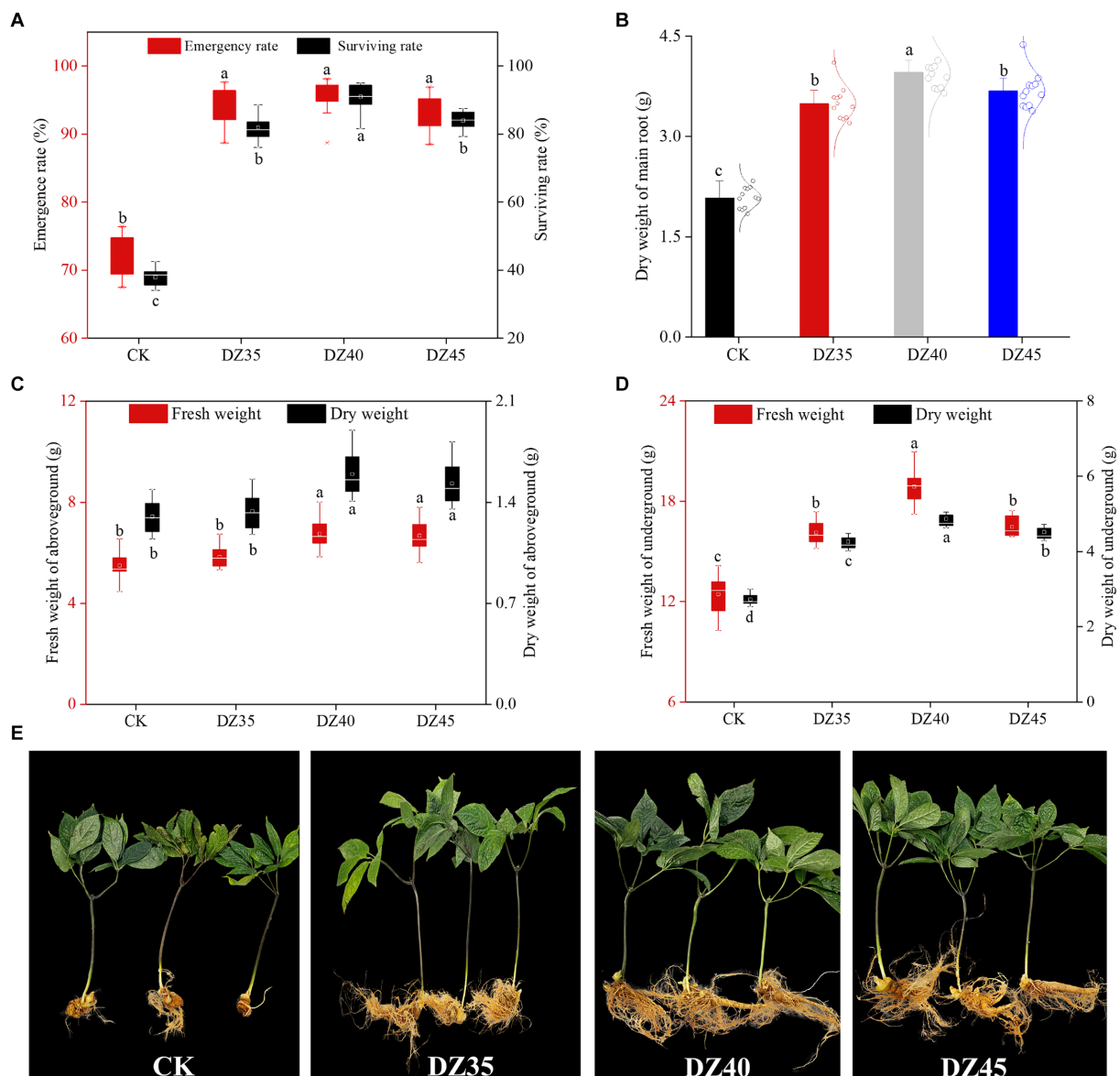


FIGURE 1
Effects of different concentrations of dazomet fumigation on emergence rate and survival rate (A), main root weight (B), aboveground weight (C), underground weight (D) and plant growth (E) of *P. notoginseng*. Lowercase letters indicate significance at the $p < 0.05$ level ($n = 15$).

respectively (Figures 1C,D). These results indicated that DZ fumigation enhanced *P. notoginseng* yield, with the largest effect produced by the DZ40 treatment.

3.2 Effects of DZ fumigation on the main saponins content of the main root

DZ fumigation can alter the main saponins contents in the main roots of *P. notoginseng*. Compared to the CK, the content of R₁, Rg₁, and Rd. in the main roots increased by 11.63, 21.71, and 22.26%, respectively, with DZ35 treatment; by 23.8, 43.18, and 32.25%, respectively, with DZ40 treatment; and by 46, 25.96, and 10%, respectively, with DZ45 treatment. The content of Re and Rb₁ changed by −5.9, 19.9, and −17.44% and 2.27, 5.31, and −9.57% (Figure 2).

Under DZ40 and DZ45 treatments, the total saponin content (PNS, sum of R₁, Rg₁, Rd., Re, and Rb₁) in the main roots was 8.80 and 7.96%, respectively, which were 24.06 and 12.15% higher than CK (Figure 2F). These results indicated that the PNS content increased after DZ fumigation, and DZ40 treatment showed the most significant promotion.

3.3 Effects of DZ fumigation on the levels of nitrogen, phosphorus, and potassium nutrients in plant and soil

DZ fumigation could significantly promote the accumulation of nutrient elements in the soil. Compared to the CK, after 18 months of fumigation, the contents of TN, TP, TK, AK, AP, and NH₄⁺-N in the

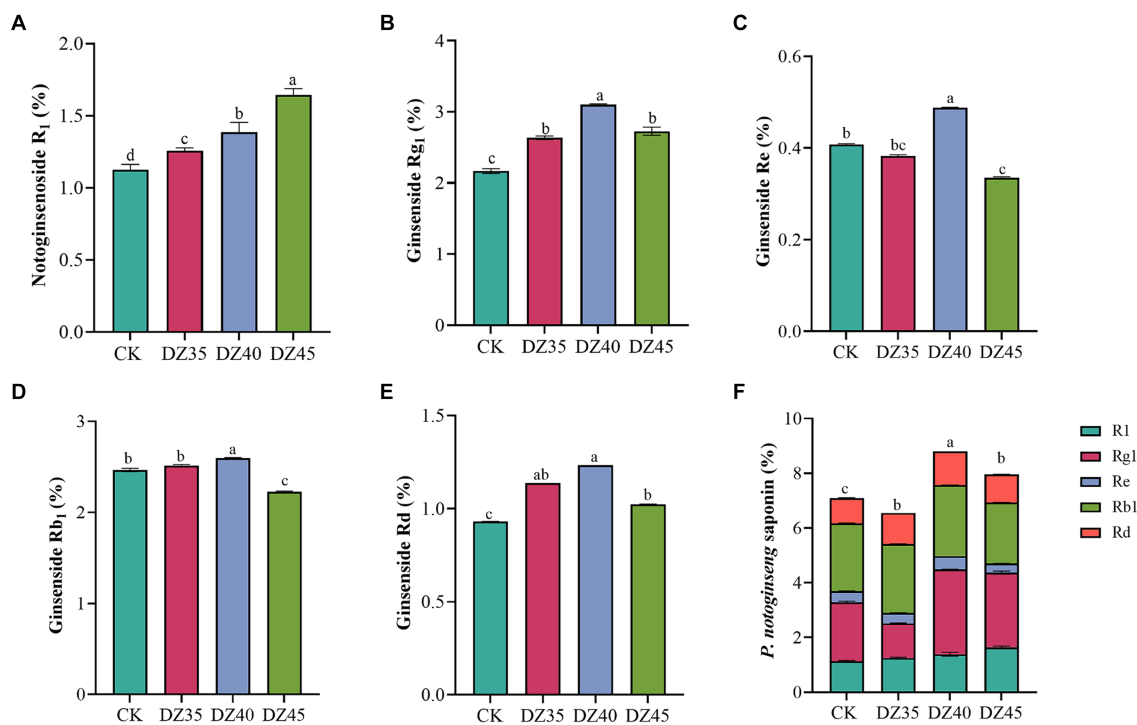


FIGURE 2
Effects of dazomet fumigation on the main saponins contents of *P. notoginseng*. Lowercase letters indicate significance at $p < 0.05$ level ($n = 3$).

soil were significantly increased after DZ fumigation, and the enhancement effect increased with the DZ application amount (Figure 3). One month after fumigation, compared to the CK, the content of NO_3^- -N in the soil significantly decreased, and a higher DZ concentration correlated with lower NO_3^- -N content. However, after 18 months of fumigation, DZ fumigation showed a significant promoting effect on the content of NO_3^- -N in the soil (Figure 3B). This indicated that over time, the inhibitory impact of DZ fumigation on soil nitrification gradually diminishes, ultimately facilitating soil nitrification.

DZ fumigation promoted the accumulation of nitrogen, phosphorus, and potassium in *P. notoginseng* plants. After DZ fumigation, the content of nitrogen, phosphorus, and potassium in the main root, leaf, rhizome, rootlet, and stems was significantly higher than that in the untreated control (CK), especially in the DZ40 treatment. The contents of nitrogen in the main root, leaves, rhizomes, rootlet, and stems increased by 54.36, 19.68, 25.62, 19.56, and 84.81% respectively, and the contents of phosphorus increased by 98.29, 16.28, 43.64, 24.52, and 122.43% respectively, and the contents of potassium increased by 15.91, 26.57, 21.45, 25.01, and 27.25%, respectively, compared to the CK (Figure 4).

3.4 Effect of DZ fumigation on soil microorganisms

3.4.1 Effect of DZ fumigation on the α -diversity of soil microbial communities

At 1 month following DZ fumigation, the Chao1 and Shannon indices of soil bacteria were significantly increased compared to CK. A decrease was observed with the increase in DZ application amount

(Figures 5A,B). DZ fumigation also resulted in increased Chao1 and Shannon indices of soil fungi, except for the DZ45 treatment, which showed a decrease with the increase in DZ concentration (Figures 5C,D).

At 18 months following DZ fumigation, the Chao1 and Shannon indices of bacteria and fungi in the soil were significantly lower than CK. The bacterial Chao1 and Shannon indices under DZ40 treatment were slightly higher than that of DZ35 and DZ40 (Figures 5E,F). The Chao1 index of fungi in the soil decreased overall with the increasing application amount of DZ, while the Shannon index had an overall increase with increasing application amount of DZ (Figures 5G,H).

3.4.2 Effect of DZ fumigation on the composition of bacterial and fungal genera in soil

The dominant bacterial genera in the soil included *DA101*, *Candidatus-Koribacter*, *Bradyrhizobium*, and *Rhodoplanes* (Figure 6A). DZ fumigation increased the relative abundance of *Candidatus-Koribacter* and *Rhodoplanes*, while decreasing the relative abundance of *DA101* and *Bradyrhizobium*. One month after fumigation, compared to CK, the relative abundance of *Candidatus-Koribacter* increased by 77.89, 63.86, and 77.55% with the DZ35, DZ40, and DZ45 treatments, respectively. The relative abundance of *Rhodoplanes* increased by 80.85, 137.89, and 74.67% in the same treatments. The relative abundance of *DA101* decreased by 53.99, 61.03, and 59.40%, and the relative abundance of *Bradyrhizobium* decreased by 68.44, 64.15, and 64.06%. DZ fumigation also promoted the growth of beneficial bacteria, such as *Rhodanobacter*, *Ramlibacter*, and *Burkholderia* (Figures 6C–E).

At 18 months after fumigation, compared to the CK, the relative abundance of *Candidatus-Koribacter* continued to increase, while the relative abundances of *DA101*, *Bradyrhizobium*, and *Rhodoplanes* showed decreasing trends. Specifically, compared to

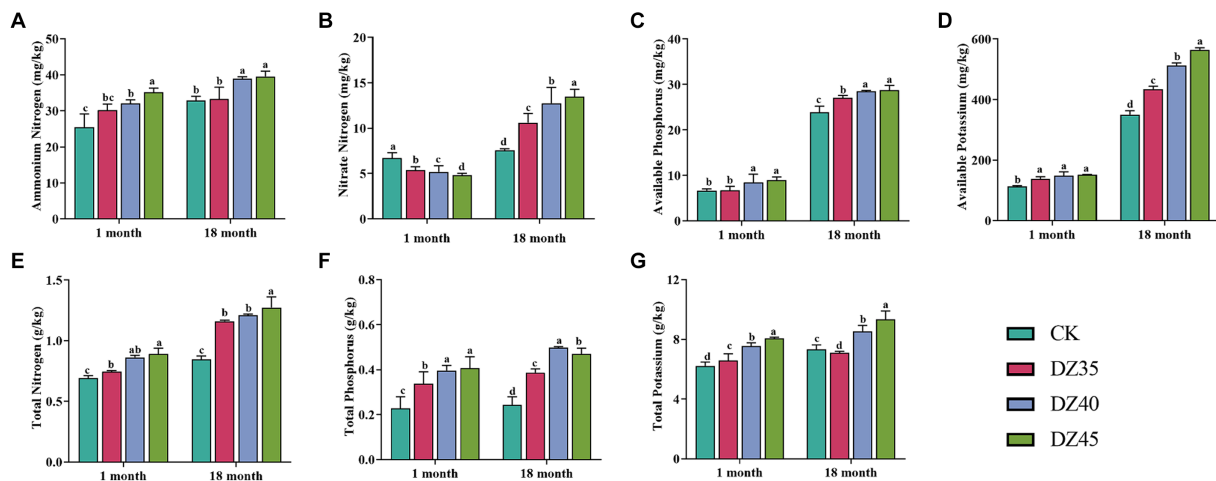


FIGURE 3

Effects of dazomet fumigation on the levels of ammonium nitrogen (A), nitrate nitrogen (B), available phosphorus (C), available potassium (D), total nitrogen (E), total phosphorus (F) and total potassium (G) in soil. Lowercase letters indicate significance at $p < 0.05$ level ($n = 3$).

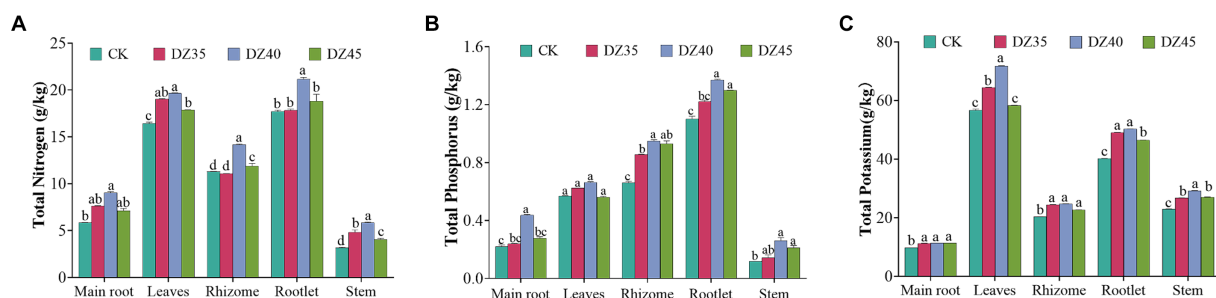


FIGURE 4

Effects of dazomet fumigation on the content of total nitrogen (A), total phosphorus (B) and total potassium (C) of *P. notoginseng*. Lowercase letters indicate significance at $p < 0.05$ level ($n = 3$).

the CK, the relative abundance of *DA101* decreased by 4.16, 2.63, and 2.55% in the DZ35, DZ40, and DZ45 treatments, respectively. The relative abundance of *Bradyrhizobium* changed by -7.01, 50.44, and 43.01%, while that of *Rhodoplanes* changed by -11.65, 27.55, and 30.93%. In contrast, the relative abundance of *Candidatus-Koribacter* increased by 64.01, 150.91, and 74.85%, compared to CK (Figure 6A). The relative abundances of *Rhodanobacter* and *Ramlibacter* significantly increased compared to the CK, while the relative abundance of *Burkholderia* decreased by 89.66 to 99.60%. The highest relative abundance of *Rhodanobacter*, *Ramlibacter*, and *Burkholderia* were observed in the DZ40 treatment (Figures 6F–H).

Mortierella is a dominant soil fungus. DZ fumigation significantly reduced its relative abundance, and the relative abundance decreased with increasing DZ application. One month after fumigation, compared to CK, the relative abundance of *Mortierella* in the DZ35, DZ40, and DZ45 treatments decreased by 23.98, 51.13, and 56.43%, respectively (Figure 6B). DZ fumigation reduced the relative abundance of soil pathogens *Fusarium*, *Plectosphaerella* and *Ilyonectria*. Compared to CK, the relative abundance of *Fusarium* decreased by 70, 70.81, and 85.08% in the DZ35, DZ40, and DZ45 treatments, respectively. *Plectosphaerella* decreased by 66.49, 76.95,

and 68.43% while *Ilyonectria* decreased by 61.44, 92.25, and 100% (Figures 6I–K).

At 18 months following fumigation, the relative abundance of *Mortierella* in the DZ35 treatment increased by 34.93% compared to the CK treatment, while the DZ40 and DZ45 treatments also showed suppressive effects on its relative abundance (Figure 6B). A strong inhibitory effects were still observed on the relative abundance of *Fusarium* and *Plectosphaerella* after DZ fumigation, with the relative abundance of *Fusarium* decreased by 99.03–99.66%, and the *Plectosphaerella* decreased by 28.29–74.36%, compared to the CK (Figures 6L–N).

3.4.3 Correlation analysis of soil microbial communities

In Figure 7A, the X and Y axes accounted for 33.57 and 24.2%, respectively, of the variation in the community structure at the bacterial genus level. Environmental factors such as TN, TP, AK, AP, AN, and NN had p values less than 0.01, indicating a significant correlation with the community structure. The pH and TK factors had p values of 0.015 and 0.016, respectively, which were considered statistically significant. However, EC, with a p value of 0.18, was not significant. This suggested that all environmental factors, except EC,

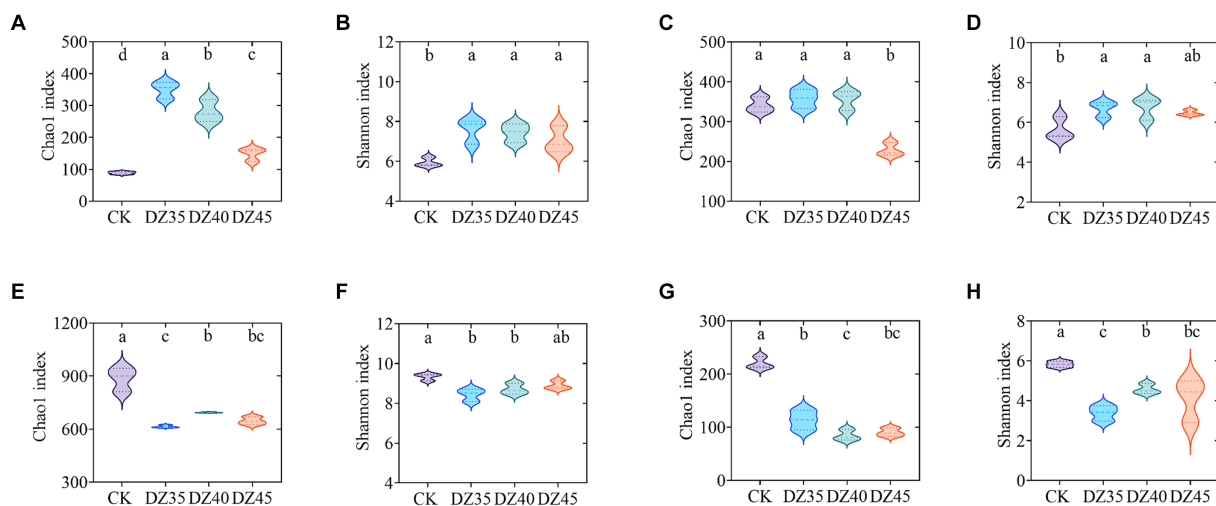


FIGURE 5

Alpha diversity indices of soil bacteria and fungi within dazomet fumigation. (A) The bacterial Chao1 index for one month; (B) The bacterial Shannon index for one month; (C) The fungal Chao1 index for one month; (D) The fungal Shannon index for one month; (E) The bacterial Chao1 index for 18 month; (F) The bacterial Shannon index for 18 month; (G) The fungal Chao1 index for 18 month; (H) The fungal Shannon index for 18 month. Lowercase letters indicate significance at $p < 0.05$ level ($n = 3$).

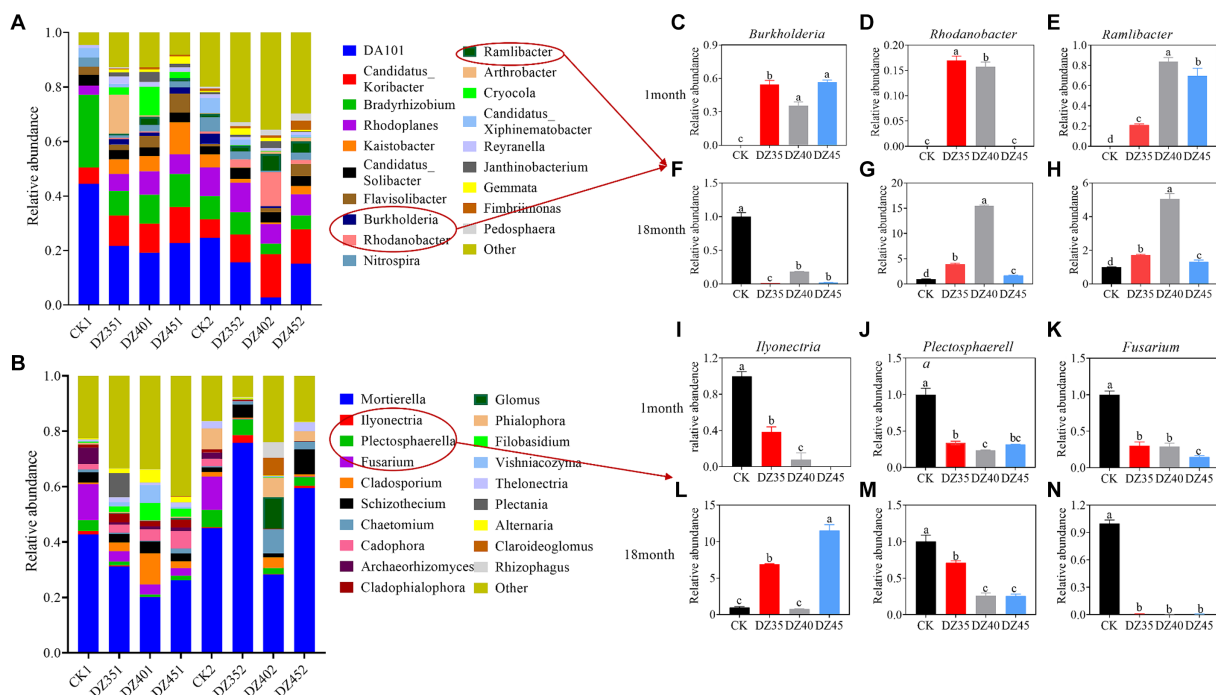


FIGURE 6

Effects of dazomet fumigation on the composition of soil bacteria (A) and fungi (B) at the genus level (TOP 20). The relative abundance of Burkholderia (C), Rhodanobacter (D), and Ramlibacter (E) for one month; The relative abundance of Burkholderia (F), Rhodanobacter (G), and Ramlibacter (H) for 18 month; The relative abundance of Ilyonectria (I), Plectosphaerella (J), and Fusarium (K) for one month; The relative abundance of Ilyonectria (L), Plectosphaerella (M), and Fusarium (N) for 18 month. Lowercase letters indicate significance at $p < 0.05$ level ($n = 3$).

significantly impacted the bacterial community structure. Among these factors, soil TN and NN content exhibited the strongest correlation, followed by AP, AK and TP. The correlation heatmap revealed a positive relationship between most bacterial genera and soil physicochemical properties, particularly with NN, AK, and AP. These findings were consistent with the results of the RDA

analysis in Figure 7C. For instance, *Pedobacter* showed highly significant positive correlations with NN, AK, AP, TN, AN, and EC; *Opitutus*, *Phenylobacterium*, *Cupriavidus*, *Bosea*, *Pedosphaera*, and *Afipia* showed highly significant positive correlations with NN, AK, and AP; *Fimbrimonas* and *Hyphomicrobium* showed highly significant positive correlations with AP and AK; *Dyella* showed a

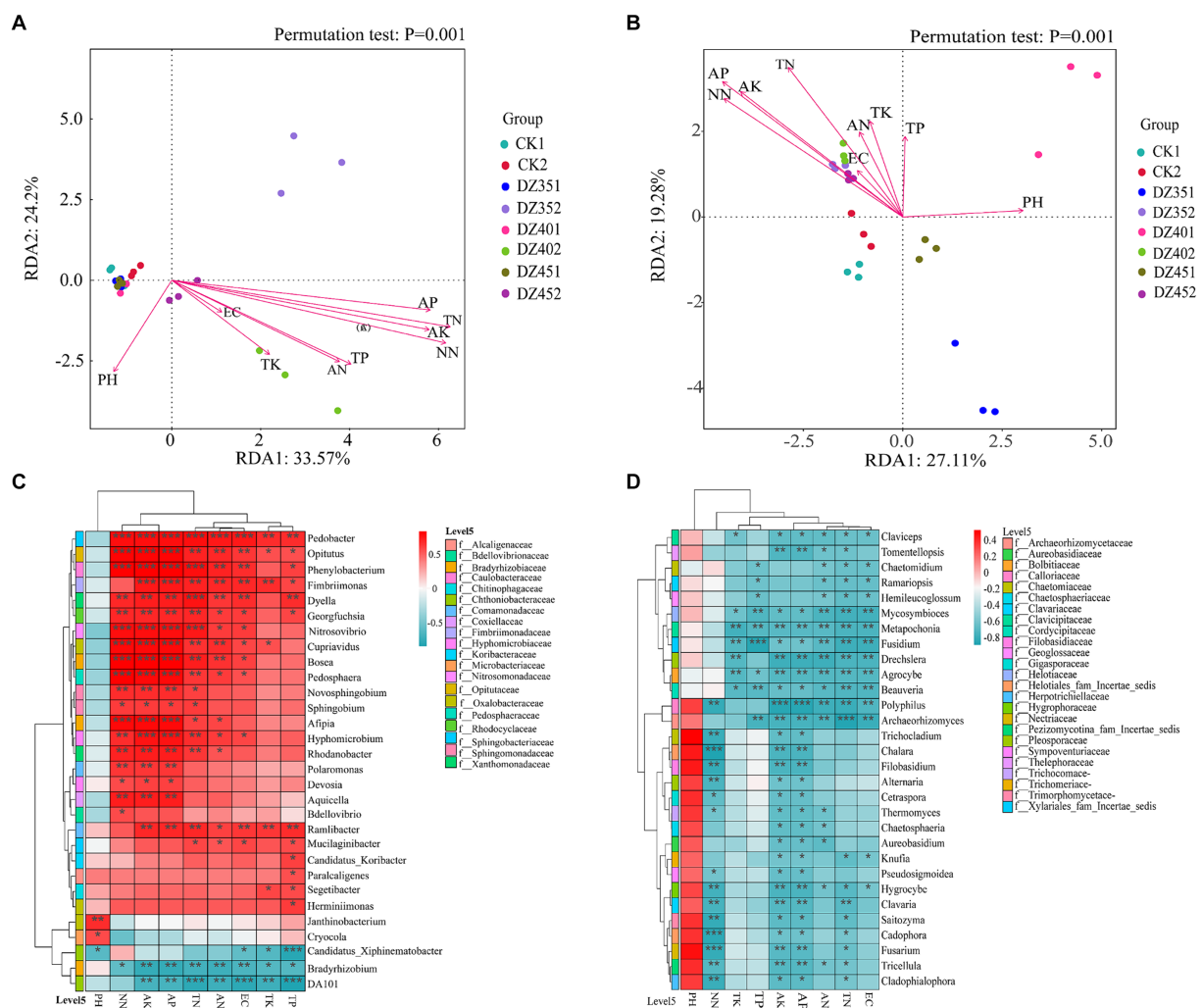


FIGURE 7

Redundancy analysis (RDA) and correlation heatmap analysis of soil physicochemical with microorganisms (A/C: bacteria, B/D: fungi).

highly significant positive correlation with TN; *Phenyllobacterium* and *Nitrososporium* showed highly significant positive correlations with NN, AK, AP and TN; *Janthinobacterium* and *Cryocella* showed significant correlations with pH. Conversely, *Bradyrhizobium* and DA101 had significant negative correlations with AK, AP, TN, AN, EC, TK and TP.

In Figure 7B, the X and Y axes accounted for 27.11 and 19.28%, respectively, of the variation in the horizontal community structure of fungi. Except for TP and EC, other environmental factors significantly explained this variation, with pH, TN, AP, AK and NN having p values less than 0.01, indicating a highly significant relationship. Specifically, AP content in the soil showed the strongest correlation with fungal community structure, followed by NN, AK and TN. The correlation heatmap analysis in Figure 7D demonstrated that most fungal genera have negative correlations with soil physicochemical indicators, except for pH. Notably, *Chalara*, *Cadophora* sp., and *Fusarium* showed significant negative correlations with NN; *Fusidium* had a negative correlation with TP; *Polyphillus* exhibited positive correlations with AK and AP; and *Archaeorhizomyces* showed a negative correlation with TN.

3.4.4 Functional prediction of soil microorganisms

After fumigation, soil bacterial functions in Level 1 of the KEGG database were primarily categorized into metabolism, cellular processes, genetic information processing, and human diseases. Metabolism-related pathways constituted 59.09 to 59.12%, while cellular processes made up 30.44 to 30.78%, with the lowest percentage associated with human diseases and genetic information processing (Figure 8). At 1 month after fumigation, there was an increase in the relative abundance of certain functional bacterial communities participating in metabolism and genetic information processing. However, the majority of functional bacterial communities declined in relative abundance. Compared to CK, bacterial groups linked to carbohydrate metabolism, cofactor and vitamin metabolism, nucleotide metabolism, biosynthesis of other secondary metabolites, amino acid metabolism, replication and repair, and translation showed increases, with DZ40 treatment displaying the highest relative abundance (Figure 8A). At 18 months after fumigation, bacterial groups associated with amino acid metabolism, cofactor and vitamin metabolism, nucleotide metabolism, other amino acids metabolism,

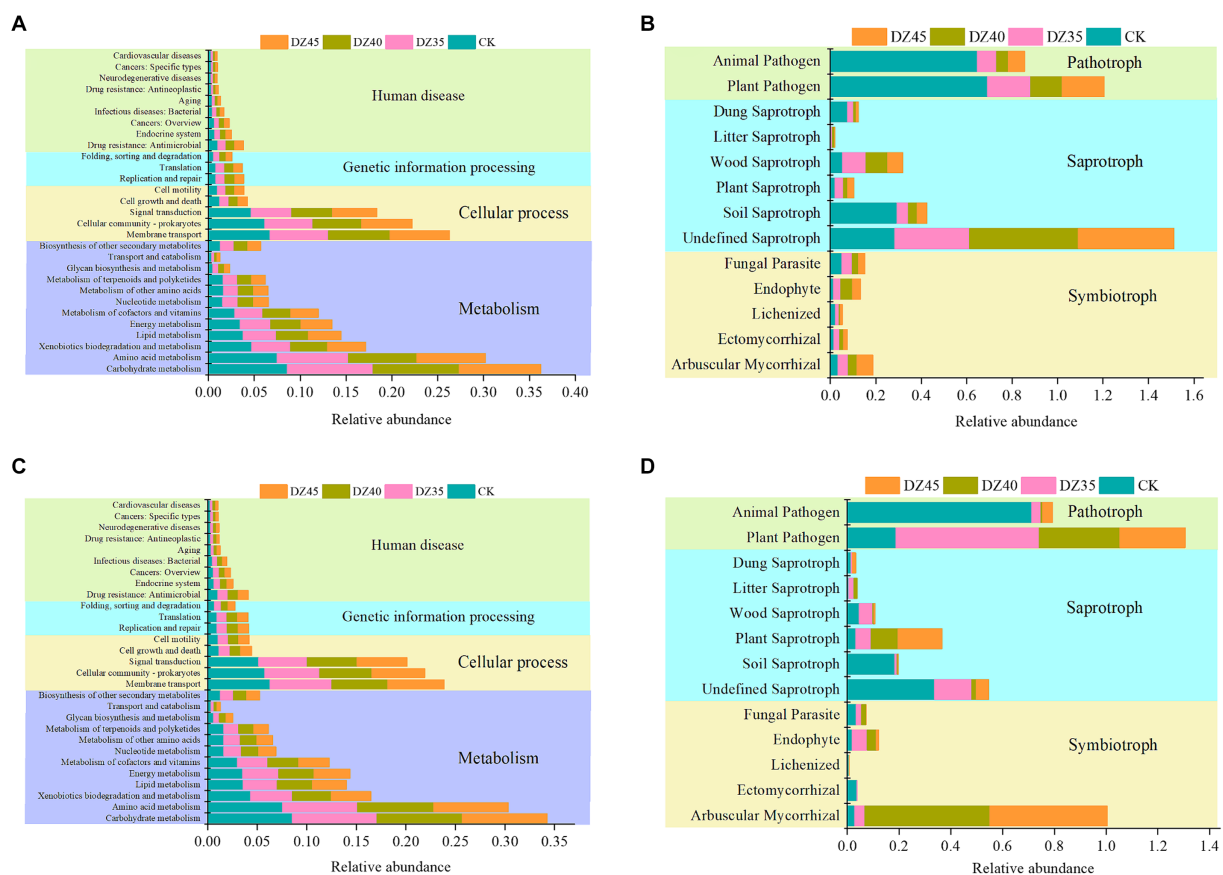


FIGURE 8

Prediction analysis of soil microorganisms at 1 month (A: bacteria, B: fungi) and 18 months after fumigation (C: bacteria, D: fungi).

transport and catabolism, and biosynthesis of other secondary metabolites showed a higher relative abundance in the DZ40 treatment compared to CK, DZ35, and DZ45 treatments (Figure 8C). Bacterial groups involved in biosynthesis of other secondary metabolites exhibited higher relative abundance compared to other treatments, correlating with the higher PNS content of secondary metabolites in the DZ40 treatment compared to other treatments.

Soil fungi can be categorized, based on their nutrition modes, into pathotroph pathogen, symbiotroph pathogen, and saprotroph pathogen. In this study, the fungal communities in fumigated soil revealed shifts in their functional composition because of DZ fumigation. One month after fumigation, the relative abundances of arbuscular mycorrhizal-ectomycorrhizal fungi and undefined-plant-wood-litter saprotrophic fungi increased, while the relative abundance of plant-animal pathogen fungi significantly decreased. In comparison to the CK, the relative abundances in the DZ35, DZ40, and DZ45 treatments declined by 79.59, 85.78, and 80.65%, respectively, (Figure 8B). This indicated that DZ fumigation enhanced the proliferation of soil saprotrophic fungi involved in decomposing plant residues, promoting carbon and nitrogen cycling and concurrently reducing the relative abundance of pathogenic fungi. These changes reduced the risk of infection and incidence of soil-borne disease.

At 18 months after fumigation, the relative abundance of arbuscular mycorrhizal-ectomycorrhizal fungi in the soil remained higher than that in the CK treatment. The relative abundance of these fungi in the DZ40 and DZ45 treatments was 642.92 and 599.32%

higher than in the CK treatment, respectively. Conversely, the relative abundances of saprotrophic fungi and plant-animal pathogenic fungi were lower than those in the CK treatment. With an increasing DZ application amount, the relative abundance of plant-animal pathogenic fungi in the soil decreased (Figure 8D).

4 Discussion

Soil microbes regulate soil ecology and soil immunity, which are critical for maintaining soil health. Significant negative plant-soil feedback (NPSF) exists between *P. notoginseng* and soil. Throughout *P. notoginseng* growth, the accumulation of root exudates in the rhizosphere soil can inhibit the growth of beneficial bacteria and facilitate the proliferation of pathogenic fungi. This leads to an imbalance in rhizosphere microbial ecology, which is the main driver of *P. notoginseng*-soil NPSF (Wei et al., 2018; Luo et al., 2019). Fumigation can alter the ratio of pathogenic and beneficial bacteria and reshape the ecological balance of soil microorganisms (Ibekwe et al., 2001). *Fusarium*, *Plectosphaerella* and *Ilyonectria* are pathogens that cause plant wilt, root rot, and rust diseases. These species pose challenges for consecutive cropping of tomato and *P. notoginseng* (Gao et al., 2021; Luo et al., 2022). The present study revealed that DZ fumigation significantly inhibited the growth of *Fusarium*, *Plectosphaerella*, and *Ilyonectria* in the test soil, with the inhibitory effect persisting for up to 18 months after fumigation. DZ

fumigation also increased the relative abundance of beneficial bacteria, including *Ramlibacter*, *Rhodanobacter* and *Burkholderia* in the soil, with the levels of *Ramlibacter* and *Rhodanobacter* remaining significantly elevated 18 months after fumigation (Figure 6). The main reason for this phenomenon is that the degradation product of DZ in soil (methyl isothiocyanate) can kill *Fusarium* by interfering with its reactive oxygen species scavenging capacity (Zhang et al., 2024), and *Rhodanobacter* has an antagonistic effect on *Fusarium*, which can effectively inhibit the growth of *Fusarium* (Huo et al., 2018). *Burkholderia* as a soil biocontrol bacterium, which can alleviate the NPSF and improve the emergence rate and fresh weight of *P. notoginseng* plants (Santos et al., 2004; Elshafie et al., 2012; Esmaeel et al., 2019; Luo et al., 2019). *Ramlibacter* and *Rhodanobacter* played significant roles in the soil nitrogen cycle, contributing to the enhancement of soil fertility (Yu et al., 2019). Overall, DZ fumigation improved the soil microecological environment, alleviated the NPSF of *P. notoginseng* and promoted its growth and development.

The soil environment, encompassing pH, N, P, K, temperature, and humidity, is crucial in shaping the structure and diversity of soil microbial communities. Strong associations exist between specific microbial groups and soil variables (Hermans et al., 2020). Our results showed that soil AP, AN, NN were significantly associated with the taxonomic structure of fungal genera, and the pathogenic fungus *Fusarium* exhibited a highly significant negative correlation with soil NN (Figure 7B). An increase in NN content in the soil effectively regulated the incidence of root rot disease in *P. notoginseng*, consistent with previous research (Ren et al., 2021; Zhao et al., 2023). These data suggest that managing NN levels can help control *P. notoginseng* diseases induced by *Fusarium* and other fungi positively correlated with *Fusarium*. This management could reduce pesticide usage and promote sustainable cultivation of *P. notoginseng*.

The nutritional environment in the soil is critical in influencing the distribution and composition of soil microbiota, which, in turn, drives soil nutrient cycling. Soil microbiota are involved in nutrient transformation and regulation of nutrient types and quantities. DZ fumigation can modify the abundance and community structure of denitrifying bacteria, suppress nitrification, and promote the conversion of nitrate into ammonium nitrogen (Fang et al., 2020; Sennett et al., 2022). In the present study, the TN and $\text{NH}_4^+\text{-N}$ contents in the soil after DZ fumigation were significantly elevated compared to CK (Figures 3A,B,E). This increase may be attributed to the increased abundance of *Rhodanobacter* and *Ramlibacter* in the soil following DZ fumigation, which enhance denitrification and nitrate assimilation reduction processes and increase $\text{NH}_4^+\text{-N}$ content (Lee et al., 2007; Prakash et al., 2012; Huang et al., 2021; Hu et al., 2023).

Soil microbial activity is the primary force driving phosphorus transformation and cycling. DZ fumigation decreases the abundance and diversity of soil microorganisms carrying *phoD*, promotes phosphorus mineralization and results in a rapid increase of AP content (Huang et al., 2020). We also found that DZ fumigation augmented the levels of AP and TP in the soil (Figures 3C,F). The release of phosphorus-containing substances such as nucleic acids post-microbial death resulting from fumigation, combined with the increased relative abundance of phosphate-solubilizing bacteria like *Burkholderia* due to DZ fumigation, facilitates the dissolution of insoluble phosphates. This, consequently, elevates soil phosphorus content (Lin et al., 2006; Weisskopf et al., 2011).

Despite previous reports concluding that DZ fumigation did not significantly affect soil potassium content and even decreased the AK level (Li et al., 2017, 2023; Chen et al., 2022), we observed an increase in AK and TK levels in the soil after DZ fumigation (Figures 3D,G). This rise may be attributed to the enhanced relative abundance of potassium-solubilizing microbes (KSM) such as *Burkholderia* and *Arthobacter* as a result of DZ fumigation, which promoted the release and dissolution of mineral potassium (Sharma et al., 2024). Additionally, DZ fumigation led to an upsurge in the relative abundance of saprophytic fungi, which accelerated plant residue decomposition, enhanced potassium release from plants into the soil, thereby increasing soil potassium content.

Soil serves as the medium for plant growth, with soil microbial diversity crucial for maintaining plant productivity. Plant nutrient uptake and stress resistance rely heavily on soil microbes (Chen et al., 2020). Compared to the high metabolic consumption and absorption of root systems, plants typically acquire nutrients from the soil environment through fungal networks (Felderer et al., 2013). Arbuscular mycorrhizal fungi (AMF), as obligate symbiotic organisms engaging in root symbiosis, establish a mutualistic association with plant roots (Gadkar et al., 2001). AMF can enhance plant water and nutrient absorption capabilities (P and N) by improving physiological morphology and thereby promote plant growth (Ruiz-Lozano et al., 2016; Andriano et al., 2021; Jing et al., 2022). In addition, arbuscular mycorrhizal fungi can also increase the medicinal active ingredients of medicinal plants by enhancing biomass or secondary metabolic synthesis pathways. The promoting effects are most significant for flavonoids (68%) and terpenes (53%), and there is a significant increase in the content of medicinal components in underground parts (Zeng et al., 2013; Zhao et al., 2022; Ren et al., 2023; Yuan et al., 2023). We speculate that the fumigation with DZ increased the relative abundance of arbuscular mycorrhizal fungi in the soil, increased the infection rate of AMF in Sanqi, and ultimately led to a significant increase in biomass and saponin content of *P. notoginseng* (Figures 2, 4; Supplementary Table 1; Supplementary Figure 1).

Soil fumigation has been practiced in China for almost 70 years. While it helps to decrease pesticide and fertilizer usage and enhance crop yield, the continuous application of DZ can hasten its breakdown, diminishing its efficacy in managing soil-borne diseases (Di Primo et al., 2003). In regions where DZ is frequently applied, farmers may opt to escalate the dosage for improved fumigation outcomes. Nevertheless, DZ fumigation can elevate the total abundance and diversity of antibiotic resistance genes (ARGs) in the soil, with this rise positively associated with the dazomet dosage (Zhang et al., 2023). The soil-plant system is a significant pathway for the acquisition and dissemination of ARGs. Further research is needed to determine if DZ fumigation will elevate the risk of human exposure to ARGs through the *P. notoginseng* chain.

5 Conclusion

This study found that DZ fumigation before planting *P. notoginseng* reshaped the ecological balance of soil microbiota by

reducing the relative abundance of pathogenic fungi such as *Fusarium*, *Plectosphaerella* and *Ilyonectria*, while increasing the relative abundance of beneficial bacteria like *Ramlibacter*, *Burkholderia* and *Rhodanobacters*. This restructuring promoted the favorable development of soil microecology, leading to effective control of soil-borne diseases and enhancing the emergence and survival rates of *P. notoginseng*. DZ fumigation improved the soil microbial environment and increased levels of TN, TP, AP, and $\text{NH}_4^+\text{-N}$. These modifications promoted the absorption and utilization of nutrients, as well as the growth and development of *P. notoginseng*. Consequently, these changes resulted in increased saponin accumulation, thereby improving the quality of *P. notoginseng*. In conclusion, DZ fumigation effectively addresses the challenges of continuous cropping with *P. notoginseng*, enhancing both its yield and quality.

Data availability statement

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

Author contributions

Y-mL: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. M-hL: Data curation, Formal analysis, Methodology, Project administration, Validation, Writing – original draft. C-yD: Supervision, Visualization, Writing – review & editing. YL: Methodology, Visualization, Writing – review & editing. W-pZ: Investigation, Methodology, Writing – review & editing. QY: Conceptualization, Methodology, Writing – review & editing. X-mC: Resources, Writing – review & editing. YY: Conceptualization, Data curation, Funding acquisition, Resources, Writing – review & editing.

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Supplementary material

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The impact of salinization on soil bacterial diversity, yield and quality of *Glycyrrhiza uralensis* Fisch.

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Soil salinization seriously affects soil microbial diversity, and crop yield and quality worldwide. Microorganisms play a vital role in the process of crop yield and quality. Traditional Chinese medicine *Glycyrrhiza uralensis* Fisch. (licorice) can grow tenaciously in the heavily salinized land. However, the relationship between licorice plants and soil microorganisms is not clear. A field experiment was carried out to explore the effects of three different degrees of salinized soils on (i) licorice crop performance indicators, (ii) soil physical and chemical properties, and (iii) the changes in soil bacterial community structure and functional diversity in a semi-arid area of northwest China. The results showed that with the aggravation of soil salinization, the licorice yield, soil nutrients, and the bacterial abundance of Gemmatimonadetes and Myxococcota showed a downward trend, while the concentration of glycyrrhizic acid and liquiritin, and the bacterial abundance of Actinobacteria and Firmicutes showed an upward trend. The change of licorice yield mainly depended on the soil physical and chemical properties (e.g., EC and alkaline hydrolysable nitrogen). The change of licorice quality was more closely related to the change of bacterial diversity. The effect of bacterial diversity on liquiritin was greater than that on glycyrrhizic acid. Among them, Gemmatimonadetes were significantly negatively correlated with liquiritin and glycyrrhizic acid. These findings suggest that the increased soil Actinobacteria and Firmicutes or reduced Gemmatimonadetes and Myxococcota may provide a healthy and suitable living condition for the sustainable development of medicinal plant crops in a salinized soil ecosystem.

KEYWORDS

salinization, soil bacteria, Chinese medicinal materials, adaptability, yield and quality

1 Introduction

Soil salinization is one of the important driving factors of global ecosystem degradation, with an area of about 8.33×10^9 ha, and is one of the greatest challenges for the sustainable development of agriculture in the future. With the continuous disturbance of climate change and human activities, the problem of soil salinization will become more prominent. In agricultural production, most crops are not tolerant to salinity or grow poorly in saline-alkali land and their yield is reduced. Many medicinal plants can adapt to salinized and barren soil,

and have high economic and ecological benefits. At present, it is a research hotspot to analyze the relationship between medicinal plant yield and quality formation under a saline-alkali environment.

Soil bacteria are a key driver of plant productivity and play an important role in plants responding to and adapting to changes in soil salinization conditions (Zhang et al., 2023). Plants establish relationships with soil bacteria to mitigate the effects of non-favorable environmental factors on plant growth and yield, and in this process, soil bacteria play an important role (Singh et al., 2020; Omae and Tsuda, 2022; Wang Y. et al., 2022). Among them, soil bacteria which are beneficial to plants, can help resist environmental stresses such as drought and diseases, and promote the healthy growth of plants. In addition, soil bacteria can also change the soil habitat through chemical and physical methods, making the soil more suitable for plant survival (Philippot et al., 2024). Within a certain range of salinity fluctuation, some soil bacteria can regulate their own physiological metabolism to cope with salt stress. Some halophilic microbes can actively absorb salt ions (primarily potassium ions) to increase cell osmotic pressure during salt stress, sometimes by exporting sodium ions (Oren, 2008). In addition, certain amino acids and carbohydrates can be synthesized in microbial cells to balance osmotic pressure inside and outside of the cell (Rath and Rousk, 2015). At the same time, bacteria can also “help” plants to cope with salt stress. In the face of saline-alkali stress and other adversity, bacteria may improve the tolerance of plants to saline-alkali stress. Previous studies mainly focused on the effects of soil microecology on continuous cropping obstacles of medicinal materials (Zeeshan Ul Haq et al., 2023; Liao and Xia, 2024), changes in rhizosphere nutritional status and changes in root growth (Philippot et al., 2013; Solomon et al., 2024), but there were few systematic studies on the effects of plant soil microecology on the accumulation of active ingredients within the plant (Trivedi et al., 2020; Pang et al., 2021; Wang G. et al., 2022).

Licorice (Gancao in Chinese, GC) plants contain more than 400 compounds (Xiang et al., 2012), the secondary metabolites of licorice play an important role in the efficacy, liquiritin and glycyrrhizic acid are the main effective secondary metabolites of licorice (Jiao et al., 2020). It is considered an “essential herbal medicine” in traditional Chinese medicine (TCM) and is widely used in the pharmaceutical and food industries. There are many effects of licorice, such as tonifying the spleen and qi, removing heat and toxic substances, eliminating phlegm, relieving cough and pain, and harmonizing the effects of other medicines (Yan et al., 2023). Modern pharmacological studies have shown that licorice also has antibacterial, anti-inflammatory, antiviral, anti-tumor, anti-oxidation, hypoglycemic, blood lipid regulation, anti-atherosclerosis and other effects (Wahab et al., 2021; Wu et al., 2021). Licorice also has strong salt and alkali resistance.

Previous studies have shown that plants can affect their soil bacteria by synthesizing or secreting various metabolites (Jacoby et al., 2021; Koprivova and Kopriva, 2022), and in turn, soil bacteria may also affect the secondary metabolism of host plants. Researchers have found that soil bacteria can promote the formation of active ingredients of medicinal plants, such as tanshinones in *Salvia miltiorrhiza* (Chen et al., 2018; Huang et al.,

2018) and chicory acid in *Echinacea purpurea* (Maggini et al., 2019). The introduction of plant native *Bacillus subtilis* subsp. (Firmicutes) *subtilis* Co1-6 and *Paenibacillus polymyxa* Mc5Re-14 into chamomile (*Matricaria chamomilla* L.) seedlings can not only stabilize the growth performance of plants, but also increase the concentration of flavonoids apigenin-7-O-glucoside and apigenin in chamomile (Kato et al., 2008; Köberl et al., 2013). The soil bacterial groups in different plants and different strains of the same plant are also different. However, the research on the effect of plant-bacteria interaction on the secondary metabolites of host plants under saline soil environment is still weak (Pang et al., 2021).

Our overarching hypothesis is that the specific soil bacteria may provide a healthy and suitable living condition for the sustainable development of medicinal plants in salinized soil. To test this hypothesis, a field experiment was conducted in a typical farming area to compare three different degrees of salinized soil, using licorice as a test crop. The specific objectives of the current study were to determine the effects of soil salination on the licorice plant characteristics (yield and quality), soil physical and chemical properties (available nitrogen, available phosphorus, etc.), and soil bacterial structure and functional diversity in an semi-arid area of northwest China. Even though plants have evolved a range of strategies to deal with salt stress, when levels exceed a threshold, these strategies alone are no longer sufficient to sustain plant growth (Wei et al., 2023). Licorice retained a high concentration of Na^+ in roots and maintained the absorption of K^+ , Ca^{2+} , and Mg^{2+} under salt stress (Xu et al., 2021).

2 Materials and methods

2.1 Site description

The experimental site is located in Pingluo County, Shizuishan City, Ningxia Hui Autonomous Region (Lat 38° 51' N, Long 106° 31' E). Long term unreasonable irrigation and low groundwater level are the main causes of soil salinity in the area. The region is characterized by a typical temperate continental climate, hot and dry in summer, cold in winter, and rainfall is concentrated in summer. The annual average temperature is 9.0°C, and the annual average precipitation is 180 mm, mainly concentrated from June to September. The potential evaporation is 1,200 mm, and the annual potential evaporation is nearly 7 times the precipitation. The average frost period was 194.6 days. The soil pH of the test site was 7.64–7.85, and the soil electrical conductivity (EC) was 0.46–2.67 mS/cm.

2.2 Experimental design and sample collection

The experiment, consisted of three treatments, and was arranged in a randomized complete block design (RCB) with three replications. The treatments were (1) FS, mild salinization (0.37 mS/cm < EC_{25} < 0.96 mS/cm), (2) SS, moderate salinization (0.96 mS/cm < EC_{25} < 1.84 mS/cm) and (3) HSS, severe salinization (1.84 mS/cm < EC_{25} < 3.02 mS/cm) were selected. The different salinization levels in these three sites were natural and the sites were selected based on EC measurements. According to the standards

TABLE 1 Yield and quality of *Glycyrrhiza uralensis* Fisch. under different soil salinity levels.

Group	Yield (t/hm ²)	Liquiritin concentration (%)	Glycyrrhizic acid concentration (%)	Total liquiritin content (kg/hm ²)	Total glycyrrhizic acid content (kg/hm ²)
FS	21.23 ± 0.23a	1.04 ± 0.03b	2.27 ± 0.02b	125.9 ± 2.4a	274.7 ± 5.2a
SS	11.80 ± 0.53a	0.76 ± 0.07b	1.77 ± 0.07c	89.7 ± 4.0b	208.9 ± 9.3b
HSS	7.61 ± 0.37b	1.55 ± 0.26a	2.48 ± 0.12a	118.0 ± 5.7a	188.6 ± 9.1c

Data are means ± SE (n = 3). Means in the same column and followed by the same lower case letter are not significantly different ($P > 0.05$) according to the Tukey's test for multiple comparisons.

(Wang et al., 1993), the value of mild salinization is $0.1 \text{ mS/cm} < \text{EC}_{25} < 0.3 \text{ mS/cm}$, while the moderate salinization is $0.3 \text{ mS/cm} < \text{EC}_{25} < 0.6 \text{ mS/cm}$ and the severe salinization is $0.6 \text{ mS/cm} < \text{EC}_{25} < 0.9 \text{ mS/cm}$ (Yang et al., 2019). One-year-old transplanted seedlings of licorice were planted in early May. Experimental plot area was $5 \text{ m} \times 6 \text{ m}$. The bulk soil samples of 0–10 cm and 10–20 cm soil layers were collected by multi-point mixing method with soil coring tube. The two soil layers were mixed together as a sample, which was sealed in a sterile plastic bag, refrigerated in an ice box and quickly brought back to the laboratory. The collected soil samples were sieved by 2 mm sieve and divided into two parts. One part was frozen at -80°C for the determination of soil microbial community. The other part was air-dried in doors and used to determine other soil physical and chemical properties.

2.3 Determination of soil physiochemical characteristic

The soil potential of hydrogen (pH) and electrical conductivity (EC) were measured by PHS-3C pH meter (Leici, Shanghai, China) and HI98304 electrical conductivity meter (HANNA, Woonsocket, Italy). Total organic carbon (TOC) and organic matter (OM) concentrations were measured by total organic carbon analyzer (Shimadzu, Kyoto, Japan). The total nitrogen (TN) concentration in soil was determined by Kjeldahl method. The soil samples were digested with concentrated sulfuric acid-hydrogen peroxide and the total phosphorus (TP) concentration was determined by spectrophotometer (Lu, 2000). The soil total potassium (TK) concentration in soil was determined by flame atomic absorption spectrometry. The soil alkaline hydrolysable nitrogen (AHN) concentration was measured by the alkaline diffusion method. The soil available phosphorus (AP) concentration was measured by NaHCO_3 leaching-AA3 flow analyzer (Seal, Norderstedt, Germany). Finally, the soil available potassium (AK) concentration was measured by NaOH fusion-flame photometry (Bao, 2000).

2.4 Soil microbial community analysis

The 16S rDNA amplicon sequencing technique was used to analyze the soil microbial community. The genomic DNA of the sample was extracted by the CTAB or SDS method, the purity and concentration of DNA were detected by agarose gel electrophoresis, and the sample was diluted into $1 \text{ ng}/\mu\text{L}$ with sterile water. The diluted genomic DNA was used as

template, and 515F and 806R were used as specific primers for PCR according to the selection of sequencing region. PCR products were detected by 2 % agarose gel electrophoresis. TruSeq[®] DNA PCR Free Sample Preparation Kit (Illumina, San Diego, USA) was used for library construction. The constructed library was quantified by Qubit and Q-PCR, and after passing the qualification test, NovaSeq6000 was used for machine sequencing.

2.5 Data analysis and processing

Excel 2020 was used for basic data processing, SPSS 23.0 statistical software was used for statistical analysis, R language tool and GraphPad Prism 9.0 software were used for mapping, one-way ANOVA was used for analysis of variance, and AI (Adobe Illustrator CS6) software was used for combination processing of images.

2.6 Data availability

All sequencing raw data have been submitted to NCBI SRA (Sequence Read Archive) and the login number is PRJNA1119953 (SUB14489355).

3 Results

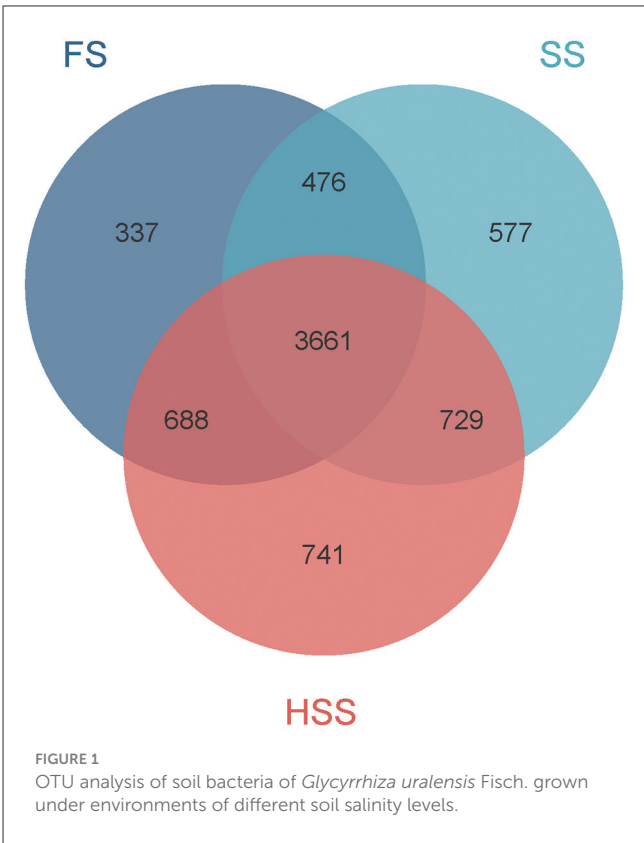
3.1 Changes of yield and quality of licorice grown under different levels of salinity

Soil salinization showed a large effect on the yield and quality of licorice (Table 1). The yield, total liquiritin concentration and total glycyrrhizic acid concentration decreased with an increase in degree of salinization (Table 1). Among all of the treatments, FS treatment exhibited the highest yield, total liquiritin concentration and total glycyrrhizic acid concentration of licorice, respectively, up to 3% and 59%, 40% and 7%, 32%, and 46%, compared with the SS and HSS treatments. The licorice yield of SS was 55% higher than that of HSS. The liquiritin was 49% and 104% higher in HSS than in the FS and SS treatments ($P < 0.05$). The greatest glycyrrhizic acid content was obtained with the HSS treatment and was 9% and 40% greater than in the SS and FS treatments.

TABLE 2 Physicochemical properties of *Glycyrrhiza uralensis* Fisch. soil under different soil salinity treatments.

Group	pH	EC25 (dS·m ⁻¹)	Total organic carbon (%)	Organic matter (g·kg ⁻¹)	Alkaline hydrolysable nitrogen (mg·kg ⁻¹)	Available phosphorus (mg·kg ⁻¹)	Available potassium (mg·kg ⁻¹)	Total nitrogen (g·kg ⁻¹)	Total phosphorus (mg·kg ⁻¹)	Total potassium (g·kg ⁻¹)
FS	7.79 ± 0.07a	0.46 ± 0.04c	1.22 ± 0.22a	21.05 ± 0.21a	72.67 ± 1.72a	30.61 ± 0.45a	264.2 ± 0.8a	1.65 ± 0.19a	559.0 ± 0.0a	21.57 ± 0.11a
SS	7.64 ± 0.05b	1.03 ± 0.06b	0.66 ± 0.02b	11.45 ± 0.38b	57.41 ± 2.80b	20.23 ± 0.14c	142.6 ± 1.4b	1.67 ± 0.10a	507.8 ± 10.5b	20.03 ± 0.04b
HSS	7.85 ± 0.03a	2.67 ± 0.30a	0.18 ± 0.02c	3.15 ± 0.40c	16.49 ± 1.01c	26.29 ± 0.52b	60.4 ± 0.8c	0.76 ± 0.18b	432.5 ± 14.7c	15.97 ± 0.31c

Data are means ± SE (n = 3). Means in the same column and followed by the same lower case letter are not significantly different ($P > 0.05$) according to the Tukey's test for multiple comparisons.



3.2 Physicochemical properties of different salinized licorice soil

The soil pH values of each treatment were weakly alkaline (Table 2). The highest soil electrical conductivity was measured in the HSS treatment, followed by SS treatment. Soil electrical conductivity of HSS and SS were higher by up to 482% and 124%, compared to the FS treatment ($P < 0.05$). The TOC, OM, AN, AP, AK, TP and TK in HSS and SS treatments were significantly lower than those in the FS treatment ($P < 0.05$). Specifically, TOC of HSS and SS treatments was lower by 85% and 46% compared with the FS treatment, respectively. The TN of HSS was 54% lower than that of FS ($P < 0.05$).

3.3 OTU analysis of bacterial communities in licorice soil with different degrees of salinization

The OTUs of bacteria detected in FS, SS, and HSS licorice soil were 5,162, 5,443, and 5,819 respectively (Figure 1). There were 3,661 common OTUs and 337, 577, and 741 unique OTUs, accounting for 7%, 11%, and 13% of the total OTUs in FS, SS, and HSS soil samples, respectively.

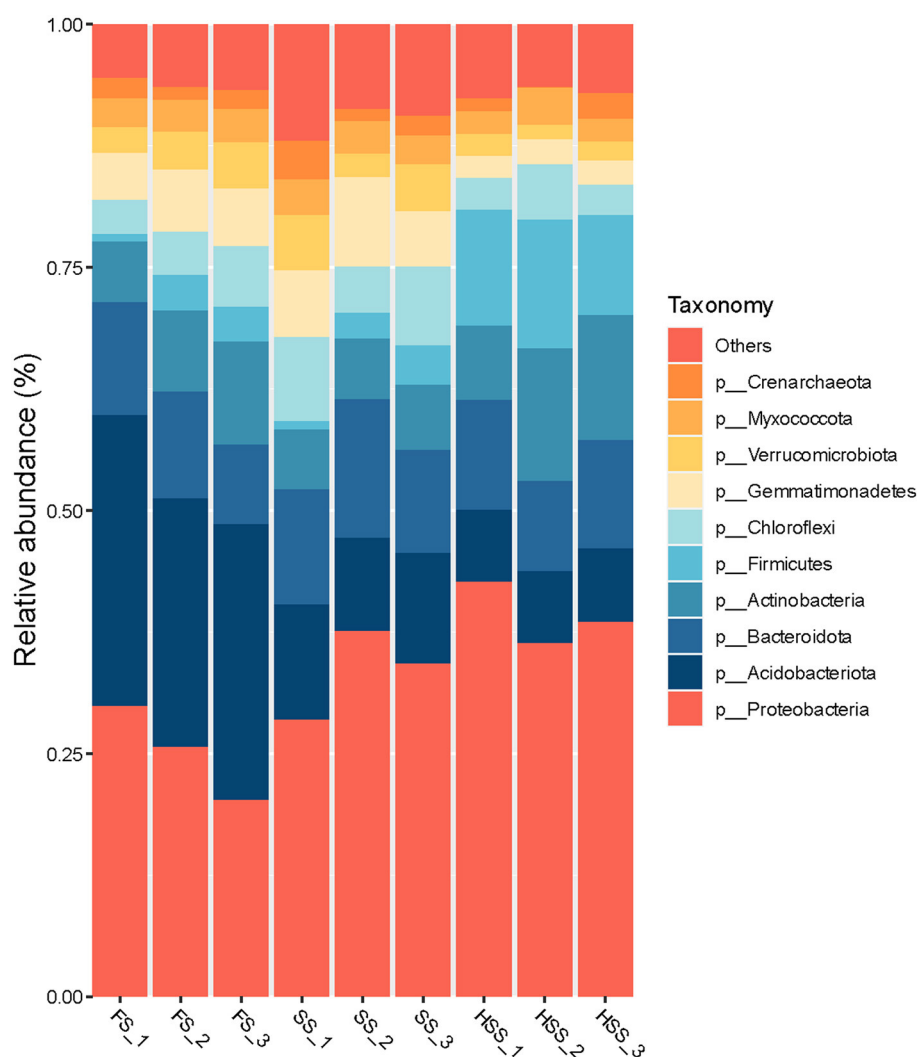


FIGURE 2
The relative abundance of bacteria at the phylum level in licorice soil under different soil salinity levels.

3.4 Soil bacterial community composition at phylum level

The remaining samples were annotated to 38 phyla according to the results of taxonomic analysis, except for 2.3%–2.9% of undetermined groups (Figure 2). The phyla with relative abundance >1% accounted for 90%–94%. The soil bacterial dominant population structure of licorice cultivated in different salinity environments was similar, but the soil bacterial abundance was different. Among all of the treatments, HSS treatment exhibited the largest relative abundance of Firmicutes, Proteobacteria and Actinobacteria, respectively by up to 341%, 55%, and 35%, compared with the FS treatment ($P < 0.05$). The highest relative abundance of Bacteroidota, Chloroflexi, Crenarchaeota, Gemmatimonadetes, Myxococcota and Verrucomicrobiota was found in SS treatment. For this treatment, the corresponding abundances were up to 20%, 51%, 35%, 26%, 2%, and 16%, respectively, greater than for the FS treatment ($P > 0.05$). The relative abundance of Acidobacteriota in HSS and SS treatments was lower. Under these two treatments, the corresponding

reduction was up to 73% and 61%, compared with the FS ($P < 0.05$).

3.5 Statistical analysis of soil bacterial diversity

3.5.1 Statistical analysis of α diversity

As shown in Table 3, the highest richness index of ACE and Chao was reached at HSS. The treatments listed in order of effect on the microbial richness were HSS > SS > FS. Shannon diversity index of SS was the highest, and the order of diversity was SS > HSS > FS. The Simpson diversity index was ranked as FS > SS > HSS. There was no significant difference between Shannon and Simpson diversity groups ($P > 0.05$).

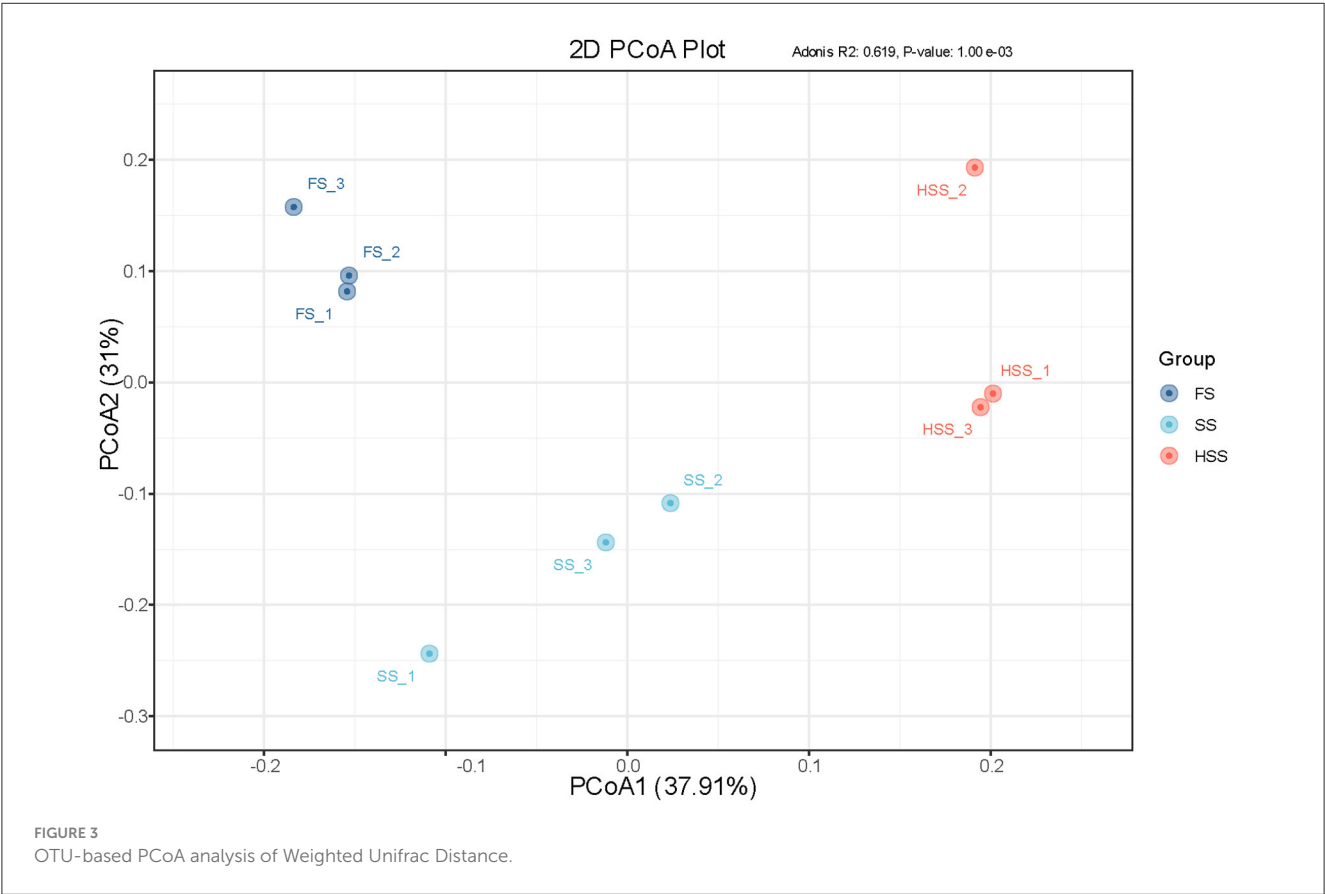
3.5.2 β diversity analysis

PCoA was used to explore the β diversity of bacterial communities in different salinized soils (Figure 3). The

TABLE 3 The species richness and diversity index of root system soil bacteria under different levels of soil salinity.

Treatment	OTUs	Alpha diversity			
		ACE	Chao	Simpson	Shannon
FS	5,159	4,472 ± 101b	4,399 ± 95b	0.9943 ± 0.0021a	6.52 ± 0.22a
SS	5,442	4,542 ± 378b	4,526 ± 354b	0.9930 ± 0.0078a	6.83 ± 0.31a
HSS	5,821	4,889 ± 31a	4,811 ± 55a	0.9917 ± 0.0076a	6.64 ± 0.46a

This analysis uses Simpson' Index of Diversity (1-D). FS, mild salinity; SS, moderate salinity; HSS, severe salinity. Means ± SE. Means in the same column and followed by the same lower case letter are not significantly different ($P > 0.05$) according to the Tukey's test for multiple comparisons.



composition of soil bacteria was in different quadrants of the biplot (Figure 3) under different salinization levels and the spatial difference between groups was large. The composition of soil bacterial communities showed significant differences under the three degrees of soil salinity.

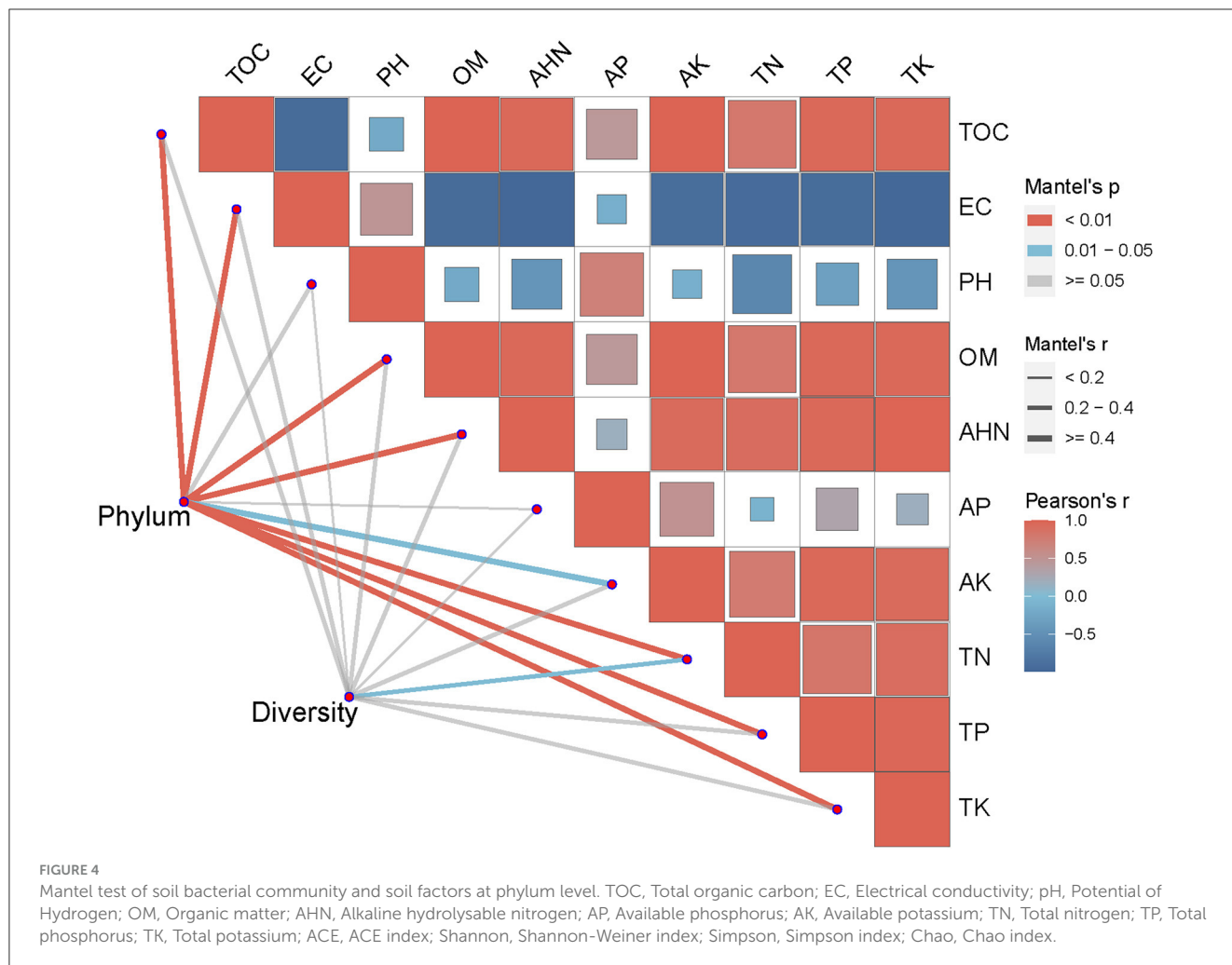
3.6 Redundancy analysis of licorice soil bacteria and soil factors in different salinized soils

As shown in Figure 4, the abundance of top 10 soil bacteria at phylum levels were more closely correlated with TOC, EC, OM, AHN, TN, TP and TK ($P < 0.01$) than with AK ($P < 0.05$), and were uncorrelated with pH and AP ($P > 0.05$). ACE index, Chao index, Simpson index and Shannon index at phylum levels were significantly correlated with TN ($P < 0.05$), and were

not significantly correlated with other soil environmental factors ($P > 0.05$).

3.7 Canonical correspondence analysis

The CCA for the entire experiment (all treatments) is given in the biplot expression of Figure 5. The first ordination axis (CCA1) explained 53.49% of the variance. The correlation coefficients of TN, TP, AP and pH were -0.91 , -1.00 , -0.38 , and 0.33 , respectively. The second ordination axis (CCA2) explained 23.70% of the variance, and the correlation coefficients of TN, TP, AP and pH were 0.41 , 0.01 , -0.92 , and -0.95 , respectively. The two axes jointly explained 77.19% of the variance of change in species composition. The abundance of bacterial community in root soil had a strong correlation with TP, AP and pH ($P < 0.01$), and significant correlation with TN ($P < 0.05$). The determination



coefficients (R^2 values) of TN, TP, AP and pH to community distribution were 0.87, 0.95, 0.97 and 0.88, respectively.

3.8 LEfSe analysis of bacterial community composition in licorice soils with different levels of salinity

The p_Acidobacteriota was significantly different among all groups, and was significantly enriched in FS (red) with the highest abundance (5.46%) (Figure 6). The LDA value of p_Acidobacteriota was greater than that of other taxa, indicating that it had a greater impact on the differences among groups.

3.9 Phenotypic analysis of soil bacterial community

BugBase analysis (Duan and Li, 2023) was used to predict the phenotypic classification of soil bacterial communities in licorice (Figure 7). There were significant differences in the phenotypes of soil bacterial communities with different degrees of salinization.

Aerobic, Anaerobic and Gram-Negative were the main bacterial community phenotypes, and there were almost no phenotypes of Contains Mobile Elements Potentially Pathogenic under different salinity treatments. Aerobic phenotype was significantly different between SS and HSS treatments ($P < 0.05$). The two phenotypes of anaerobic and facultative anaerobic bacteria had significant differences between FS and HSS ($P < 0.05$). Gram-negative and Gram-Positive phenotypes had significant differences among HSS, FS and SS ($P < 0.05$). There were no significant differences in Biofilm Formation and Stress Tolerant phenotype group among all treatments ($P > 0.05$).

3.10 Correlation analysis of licorice yield and quality with soil factors and bacteria parameters

The correlation analysis showed highly significant relationships among licorice yield, AHN, TN, TP, TK and EC for the all treatments (Figures 8A–D). All parameters were positively correlated with each other, except EC ($P < 0.01$). Licorice yield had a significant positive correlation with TOC, OM and AK ($P < 0.05$). Licorice yield had a positive

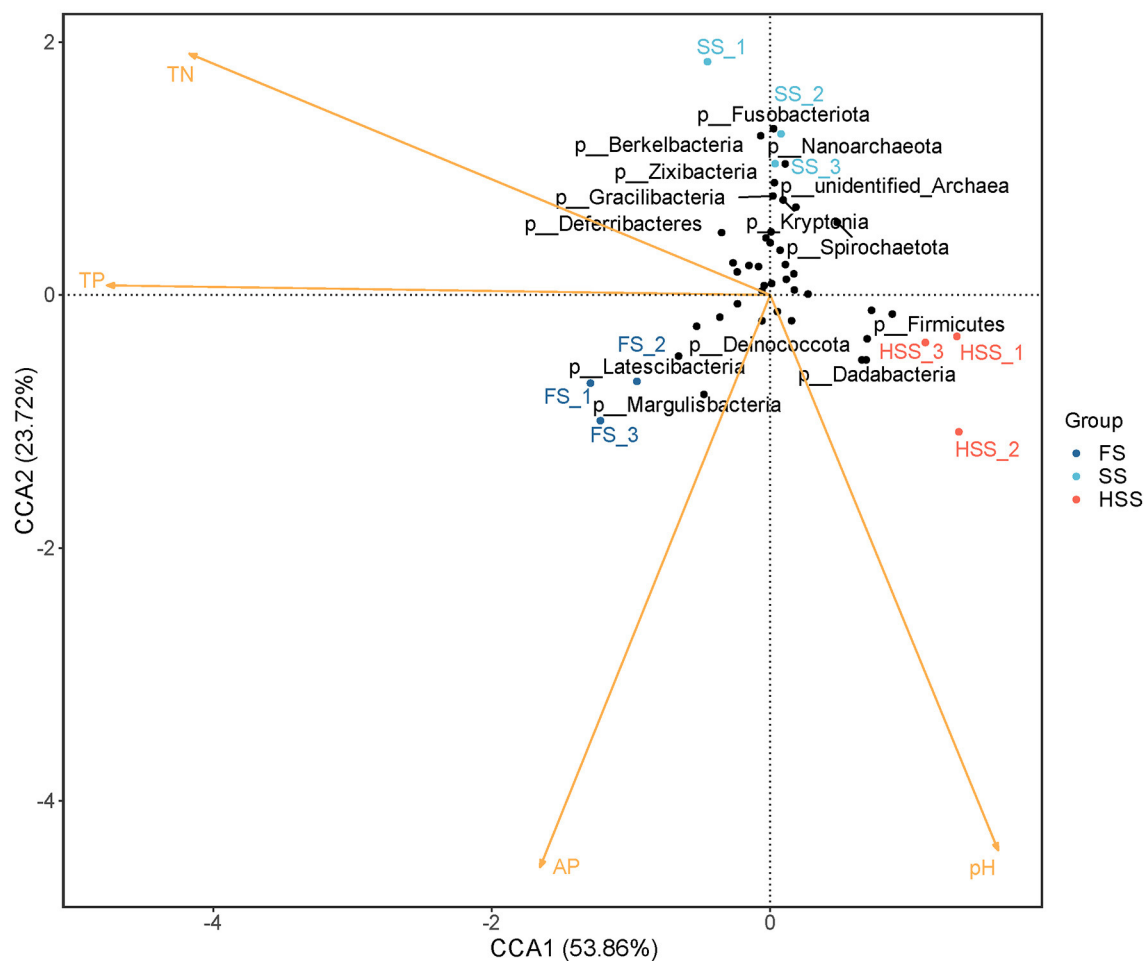


FIGURE 5
CCA analysis based on phylum level.

correlation with Gemmatimonadetes and Myxococcota, and had a negative correlation with Actinobacteria and Firmicutes ($P < 0.01$).

Liquiritin was significantly positively correlated with EC, pH and AP, and negatively correlated with TOC, OM, AHN, TN, TP and TK. Glycyrrhetic acid had a highly significant positive correlation with pH ($P < 0.01$) and had a significant positive correlation with AP ($P < 0.05$). Liquiritin was positively correlated with Firmicutes ($P < 0.01$) and Actinobacteria ($P < 0.05$), and was negatively correlated with Gemmatimonadetes ($P < 0.01$) and Verrucomimicrobiota ($P < 0.05$). Glycyrrhizic acid was negatively correlated with Gemmatimonadetes ($P < 0.01$).

Taken together, Gemmatimonadetes was positively correlated with licorice yield ($P < 0.01$), but was negatively correlated with liquiritin and glycyrrhizic acid concentration ($P < 0.01$). Licorice yield had greater correlation with soil physiochemical properties than with soil bacteria. The correlation between soil bacteria and liquiritin was stronger than that of glycyrrhizic acid.

4 Discussion

In this study, soil salinization decreased the yield of licorice (Table 1). The decreased crop performance could be associated, at least in part, with the potential contributions of the increase of Actinobacteria and Firmicutes, or the decrease of Gemmatimonadetes and Myxococcota under the degraded soil condition with the aggravation of salinization (Figure 8). Gemmatimonadetes are a group of k-strategy bacteria that can perform anaerobic photosynthesis (Mujakić et al., 2022), members of this group can survive in extreme environments, such as saline-alkali land (Guan et al., 2021). Their metabolites (Zhou et al., 2024) can supply plants with growth needs and improve the ability of plants to resist environmental pressures. Guan et al. (2021) found that Gemmatimonadetes had a strong adaptability to highly saline soil. Myxococcota contains potential phototrophic members and has photosynthetic capabilities (Li et al., 2023). Myxococcota can synthesize hundreds of carbon skeleton metabolites and has different metabolic and structural characteristics, many species of this phylum can form fruiting bodies and prey, and their

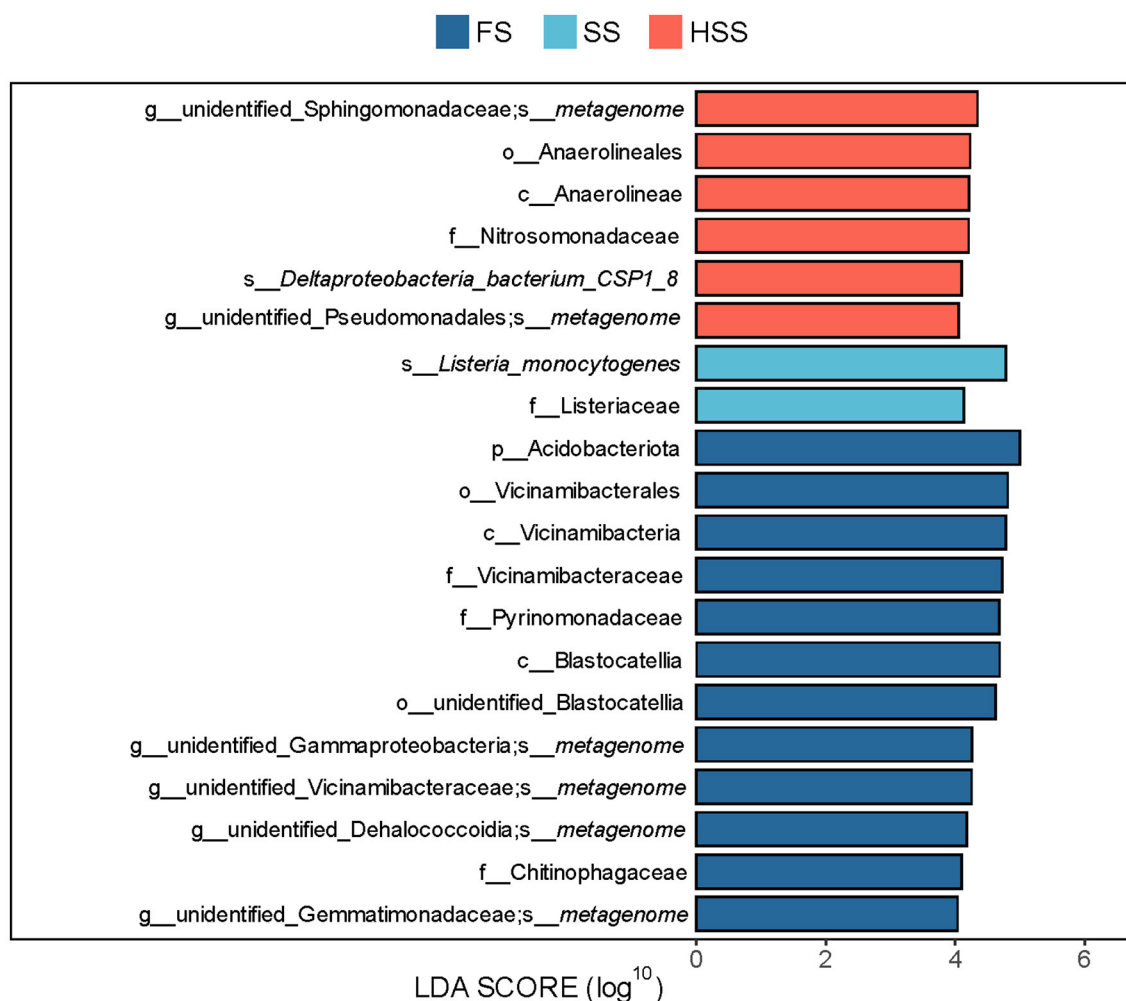


FIGURE 6
LEfSe analysis of differential species between groups. LDA value was >4. FS, mild salinity; SS, moderate salinity; HSS, severe salinity.

predation or competition process will produce a large number of secondary metabolites. This suggests that Myxococcota might primarily exist in plant-associated soil and play a vital role in the process of plant secondary metabolic synthesis (Jalal et al., 2022; Song et al., 2023). Hence one can see that with the aggravation of salinization, the dominant bacteria in the soil will change to high salt-tolerant bacteria. When the soil conditions are not conducive to plant growth, bacteria will alter the soil environment to make it conducive to plant growth or provide plants with the growth needs to help plants cope with environmental pressure.

Our study showed that soil salinization resulted in a significant improvement of licorice secondary metabolites (Table 1). Some secondary metabolites often determine the quality of plants. Plants secrete secondary metabolites to protect themselves, and to shape soil bacteria to “help” themselves face adversity under stress conditions (Berg et al., 2017). In this study, liquiritin and glycyrrhizic acid concentration were significantly negatively correlated with Gemmatimonadetes, while liquiritin and glycyrrhizic acid were significantly positively correlated with Firmicutes and Actinobacteria (Figure 8). This indicated that

recruitment of bacteria had multiple paths (Berendsen et al., 2018). Previous study found that the bacterial community accompanying plants could induce the production of plant secondary metabolites (Khade and Sruthi, 2024), and these plant metabolites have a great influence on the composition of other soil bacteria (Su et al., 2023).

Our study found that soil salinization significantly changed soil bacterial community structure and diversity. A linear increase in Firmicutes and Proteobacteria was observed with the increasing degree of soil salinization, and a similar linear reduction was observed in Acidobacteriota and Myxococcota (Figure 2, Table 3). These trends might be related to the degraded soil conditions resulting from lower input of crop residues, especially roots. Soil is an important place to shape soil bacterial community. Salinization not only changes soil physical and chemical properties, but also affects soil bacterial community structure (Bahram et al., 2018; Ren et al., 2018). As an important part of soil ecosystem, microorganisms play an important role in regulating soil nutrient cycling process (Guan et al., 2021) and plant productivity. Some studies found that plant-soil bacteria balance the relationship between soil carbon respiration and carbon stability (De Vries et al.,

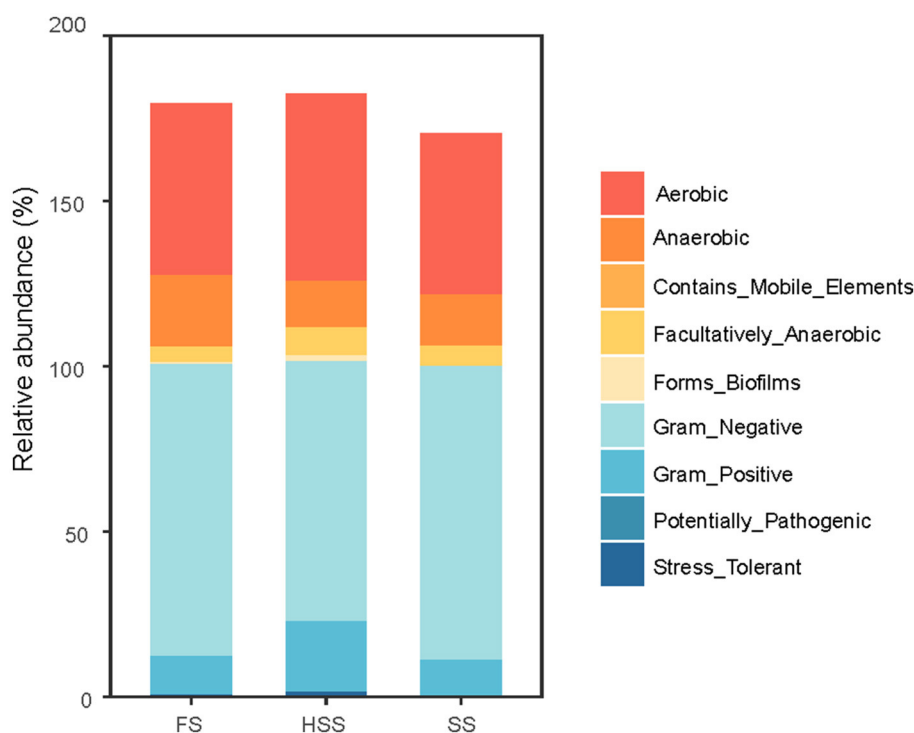


FIGURE 7
Phenotype of soil bacterial community. FS, mild salinity; SS, moderate salinity; HSS, severe salinity.

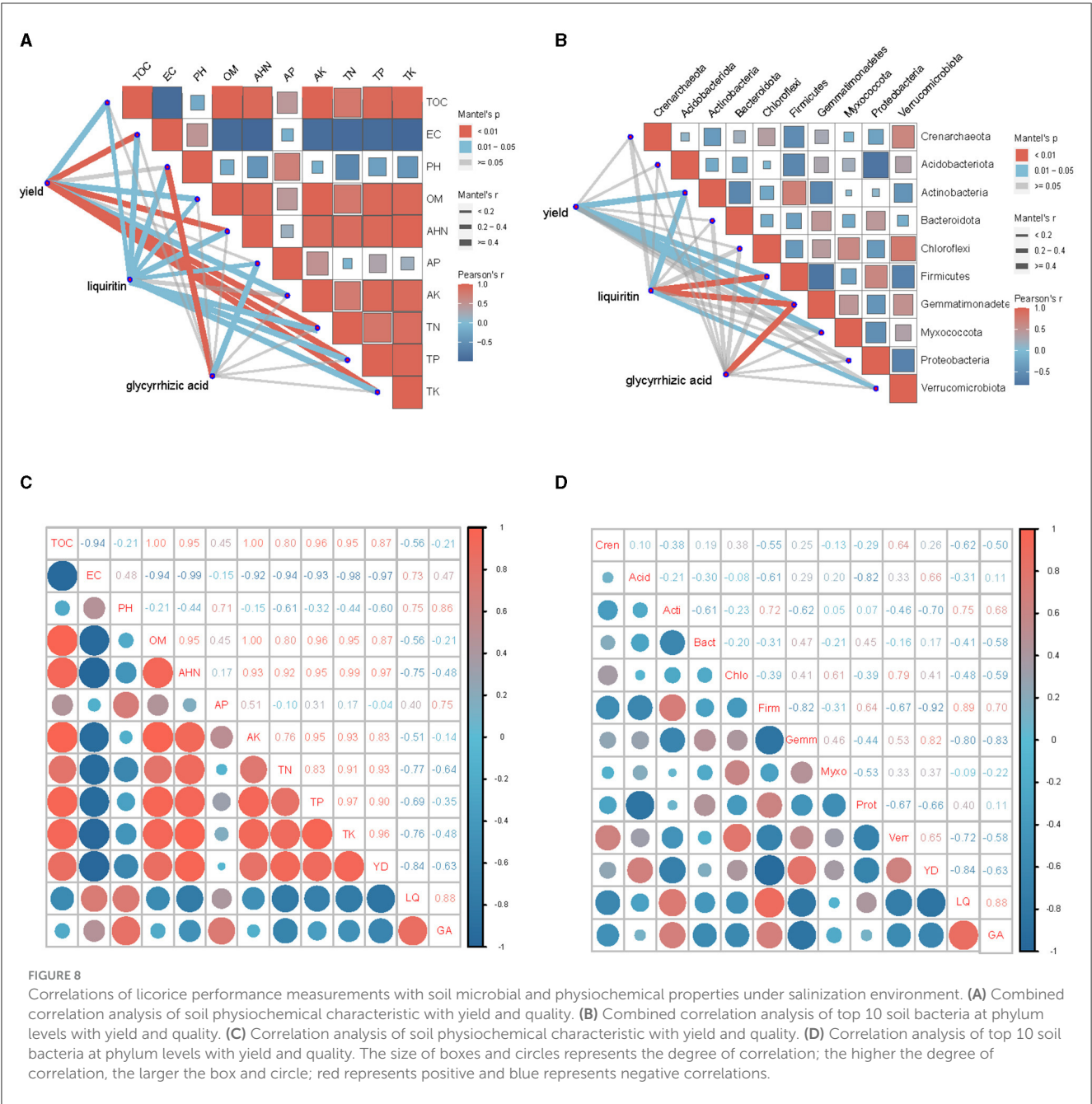
2020). For example, plants provide carbon sources to soil bacteria through root and leaf litter decomposition and root exudates, while bacteria secrete nutrients required by plants to promote plant development and crop production processes. Salinity is the main environmental factor that determines the composition of bacterial community (Zhang et al., 2019). Soil bacterial community responds to soil environment by changing its composition and diversity (Dai et al., 2023). Soil salinity significantly inhibits the effectiveness of microbial biomass carbon (MBC) (Batra and Manna, 1997), has a significant effect on soil bacterial community structure, and also reduces soil nutrient accumulation (Yuan et al., 2007). We found that the dominate bacteria in saline-alkali land are Proteobacteria, Acidobacteria, Bacteroidetes, Firmicutes (Figure 2), but the bacteria related to the yield and quality of licorice mostly do not belong to these groups (Figure 8). In other words, some bacteria need to “conquer” the soil (some halophilic bacteria or bacteria that can survive in extreme environments), and then the remaining bacteria go on to “support” plant growth and development under the soil conditions of saline-alkali land (Mallon et al., 2015; Philippot et al., 2024). It is generally agreed that bacterial communities rather than individual bacteria species can promote plant growth and development under natural conditions (Trivedi et al., 2020; Yin et al., 2022).

Crop yield of licorice could be increased by appropriately increasing nitrogen, phosphorus and potassium fertilizers, but as a Chinese herbal medicine, fertilizers may affect licorice quality, and thus its therapeutic effect. In this study, the change of licorice yield mainly depended on the physical and chemical properties of soil, and the change of licorice quality was more closely related to

the change of bacterial diversity. Soil physiochemical properties can directly act on plants and have a direct impact on their growth and development. From another point of view, changes in soil physiochemical properties can cause changes in soil bacteria, which indirectly affect plant growth processes. Therefore, improvement of the soil environment might slightly adjust the composition of soil bacteria to achieve enhanced agricultural production, such as increasing yield or increasing the concentration of secondary metabolites, and coordinating the relationship between yield and quality during the growth of Chinese herbal medicines.

As a host, plants will treat different strains differently and benefit from them in the process of symbiosis with soil bacteria (Rahman et al., 2023). Bacteria directly or indirectly affect plant growth and development through metabolic cooperation, signal hormone and nutrient exchange (Zhang et al., 2020; Pang et al., 2021), and improve plant stress resistance and resistance to pathogens (Trivedi et al., 2020). Therefore, the balance between yield and quality should be coordinated when selecting suitable planting sites and management measures.

Based on current research, future research directions will be more targeted. In the future, strains that can directly affect the yield and concentration of glycyrrhizin and glycyrrhizic acid will be screened. According to the characteristics of microorganisms in saline soil, future research needs to identify the relationships and interactions among microorganisms, liquiritin and glycyrrhizic acid. It also needs to further explore the meaningful ways in which microorganisms can be effectively utilized in agricultural production processes in saline soil, thereby accelerating the global process of recovery of saline land for agricultural use.



5 Conclusion

There is a strong correlation between soil bacteria and plant adaptation to adversity, as well as plant quality improvement, but the related research is still weak, especially in terms of functional bacteria. We found for the first time that the heavy salinization of soil decreased the licorice yield, soil nutrients, the bacterial abundance of Gemmatimonadetes and Myxococcota, but improved licorice quality with effective increases in concentrations of glycyrrhizic acid and liquiritin, and the bacterial abundance of Actinobacteria and Firmicutes. The change of licorice yield mainly depends on the physical and chemical properties of soil. The change of quality characteristics of licorice was more closely related to the change of bacterial diversity, and the effect of bacterial diversity on liquiritin was greater than that on glycyrrhizic acid. Therefore,

appropriate addition of Actinobacteria, Firmicutes in a saline condition enhances the quality of licorice while the addition of Gemmatimonadetes, Myxococcota is favorable to improve the yield of licorice. When introducing strains, we need to consider whether the strains are inherent in the native plant environments avoiding strains that will have difficulty surviving (competition or lack of carbon source food) or inhibit some of the indigenous strains.

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

YB: Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. BM: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing. NM: Conceptualization, Data curation, Supervision, Writing – review & editing. YN: Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Writing – original draft. DW: Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft. HL: Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Writing – original draft. ML: Funding acquisition, Resources, Writing – review & editing. ZS: Funding acquisition, Writing – review & editing.

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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 rhizobacterium *Bacillus megaterium* modulates the expression of antioxidant-related and drought-responsive genes to protect rice (*Oryza sativa* L.) from drought

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Global climate change poses a significant threat to plant growth and crop yield and is exacerbated by environmental factors, such as drought, salinity, greenhouse gasses, and extreme temperatures. Plant growth-promoting rhizobacteria (PGPR) help plants withstand drought. However, the mechanisms underlying PGPR–plant interactions remain unclear. Thus, this study aimed to isolate PGPR, *Bacillus megaterium* strains CACC109 and CACC119, from a ginseng field and investigate the mechanisms underlying PGPR-stimulated tolerance to drought stress by evaluating their plant growth-promoting activities and effects on rice growth and stress tolerance through *in vitro* assays, pot experiments, and physiological and molecular analyses. Compared with *B. megaterium* type strain ATCC14581, CACC109 and CACC119 exhibited higher survival rates under osmotic stress, indicating their potential to enhance drought tolerance. Additionally, CACC109 and CACC119 strains exhibited various plant growth-promoting activities, including phosphate solubilization, nitrogen fixation, indole-3-acetic acid production, siderophore secretion, 1-aminocyclopropane-1-carboxylate deaminase activity, and exopolysaccharide production. After inoculation, CACC109 and CACC119 significantly improved the seed germination of rice (*Oryza sativa* L.) under osmotic stress and promoted root growth under stressed and non-stressed conditions. They also facilitated plant growth in pot experiments, as evidenced by increased shoot and root lengths, weights, and leaf widths. Furthermore, CACC109 and CACC119 improved plant physiological characteristics, such as chlorophyll levels, and production of osmolytes, such as proline. In particular, CACC109- and CACC119-treated rice plants showed better drought tolerance, as evidenced by their higher survival rates, greater chlorophyll contents, and lower water loss rates, compared with mock-treated rice plants. Application of CACC109 and CACC119 upregulated the expression of antioxidant-related genes (e.g., *OsCAT*, *OsPOD*, *OsAPX*, and *OsSOD*) and drought-responsive genes (e.g., *OsWRKY47*, *OsZIP23*, *OsDREB2*, *OsNAC066*, *OsAREB1*, and *OsAREB2*). In conclusion, CACC109 and CACC119 are promising biostimulants for enhancing plant growth and conferring resistance to abiotic stresses in crop production. Future studies should conduct field trials to validate these findings under real agricultural conditions, optimize inoculation methods

for practical use, and further investigate the biochemical and physiological responses underlying the observed benefits.

KEYWORDS

Bacillus megaterium, drought, plant growth-promoting rhizobacteria, rice, stress-responsive gene

1 Introduction

Global population growth and extreme climate change pose important agricultural challenges that may cause insufficient food supply worldwide (FAO, 2017). Rice (*Oryza sativa* L.), a crucial staple feeding nearly half of the global population, is grown on around 163 million hectares of land worldwide (Fukagawa and Ziska, 2019; Shahbandeh, 2024). Despite its importance, rice productivity is affected by abiotic and biotic stresses. Drought and high salinity are abiotic stresses that severely affect rice growth, development, and production. Specifically, drought is considered the most important constraint to rice production, affecting approximately 19–23 million hectares of rice fields (Kumar, 2017).

Under drought, plants exhibit changes in various properties, including physiological (e.g., photosynthesis, osmotic balance, transpiration, leaf water content, and stomatal water transmission activity), morphological (e.g., leaf area, leaf number, root length, leaf aging, maturation, and growth stage), and biochemical (e.g., antioxidant content, chlorophyll content, proline accumulation, hormonal content, and secondary metabolites) (Anjum et al., 2011; Sharma et al., 2012; Haworth et al., 2013; Ammar et al., 2015; Conesa et al., 2016; Tiwari et al., 2021; Yang et al., 2021). Although these mechanisms enable plants to minimize water loss and usage, they can adversely affect plant growth and development, consequently reducing yield (Nakabayashi and Saito, 2015).

With the urgent need to support crop growth to meet food demands amid limited water resources, interest in enhancing drought tolerance and discovering sustainable solutions to related food security challenges has increased. The acquisition of essential nutrients under drought is a significant challenge for plants, often limiting their growth and productivity. Plant growth-promoting rhizobacteria (PGPR) represent a wide variety of beneficial soil bacteria that colonize the rhizosphere and plant roots, and can grow in, on, or around plant tissues. PGPR can increase nutrient availability and uptake, directly and indirectly supporting plant growth and resilience under drought (Backer et al., 2018). Under drought, PGPR play pivotal roles in nutrient solubilization and uptake. These bacteria solubilize phosphorus and other essential nutrients, making them more accessible to plants, while also improving water content and potential, thereby enhancing mineral quality and grain yield (Creus et al., 2004; Richardson and Simpson, 2011; Hardoim et al., 2015). Nitrogen fixation by PGPR contributes to soil nitrogen enrichment, providing an additional nitrogen source for plants. This process is essential for nitrogen acquisition in nutrient-poor, dry soils (Hardoim et al., 2015). Siderophore production by PGPR is another vital mechanism that enhances nutrient acquisition under drought. Siderophores are iron-chelating compounds that microbes secrete to sequester iron from the soil environment, making it more accessible

to plants. This process is especially critical under drought, where nutrient availability is limited (Sharma and Johri, 2003; Ferreira et al., 2019). In addition, PGPR can improve drought tolerance by stimulating the production of drought-resistant compounds, such as bacterial exopolysaccharides (EPS), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile organic compounds (VOCs), and phytohormones, such as abscisic acid (ABA), ethylene (ET), and indole-3-acetic acid (IAA) (Vejan et al., 2016; Vurukonda et al., 2016; Tiepo et al., 2018). The production of EPS by PGPR helps retain soil moisture and stabilize soil structure, further improving the water holding capacity and nutrient availability in the soil, thereby enhancing plant growth and alleviating drought (Vurukonda et al., 2016). ACC deaminase is a PGPR-produced enzyme that can modulate plant ET levels. ET is a phytohormone associated with stress responses, and high levels of ET can inhibit plant growth. ACC deaminase lowers ET levels by breaking down its precursor, ACC, thereby mitigating stress effects and promoting root elongation and nutrient uptake under drought conditions (Glick et al., 1998; Mayak et al., 2004; Vurukonda et al., 2016). VOCs emitted by soil microorganisms also enhance the ability of plants to cope with abiotic stresses, such as drought, by inducing system resistance. VOCs such as acetoin and 2,3-butanediol improve plant growth and drought resistance by modulating stress-related pathways and enhancing water use efficiency (Ryu et al., 2003; Vejan et al., 2016). Phytohormones, including ABA and ET, are critical in plant response to drought. ABA plays central roles in regulating stomatal closure, reducing water loss, and activating stress-responsive genes. Microbial interactions can influence ABA levels, enhancing the drought response of plant. While ET is involved in the regulation of growth and stress responses, its overproduction can be detrimental. Microbial modulation of ET levels via ACC deaminase activity helps maintain a balance, promoting plant growth and stress tolerance (Glick et al., 1998; Cutler et al., 2010; Cohen et al., 2015; Vurukonda et al., 2016). PGPR produce plant growth-promoting (PGP) hormones such as auxins that enhance root growth and nutrient acquisition. The best-known auxin, IAA, modifies the plant root architecture by increasing root length, root branching, and total root surface area resulting in improved water and nutrient uptake and enhanced cellular defense against water stress (Glick, 2012; Vurukonda et al., 2016). These studies indicate the promising potential of PGPR in enhancing drought tolerance.

Plants initiate specific responses that reprogram genetic, molecular, and physiological processes to mitigate the adverse effects of drought on their growth and productivity. Many plants achieve stress resistance through PGPR-mediated activation of various stress-responsive genes. Plants inoculated with PGPR under drought stress exhibit distinct gene expression profiles compared with non-inoculated plants (Saakre et al., 2017; Kaushal, 2019). For example, inoculation of sugarcane (*Saccharum* spp.) with *Gluconacetobacter diazotrophicus*

PAL5 under drought activates ABA-dependent signaling genes, enhancing drought tolerance (Vargas et al., 2014). Colonization of plants by PGPR strains can markedly alter gene expression in response to drought. In rice, treatment with *Pseudomonas fluorescens* (Pfl) activates genes such as *Hsp20*, *bZIP1*, and *COC1* involved in the ABA signaling pathway, *PKDP* encoding a protein kinase, and *AP2-EREBP* associated with developmental and stress defense pathways (Saakre et al., 2017). Similarly, inoculation of *Arabidopsis thaliana* with GAP-P45 alters the expression patterns of genes related to polyamine biosynthesis (*ADC*, *AIH*, *CPA*, *SPDS*, *SPMS*, and *SAMDC*), leading to increased polyamine levels and reduced osmotic stress during drought (Sen et al., 2018). In wheat, PGPR strains such as *Arthrobacter protophormiae* SA3, *Bacillus subtilis* LDR2, and *Dietzia natronolimnaea* STR1 enhance drought and salinity tolerance by modifying the expression of genes and transcription factors related to the ET signaling pathway (Barnawal et al., 2019). In soybean, inoculation with *Pseudomonas simiae* AU under drought conditions results in the upregulation of genes encoding transcription factors (*DREB/EREB*), osmoprotectants (*P5CS*, *GOLS*), and water transporters (*PIP*, *TIP*), leading to improved drought tolerance compared with control plants (Vaishnav and Choudhary, 2019). Li et al. (2020) found that treating drought-stressed wheat with a cell filtrate from *Streptomyces pactum* Act12 improves growth by upregulating genes related to water deficit resistance, including those involved in cell wall expansion (*EXPA2* and *EXPA6*), proline accumulation (*P5CS*), and stomatal closure (*SnRK*) (Li et al., 2020). Additionally, application of *Bacillus subtilis* GOT9 to *A. thaliana* and *Brassica campestris* enhances drought tolerance and survival by hyper-inducing various genes responsible for improved drought tolerance phenotypes (Woo et al., 2020). Inoculation with *Pseudomonas putida* (RA) in chickpea plants under drought confers drought tolerance by differentially expressing genes involved in transcription activation (*DREB1A* and *NAC1*), stress response (*LEA* and *DHN*), reactive oxygen species (ROS) scavenging (*CAT*, *APX*, and *GST*), ET biosynthesis (*ACO* and *ACS*), salicylic acid (*PR1*), and jasmonate (*MYC2*) signaling (Tiwari et al., 2021). Similarly, potted rice plants inoculated with *Bacillus endophyticus* PB3, *Bacillus altitudinis* PB46, and *Bacillus megaterium* PB50 exhibit significant drought tolerance, particularly with strain PB50, by upregulating genes such as *LEA*, *SNAC1*, *HSP70*, *RAB16B*, and *bZIP23*, along with increased osmolyte accumulation (Devarajan et al., 2021). Recently, Omara et al. (2022) have demonstrated that inoculating rice plants with *B. megaterium*, *Pseudomonas azotoformans*, and *Rhizobium* sp. enhances growth and drought tolerance by altering the expression of genes related to growth and stress response (*COX1*, *AP2-EREBP*, *GRAM*, *NRAMP6*, *NAM*, *GST*, and *DHN*) and expansion (*EXP1*, *EXP2*, and *EXP3*) (Omara et al., 2022).

Bacteria, similar to plants, are also affected by hydric stress during water scarcity. Therefore, identifying and selecting drought-tolerant bacteria is valuable for developing new biostimulants suitable for water-deficient regions (García et al., 2017). *Panax ginseng* is a perennial herb of significant medicinal importance in Korea (Kim, 2018). Ginseng stands out among crops due to the high commercial value of its roots, and maintaining healthy root development is essential throughout its 4–6-year cultivation period (Durairaj et al., 2018; Ding et al., 2022). However, ginseng cultivation is frequently challenged by biotic factors, such as microbial pathogens and pests, and abiotic factors, including drought, salinity, and temperature (Foyer et al., 2016), leading to changes in microbial communities.

Notably, a close relationship exists between changes in microbial communities and edaphic factors in soils where ginseng is cultivated (Punja et al., 2008; Li et al., 2018). Ginseng roots and the surrounding soil harbor a diverse microbial community, including PGPR (Ji et al., 2021). However, the specific mechanisms underlying PGPR–plant interactions and their optimization for sustainable agricultural practices remain unclear.

Thus, this study aimed to investigate the effects of bacterial strains isolated from the rhizosphere and soil of ginseng plants growing in a drought-stressed field on the growth and drought resistance of rice and elucidate the underlying mechanisms. Two bacterial strains, *Bacillus megaterium* CACC109 and CACC119, were identified for their osmotic stress tolerance and PGP traits. Various parameters, including seed germination, shoot and root growth, physiological properties, osmolyte production, expression of antioxidant-related genes and drought-responsive genes (DRGs), and soil composition, were compared between rice treated with these strains and control plants. We hypothesized that rice plants inoculated with bacterial strains isolated from the rhizosphere under drought would exhibit enhanced growth and improved drought resistance due to the beneficial impact of the bacteria on plant metabolism.

2 Materials and methods

2.1 Isolation and characterization of rhizosphere bacteria

Ginseng roots and soil were collected from a drought-stressed ginseng field (36°8'15.1"N, 127°28'3.0"E) in South Korea. The roots were washed in sterile distilled water to remove bulk soil and then transferred to new sterilized bottles containing 100 mL of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.7). The mixture was shaken at 200 rpm for 30 min at 27°C. Subsequently, the roots were carefully removed, and the shaken solution was stored as the rhizosphere sample. The rhizosphere solution was then centrifuged at 500 rpm for 5 min at 27°C, and the resulting supernatant was transferred to a new 50 mL conical tube and centrifuged at 8000 rpm for 15 min. The supernatant was discarded, and the resulting pellet was resuspended in 30 mL of sterile water. Bacteria were isolated using the agar plate method in combination with serial dilutions. Rhizosphere samples were diluted with sterile distilled water at a ratio of 1:10, and each dilution (0.1 mL) was cultured on Luria–Bertani (LB) broth at 30°C for 2 days. Each colony was selected and streaked onto LB agar for purification (Choi et al., 2020). The various bacterial isolates obtained from the samples were designated as “CACC” (Cialm Agricultural Cultures Collection), followed by their respective isolation numbers. The isolated bacterial cells were then grown in LB medium and stored at –80°C.

The isolated bacteria were identified based on 16S rRNA gene sequences. They were cultured in LB broth at 30°C in a shaker at 200 rpm, and the cells were collected by centrifugation at 5,000 rpm for 10 min and 4°C. Genomic DNA was extracted using the ExiPrep™ Plus Bacteria Genomic DNA Kit (BIONEER, Daejeon, South Korea) following the manufacturer's protocols. The 16S rRNA gene was amplified from the genomic DNA using the universal primers 518F (CCAGCAGCCGCGGTAATAC) and 805R (GACTACCAGGGTA TCTAATC), followed by a BLAST search in the National Center for

Biotechnology Information (NCBI) database to identify sequence homologies.

2.1.1 Phylogenetic analysis of *Bacillus* spp.

The full-length 16S rRNA gene sequences from the bacterial strains were compared with the sequences available in the NCBI database.¹ The sequences were aligned using Multiple Sequence Comparison by Log-Expectation (Edgar, 2004). A phylogenetic tree was constructed using the neighbor-joining method, and the relative stability of the branches was evaluated by bootstrap analysis of 1,000 datasets using MEGA11 software (Tamura et al., 2021).

2.1.2 Phosphate solubilization

To quantify phosphate solubilization, bacterial strains were inoculated into a National Botanical Research Institute Phosphorus (NBRIP) medium consisting of 10 g glucose, 5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.1 g $(\text{NH}_4)_2\text{SO}_4$, and 5 g tricalcium phosphate as the sole P source in 1 L of distilled water (Olsen et al., 1954). Cultured bacterial strains (0.3 mL) were inoculated into 30 mL of NBRIP medium in Erlenmeyer flasks. Controls were prepared using the NBRIP medium only. After incubation for 7 days at 30°C and 150 rpm, the culture medium was centrifuged to quantify soluble phosphorus using the MARS 6 microwave instrument from CEM (Kamp-Lintfort, Germany). For the digestion, 5 mL of the supernatant was transferred to a 110 mL vessel, and 15 mL of 70% nitric acid solution was added. The mixture was subjected to a temperature–time ramp, reaching a final temperature of 180°C within 15 min, and this temperature was maintained for 20 min to ensure complete acid digestion. Following digestion, the 70% nitric acid was evaporated with heat, and the residue was diluted with 3% nitric acid solution for analysis. The diluted sample was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) on an iCAP-RQ instrument (Thermo Fisher Scientific, Waltham, MA, United States) equipped with an ASX-560 autosampler (TELEDYNE, Englewood, CO, United States). The analysis utilized the ^{31}P isotope for phosphorus quantification. A kinetic energy discrimination mode with helium gas was employed to mitigate interference from $^{14}\text{N}^{16}\text{OH}^+$ polyatomic ions (Fleischer et al., 2014).

2.1.3 Nitrogen fixation

Molecular nitrogen fixation was assessed using a nitrogen-free medium (Nfb) and the phenate method to detect $\text{NH}_4\text{-N}$ production (Ahmad et al., 2013). A bacterial colony was inoculated into 25 mL of Nfb and cultured at 30°C for 7 days. The culture medium was centrifuged at 4°C and 8,000 rpm for 20 min to obtain a supernatant. The supernatant was then diluted 10 times into 25 mL portions and added with 1 mL of phenol, 1 mL of sodium nitroprusside, and 2.5 mL of oxidizing solution. The mixed solution was incubated for 1 h, and the absorbance was measured at 640 nm to quantify $\text{NH}_4\text{-N}$ production using an ammonium standard solution (0.1, 0.2, 0.5, and 1 mg N/L) for calibration. Distilled water was used as a blank.

2.1.4 IAA production

The colorimetric determination of IAA was performed as previously described (Meudt and Gaines, 1967). The bacterial strains were cultured in DF medium [2 g $(\text{NH}_4)_2\text{SO}_4$, 1 g KH_2PO_4 , 15 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 μg H_3BO_3 , 11 μg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 125 μg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 78 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 17 μg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per liter] supplemented with 0.5 g tryptophan and then incubated at 30°C and 180 rpm for 5 days. After centrifugation at 5000 rpm for 20 min, 1 mL of the supernatant was mixed with 2 mL of modified Salkowski reagent (50 mL of perchloric acid and 1 mL of 0.05 M FeCl_3) and left in the dark at 24°C for 30 min. The optical density (OD) was measured at 530 nm using microplate reader (SPARK, TECAN, Männedorf, Switzerland), and the IAA concentration was calculated from a standard curve prepared using IAA solutions ranging from 0 to 100 ng/mL.

2.1.5 Siderophore production

Siderophore production was assessed using King's B broth (20 g protease peptone, 10 mL glycerol, 1.5 g KH_2PO_4 , and 1.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L). The medium was iron-deficient to induce siderophore synthesis. For quantitative analysis, 0.4 mL of the bacterial cultures were inoculated into 40 mL of King's B broth and incubated at 30°C for 4 days as previously described (Nagarajkumar et al., 2004; Cherif-Silini et al., 2016). After centrifugation at 5000 rpm for 30 min, the supernatant was adjusted to pH 2.9, mixed with an equal volume of ethyl acetate, shaken for 30 min, and then recentrifuged at 5,000 rpm for 30 min. The upper ethyl acetate layer was collected and mixed with Hathway reaction solution (1 mL of 0.1 M FeCl_3 in 0.1 N HCl added to 100 mL distilled water plus 1 mL of potassium ferricyanide). After 20 min, the absorbance was measured at 700 nm using microplate reader (SPARK, TECAN). Siderophore production was quantified based on the measurements, and a standard curve was prepared using dihydroxybenzoic acid.

2.1.6 ACC deaminase production

The ACC deaminase activity was evaluated using a previously described method (Misra and Chauhan, 2020), which measures α -ketobutyrate production resulting from ACC cleavage by ACC deaminase. Briefly, to measure ACC deaminase production, 20 μL of 0.5 M ACC was added to 0.2 mL of cell pellets obtained by inoculating the isolated strain into DF salts minimal medium containing 3 mM ACC as the sole nitrogen source [replacing $(\text{NH}_4)_2\text{SO}_4$]. The reaction was allowed to proceed for 15 min. After the addition of 0.56 M HCl and 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl), the mixture was stirred for 30 min and then added with 2 mL of 2 N NaOH. Absorbance was measured at 540 nm, and ACC deaminase activity was quantified by comparing the absorbance with a standard curve of known α -ketobutyrate concentrations.

2.1.7 Bacterial growth and exopolysaccharide formation

Bacterial growth was evaluated at different water potentials of 0, −0.15, and −0.49 MPa, which were generated using LB liquid medium supplemented with polyethylene glycol (PEG) 6,000 at concentrations of 0, 10, and 20%, respectively. The selected strains were inoculated into the medium at an initial OD of 0.01 and then cultured at 30°C with shaking at 180 rpm for 48 h. Bacterial growth was monitored by

¹ <http://www.ncbi.nlm.nih.gov>

measuring the OD_{600nm} using microplate reader (SPARK, TECAN). Growth at 0 MPa was considered 100%, whereas growth at −0.15 and −0.49 MPa was expressed as a percentage relative to that at 0 MPa.

The production of EPS (total biopolymers) at different water potentials (0, −0.15, and −0.49 MPa) was determined in broth cultures of the same strains. After inoculation into nutrient broth supplemented with 5% glucose and incubation at 30°C and 120 rpm for 40 h, the supernatant was collected by centrifugation at 12,000 rpm for 20 min at 4°C. Acetone was added to the supernatant in a ratio of 1:2 (v:v), and the mixture was allowed at 4°C for 24 h to precipitate the EPS. The precipitate was filtered through filter paper and dried at 24°C, and the weight of the filter paper was measured. The blank was measured using untreated filter paper for value calculation. Each treatment was performed in triplicate in two biological experiments, including control samples inoculated with *B. megaterium* ATCC14581.

2.1.8 Whole genome sequencing, gene prediction, and annotation

The genomes of CACC109 and CACC119 were assembled *de novo* from PacBio sequencing data by CJ Bioscience, Inc. (South Korea). Contigs were assembled and circularized using SMRT Link (Pacific Biosciences, Menlo Park, CA, United States) and Circlator 1.4.0 (Sanger Institute, Essex, United Kingdom). Gene prediction and functional annotation were performed using the EzBioCloud genome database, Prodigal 2.6.2 (CDSs) (Hyatt et al., 2010), tRNAscan-SE 1.3.1 (tRNA) (Schattner et al., 2005), and Rfam 12.0, (rRNA and other non-coding RNAs) (Nawrocki et al., 2015). CRISPR detection was conducted using PilerCR 1.06 (Edgar, 2007) and CRT 1.2 (Bland et al., 2007). Functional classification was performed using EggNOG 4.5.² Additional annotation was achieved by comparing the predicted CDSs with the Swissprot (The UniProt Consortium, 2015), KEGG (Kanehisa et al., 2014), and SEED databases (Overbeek, 2005) using the UBLAST program (Edgar, 2010).

2.2 Effect of *B. megaterium* CACC109 and CACC119 on drought tolerance

2.2.1 Effects of bacterial strains on rice germination and growth under osmotic-stressed and non-stressed conditions

Germination and seedling growth assays were performed on rice seeds to evaluate the efficacy of the isolated bacterial strains in improving osmotic stress tolerance. Saechungmu rice (*Oryza sativa* L.) seeds were surface sterilized with 2% sodium hypochlorite and 70% ethanol and then washed multiple times with distilled water. The seeds were filled to the 20 mL mark in a 50 mL conical tube and either 1×10^7 CFU (colony-forming unit)/mL of bacterial suspension or 0.85% saline (mock) was added up to the 40 mL mark, ensuring the seeds were completely submerged. The seeds were then gently shaken for 8 h. Twenty seeds from each treatment were evenly distributed among three Petri dishes each containing Murashige & Skoog (MS) medium (DUCHEFA BIOCHEMIE, The Netherlands) with different water potentials (0, −0.15, and −0.49 MPa) achieved with PEG 6000.

Germination was carried out at 27°C in the dark for 5 days, and then the germination rate, root length, and growth parameters were measured. The germination rate was calculated by dividing the number of germinated seeds by the total number of seeds and expressed as a ratio (Islam et al., 2016).

A similar assay was conducted using an iso-osmotic solution of mannitol to assess the effect of the isolated bacterial strains on osmotic stress tolerance. Seeds treated with bacteria or mock solution were germinated in a dark box at 27°C for 3 days. Uniformly germinated seeds were then transferred to MS agar supplemented with 0, 150, and 200 mM mannitol and grown under a 12-h light–dark cycle at 27°C for 3 days. Seedling root length was measured after 3 days. Each assay consisted of 10 seeds arranged in Petri dishes, with three replicates per treatment, and securely sealed with Parafilm.

2.2.2 Experimental design for plant growth and drought study

To study the effects of bacterial strains on rice growth and various physiological, molecular, and biochemical responses under drought and non-drought conditions, Saechungmu rice seeds were germinated at 27°C in the dark for 3 days. Uniformly germinated seedlings, 5 or 30, were selected and planted in regular or Wagner pots, respectively, filled with autoclaved potting soil for rice plants (Punong, Gyeongbuk, South Korea). At 7 days post-planting, the plants were treated weekly for 4 weeks with either 5 mL of 1×10^7 CFU/mL *B. megaterium* CACC109, 1×10^7 CFU/mL *B. megaterium* CACC119, or 0.85% saline (mock) per plant. A 1,000-fold dilution of Hyponex fertilizer was applied to the plants at 10 days post-planting. The plants were maintained in a growth chamber with a constant temperature of 27°C, a 12-h light–dark cycle, and approximately 70% relative humidity. After 6 weeks of growth, watering was halted for half of the mock-, CACC109-, and CACC119-treated rice plants, while the remaining plants continued to grow under well-watered conditions, serving as the control group. Soil moisture content was monitored daily using a moisture analyzer MA37 (Sartorius, Göttingen, Germany) to ensure a consistent water retention rate of 20–40% throughout the drought.

Chlorophyll content was assessed by measuring 20 leaves from mock-, CACC109-, and CACC119-treated independent plants in both the well-watered and water-stressed groups using a SPAD-502 meter (Konica Minolta, Tokyo, Japan). The height, weight, and water retention of 20 plants from each treatment group were assessed 15 or 37 days after inducing drought in the well-watered and water-stressed groups. Each plant, including its roots, was weighed to determine the fresh weight, followed by drying at 65°C for 3 days and subsequent weighing to obtain the dry weight. Water retention for each plant was calculated as the difference between the fresh and dry weights.

For gene expression analysis, leaf samples were collected from the mock-, CACC109-, and CACC119-treated plants grown under well-watered and water-stressed conditions at three time intervals (early, 10 days; middle, 20 days; and late, 35 days post-drought). For each treatment group, four biological replicates were sampled, with each biological replicate being a pool of three leaf tissues from independent plants. The collected samples were immediately flash-frozen in liquid nitrogen and stored at −80°C.

2.2.3 Estimation of proline content

Total proline accumulation in the leaf samples was quantified as previously described (Bates et al., 1973). Briefly, 150 mg of leaf samples

² <http://eggnogetdb.embl.de>

were ground with liquid nitrogen and homogenized in 2 mL of 3% sulfosalicylic acid. After centrifugation at 12,000 rpm for 10 min, the supernatant was transferred to a new tube. Then, 0.5 mL of the supernatant was mixed with 0.5 mL of ninhydrin and 0.5 mL of glacial acetic acid in a 15 mL conical tube. The mixture was boiled for 45 min and immediately cooled on ice for 30 min. The samples were mixed with 1.5 mL of toluene and centrifuged at 700 rpm for 5 min to separate the total proline. Total proline content was measured at 520 nm against a blank using a standard curve prepared with proline.

2.2.4 Total RNA extraction and gene expression analysis

Total RNA from leaf tissue was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, United States). The RNA was treated with DNase I (Promega, Madison, WI, United States), cleaned using UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25, 24:1, v/v) (Thermo Fisher Scientific), and checked for quality and quantity using NANODROP ONE (Thermo Fisher Scientific). For each line, cDNA was synthesized from 2 µg of total RNA using M-MLV reverse transcriptase (Promega). Quantitative PCR analyses were conducted on a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, United States) using SYBR Green Supermix (containing hot-start iTaq DNA polymerase, dNTPs, MgCl₂, SYBR® Green I dye, enhancers, and stabilizers from Bio-Rad Laboratories) and gene-specific primers. Samples were prepared in triplicate, with a total reaction volume of 20 µL containing the following components: 2 µL of cDNA, 1 µL of each primer (forward and reverse, at 10 pM each), 10 µL of SYBR Green Supermix, and 6 µL of double-distilled water. Amplification reactions were initiated with a denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s. Melting curves were recorded over a temperature range of 65°C to 95°C in 0.5°C increments. Rice *Actin* served as an endogenous control for normalization. Expression levels were determined using the comparative cycle threshold (Ct) method. Details of the quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers used are listed in [Supplementary Table S1](#).

2.2.5 Analysis of chemical properties of soil

Soil samples (200 g each) were collected from each pot after the drought stress experiment. The samples were air-dried at room temperature, crushed to pass through a 2 mm mesh sieve, and thoroughly mixed. The pH was measured in distilled water at a ratio of 1:5 (soil sample: distilled water) using a glass electrode pH meter. Exchangeable cations from the soil were extracted with 0.05 N NH₄OAc (pH 7.0) and determined using iCAP™ RQplus ICP-MS (Thermo Fisher Scientific). Available phosphoric acid was extracted following the Lancaster method and determined using iCAP™ RQplus ICP-MS (Thermo Fisher Scientific).

2.3 Statistical analysis

Statistical analyses were performed using the JMP Pro 16 software package. Two-way analyses of variance (ANOVA) with Tukey's honest significant difference (HSD) test were used to assess the statistical significance of the bacterial growth, EPS production, seed germination, plant height, root length, chlorophyll content, proline production, and gene expression assay. Students' *t*-test was used to

compare significant differences in plant growth-promoting traits (phosphate solubility, nitrogen fixation, IAA productivity, siderophore productivity, and ACC deaminase activity), plant weight, water retention, survival rate under salinity stress, and soluble organic components in soil. The significance level was determined at a $p < 0.05$.

3 Results

3.1 PGPR isolated and screened from the ginseng rhizosphere

Five ginseng rhizosphere soil samples were collected from ginseng cultivation fields in South Korea to isolate PGPR with osmotic stress tolerance. A total of 31 isolates were obtained from the samples and identified based on their 16S rRNA gene sequences ([Supplementary Table S2](#)). Subsequently, the isolates were screened for osmotic stress tolerance in LB medium supplemented with PEG 6000. Two isolates exhibited better growth than the *B. megaterium* type strain ATCC14581 under osmotic stress ([Figure 1A](#)), indicating their efficacy as stress-tolerant isolates. Therefore, these strains were selected for further studies.

Identification of these two isolates by 16S rRNA gene sequencing revealed that they belonged to *B. megaterium* and thus were designated as *B. megaterium* CACC109 and CACC119. The obtained sequences of CACC109 and CACC119 were submitted to NCBI GenBank under accession numbers CP117689 and OR915158, respectively. A phylogenetic analysis was conducted using 16S rRNA gene sequences. The sequences of CACC109 and CACC119, along with their closest relatives from NCBI GenBank, were used to construct a phylogenetic tree with the maximum-likelihood method. The analysis showed that CACC109 was most closely related to *B. megaterium* DSM319, whereas CACC119 was most closely related to *B. megaterium* NBRC15308 ([Figure 1B](#)).

The EPS production of the bacterial strains subjected to different levels of osmotic stress induced by PEG6000 was quantified. Under non-osmotic stress, CACC109 and CACC119 produced 4.28 and 4.3 mg mL⁻¹ EPS, respectively, which were significantly higher than the 2.15 mg mL⁻¹ EPS produced by ATCC14581 ([Figure 1C](#)). However, EPS production decreased under -0.15 MPa osmotic stress. Remarkably, CACC109 demonstrated the highest EPS production, reaching 8.96 mg mL⁻¹ at -0.49 MPa, whereas CACC119 exhibited substantially lower levels than CACC109 and similar levels to ATCC14581 ([Figure 1C](#)).

Analysis of the PGP properties of osmotic-tolerant strains CACC109 and CACC119 revealed that CACC109 had a significant ability to solubilize phosphate, with a maximum soluble P₂O₅ concentration of 119 µg/mL, whereas CACC119 did not ([Figure 1D](#)). In terms of nitrogen fixation, CACC109 and CACC119 produced 0.372 and 0.347 µg/mL NH₄, respectively ([Figure 1E](#)). In terms of IAA production, CACC109 and CACC119 produced significantly higher concentrations of indole compounds than ATCC14581 ([Figure 1F](#)). Although both strains tested positive for siderophore production, CACC119 exhibited significantly greater production of siderophores than CACC109 ([Figure 1G](#)). Meanwhile, CACC109 exhibited significant ACC deaminase activity, whereas CACC119 did not ([Figure 1H](#)).

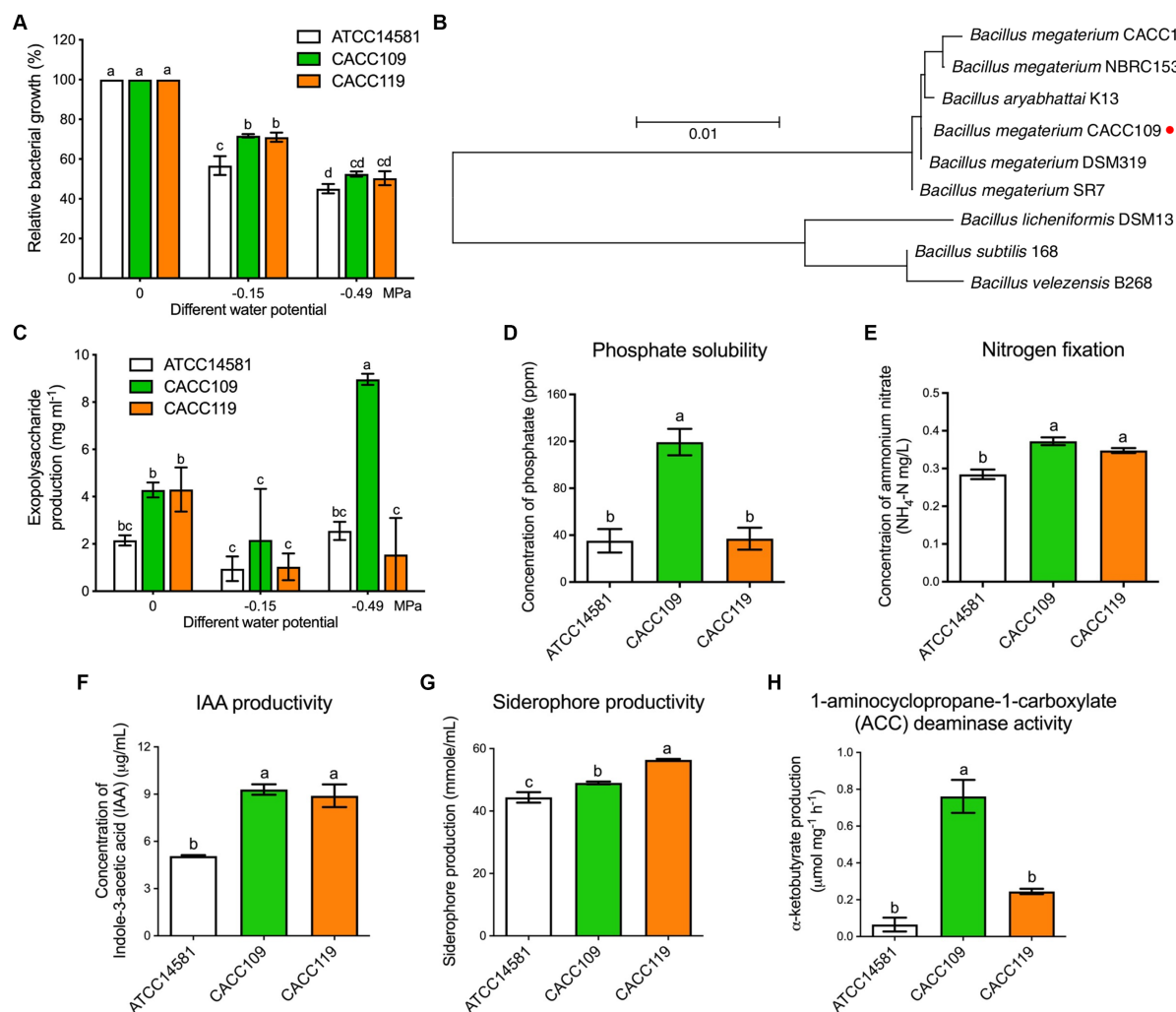


FIGURE 1

Isolation, characterization, and plant growth-promoting properties of water stress-tolerant *Bacillus megaterium* strains CACC109 and CACC119. (A) Relative growth rates of *B. megaterium* strains CACC109 and CACC119 *in vitro* at different water potentials. Growth was measured 24 h after incubation. Growth at 0 MPa was converted to 100%, and the growth values at -0.15 and -0.49 MPa were expressed as a percentage relative to that at 0 MPa. Data are expressed as the mean $\pm$ SD of three biological replicates. Different letters indicate significant differences among the bacterial strains in the various water potentials ($p < 0.05$, two-way ANOVA and Tukey's test). *B. megaterium* ATCC14581 was used as a control. (B) Evolutionary relationships of CACC109 and CACC119 with other known *Bacillus* strains. Sequences were aligned through "Clustal W" using MEGA 11. The maximum-likelihood method with the Tamura-Nei model was used to construct the phylogenetic tree based on 16S rRNA gene nucleotide sequences. Bootstrapped consensus tree derived from 1,000 replicates. Evolutionary analysis was performed in MEGA 11. Red dots indicate the positions of CACC109 and CACC119. (C) EPS production of CACC109 and CACC119 *in vitro* at different water potentials 40 h after inoculation. Bars represent the mean $\pm$ SE of three biological replicates. Different letters indicate significant differences among the bacterial strains in the various water potentials ($p < 0.05$, two-way ANOVA and Tukey's test). (D) Phosphate solubilization, (E) nitrogen fixation, (F) indole-3-acetic acid (IAA) production, (G) siderophore production, and (H) 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Data in (D–H) are expressed as mean $\pm$ SD of three biological replicates. Different letters indicate significant differences ($p < 0.05$, Student's *t*-test). *B. megaterium* ATCC14581 type strain was used as a control. *B. megaterium* ATCC14581 was used as a control. Accession numbers: *Bacillus megaterium* CACC119 (OR915158), *Bacillus megaterium* NBRC15308 (CP009920), *Bacillus aryabhattai* K13 (CP024035), *Bacillus megaterium* CACC109 (CP117689), *Bacillus megaterium* DSM319 (CP001982), *Bacillus megaterium* SR7 (CP022674), *Bacillus licheniformis* DSM13 (AE017333), *Bacillus subtilis* 168 (CP053102), *Bacillus velezensis* B268 (CP053764).

3.2 Effects of CACC109 and CACC119 on seed germination and root growth

The effects of CACC109 and CACC119 on the germination and root growth of rice seeds were examined under osmotic-stressed and non-stressed conditions. Under non-stressed conditions (0 MPa), the CACC109-, CACC119-, ATCC14581-, and mock-treated seeds showed identical germination ratios, with the maximum germination observed on day three (Figure 2A). At -0.15 MPa, no significant

differences in germination rates were observed among the treatment groups (Figure 2A). However, the germination rates of the CACC109- and CACC119-treated seeds were significantly higher than those of the mock- and ATCC14581-treated lines at -0.49 MPa, indicating that both PGPR strains supported seed germination in rice under osmotic stress challenge (Figure 2A). In addition, the CACC109- and CACC119-treated seeds had significantly greater root growth than the mock- and ATCC14581-treated seeds under osmotic-stressed and non-stressed conditions (Figure 2B).

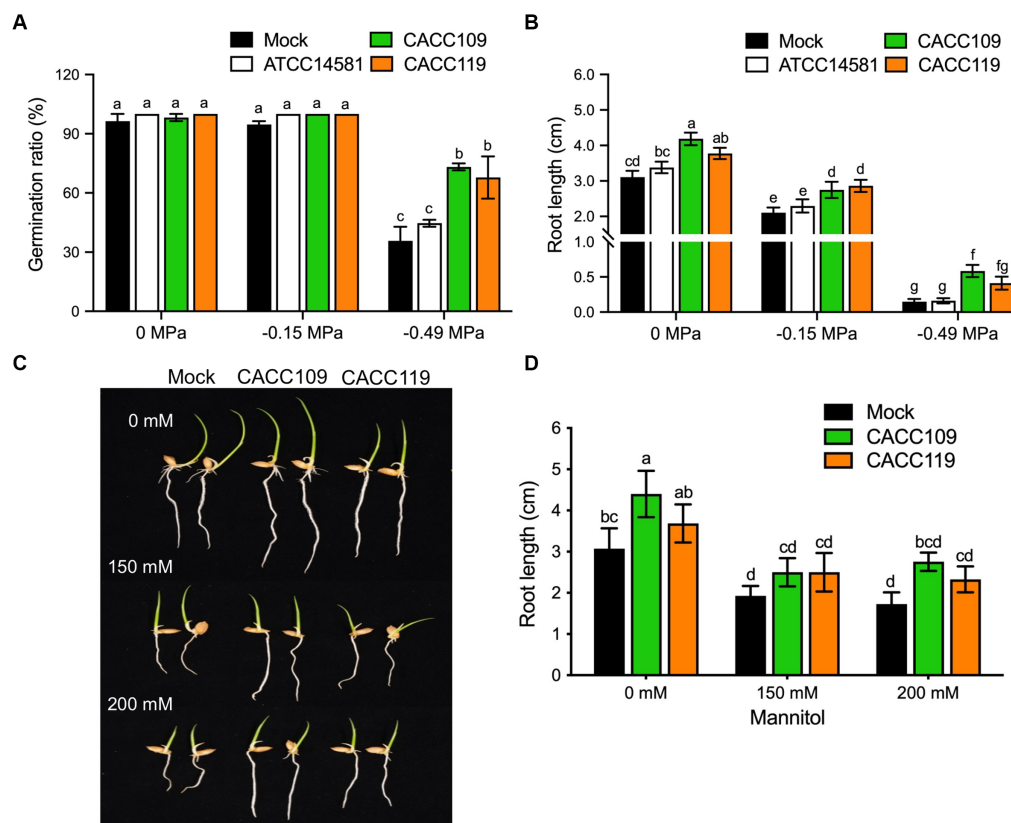


FIGURE 2

Effects of CACC109 and CACC119 on the osmotic stress resistance in rice seedlings. (A) Germination rate and (B) root length in rice seeds treated with mock, CACC109, or CACC119 at different water potentials. (C) Root growth of rice seedlings treated with mock, CACC109, or CACC119 grown in Murashige & Skoog (MS) media containing 0, 150, and 200 mM mannitol for 3 days. (D) Root length in (C) ($n > 30$). In (A,B), seeds were soaked in mock, CACC109, or CACC119 for 8 h and incubated at 30°C for 5 days under different water potentials created with PEG6000. In (C,D), for the mannitol assays, rice seeds were soaked in mock, CACC109, or CACC119 for 10 h, germinated in the magenta box, and similar-sized 3-day-old seedlings were transferred to MS media containing 0, 150, and 200 mM mannitol. Images and measurements were taken 3 days after growth on the MS medium. Assays were performed using 1×10^7 CFU/mL of CACC109 and CACC119. Data represent the mean $\pm$ SE of three independent experiments. Different letters indicate significant differences among the treatments in the various osmotic stresses ($p < 0.05$, two-way ANOVA and Tukey's test).

To validate the effects of CACC109 and CACC119 on rice root growth, we germinated rice seeds and selected evenly germinated seedlings. The seedlings were then exposed to different concentrations of mannitol to induce osmotic stress (Claeys et al., 2014). The root lengths of the mock-, CACC109-, and CACC119-treated seedlings gradually decreased with increasing osmotic stress. This effect is similar to the observed effects of PEG-induced osmotic stress. In the absence of osmotic stress, the CACC109- and CACC119-treated seedlings had significantly longer roots than the mock-treated seedlings (Figures 2C,D). When exposed to 150 or 200 mM mannitol, the CACC109- and CACC119-treated seedlings had significantly longer roots than the mock-treated seedlings, indicating that both CACC strains promoted root growth of the rice seedlings under normal and drought conditions (Figures 2C,D). Collectively, these data indicated that CACC109 and CACC119 enhanced osmotic stress tolerance, promoted seed germination, and facilitated root development in rice seedlings.

3.3 Effects of CACC109 and CACC119 on plant growth and drought resistance

We investigated the effects of PGPR CACC109 and CACC119 on rice growth and drought stress resilience. Under well-watered

conditions, the CACC109- and CACC119-treated rice plants had significantly greater shoot and root growth than the mock-treated plants (Figures 3A–D). Treatment with CACC109 and CACC119 notably increased the rice leaf width (Figures 3E,F). The CACC109- and CACC119-treated plants exhibited 64 and 40% higher shoot weight, respectively, than the mock-treated plants (Figure 3G). Compared with the mock treatment, CACC109 considerably increased root biomass due to axial and lateral root development, whereas CACC119 had a less pronounced effect (Supplementary Figure S1). These results indicated that CACC application significantly promoted plant growth in rice.

After 15 days of water stress, all mock-treated rice plants displayed typical severe dehydration symptoms with significant wilting, and only a few leaves remained green. By contrast, the leaves of most CACC109-treated plants appeared less dehydrated and remained green. The CACC119-treated plants exhibited an intermediate drought stress phenotype relative to the mock- and CACC109-treated plants (Figure 3A). Additionally, water stress caused growth retardation in the mock- and PGPR-treated plants, although this effect was more pronounced in the mock-treated plants (Figures 3A–D). Under drought, the CACC109- and CACC119-treated plants had greater shoot weights than the mock-treated plants (Figure 3G).

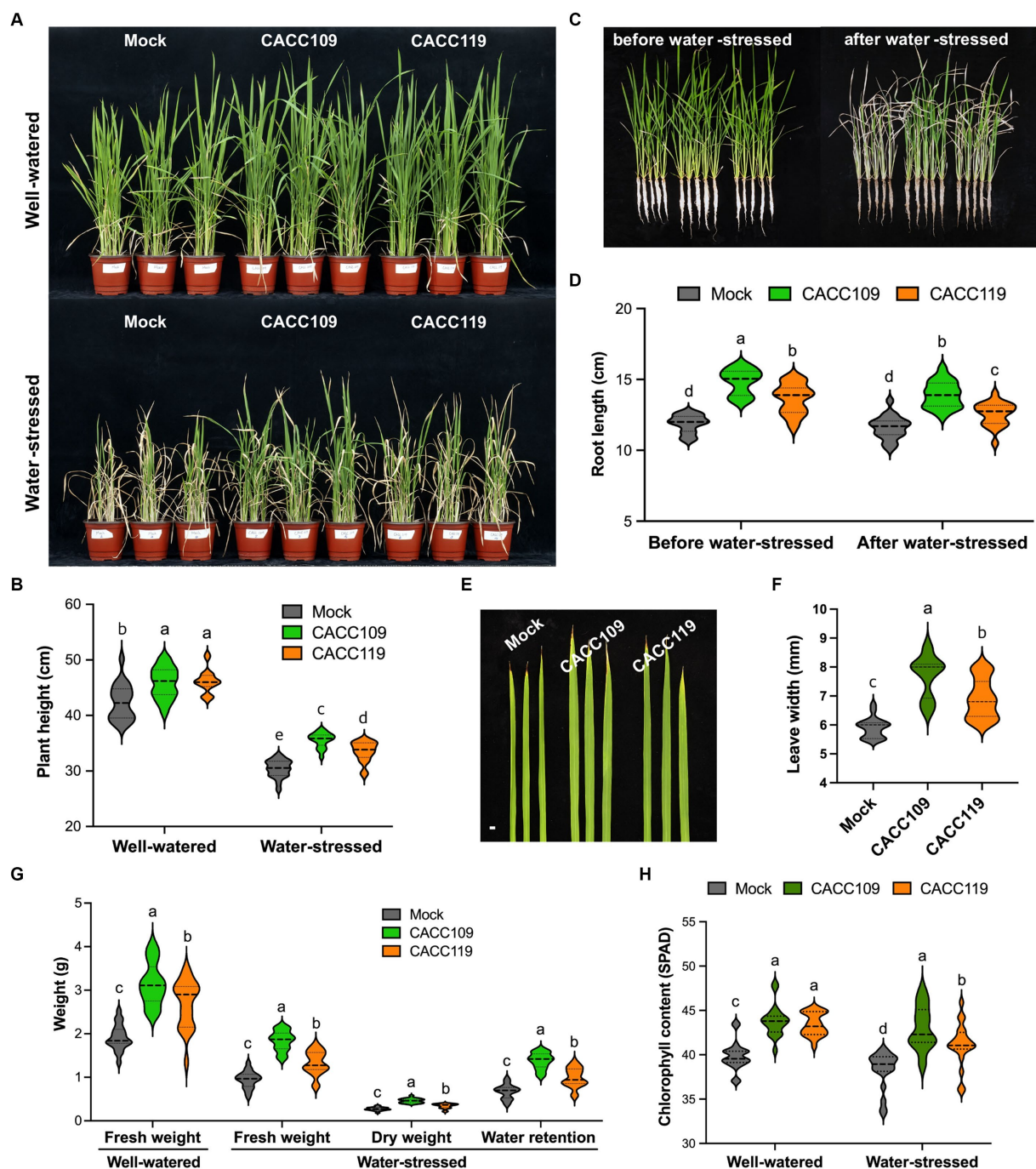


FIGURE 3

Growth promotion and drought resilience in rice by CACC109 and CACC119. (A) Rice plants treated with mock, CACC109, or CACC119 under well-watered and water-stressed conditions. The 6-week-old mock-, CACC109-, or CACC119-treated plants (25 plants for each line) were subjected to drought with 20–40% soil water potential for 15days. (B) Height of rice plants treated with mock, CACC109, or CACC119 under well-watered and water-stressed conditions. (C) Aboveground shoot and root lengths of rice plants treated with mock, CACC109, or CACC119 before water stress (6weeks old) or after water stress (15days under drought). (D) Root length in (C). (E) Flag leaf morphology of 7-week-old rice plants treated with mock, CACC109, or CACC119 under well-watered conditions, bar=5mm. (F) Leaf width in (E). (G) Fresh weight of mock-, CACC109-, or CACC119-treated rice plants grown under well-watered conditions for 8weeks. Fresh weight, dry weight, and water retention (fresh weight – dry weight) of mock-, CACC109-, or CACC119-treated rice plants after 15days of drought. (H) Chlorophyll content of mock-, CACC109-, or CACC119-treated rice plants grown under well-watered or water-stressed conditions. In (B–H), values are means $\pm$ SD ($n=20$, individual plants). Different letters indicate significant differences ($p<0.05$, two-way ANOVA and Tukey's test in B,D,H; Student's t -test in F,G). Control plants (mock) were treated with the same amount of saline solution as a negative control. Chlorophyll and height were measured on mature leaves. The results presented here are representative of three independent experiments.

Under water-stressed conditions, the CACC109-treated plants showed significantly higher shoot dry weight than the mock-inoculated plants. However, no significant difference in shoot dry weight was found between the CACC119- and mock-treated plants (Figure 3G). Drought is typically characterized by decreased water and chlorophyll contents (Bhandari et al., 2023). The CACC109- and CACC119-treated plants had higher water retention than the mock-treated plants (Figure 3G). After 10 days of drought, the chlorophyll content in the mock-treated plants decreased by 7%, whereas those in the CACC109- and CACC119-treated plants decreased by only 3 and 3.5%, respectively, compared with their respective controls (Figure 3H). These data were consistent with the phenotypes of rice plants under drought in each treatment group. Taken together, these results indicated that CACC109 and CACC119 significantly improved the growth and the drought tolerance of rice plants.

3.4 Evaluation of CACC109 and CACC119 on drought tolerance in rice

Additional pot experiments were conducted to evaluate the effects of PGPR CACC109 and CACC119 on the drought tolerance in rice plants. These studies were conducted using a large number of rice plants grown in Wagner pots to mimic conditions in rice fields. We supplied CACC109 and CACC119 in the same manner as in the small-pot test and carefully maintained water levels throughout the experiment to simulate rice field conditions. To investigate the interaction between plants and PGPR, we collected leaf tissues from the CACC109-, CACC119-, and mock-treated plants under non-stressed and water-stressed conditions at different stages of drought (early, 10 days; middle, 20 days; and late, 35 days) to examine physiological traits and gene expression.

After 37 days of water deprivation, all mock-treated plants exhibited severe drought symptoms, characterized by significant wilting and a few leaves remaining green (Figure 4A), whereas the CACC109-treated plants appeared less dehydrated and remained green. The CACC119-treated plants exhibited an intermediate drought stress phenotype compared with the mock- and CACC109-treated plants (Figure 4A). Similar results were observed in these experiments, showing that the application of CACC109 and CACC119 increased the total chlorophyll content, fresh weight, and water retention in the rice plants under drought (Figures 4B,C). The weights of the drought-stressed plants were categorized (Figure 4D). In the mock treatment group, 69.27% of the plants weighed less than 0.75 g, whereas 26.13% weighed within 0.75–0.99 g, with only 4.6% weighing over 1.0 g. By contrast, 26.53% of the CACC109-treated plants weighed within 0.5–0.749 g, 26.64% within 0.75–0.99 g, and 46.83% exceeded 1 g. Meanwhile, 41.42% of the CACC119-treated plants weighed less than 0.749 g, 49.29% weighed within 0.75–0.99 g, and 9.29% exceeded 1 g (Figure 4D). Overall, these data confirmed that CACC109 and CACC119 enhanced the drought tolerance in rice plants.

3.5 Enhanced proline accumulation and gene expression by CACC109 and CACC119 under drought stress

Plants under drought and high salinity often accumulate proline to enhance stress adaptation (Hayat et al., 2012). We evaluated the

proline levels in the leaf tissues from the CACC109-, CACC119-, and mock-treated plants under well-watered and water-stressed conditions. No significant difference in proline content was found between the PGPR- and mock-treated plants under normal growth conditions (Figures 5A,B). All plants subjected to water stress displayed significantly higher proline concentrations than the well-watered control group (Figures 5A,B), indicating that the drought treatment was successful. Interestingly, at the early stage of water stress, the CACC109- and CACC119-treated plants had significantly higher leaf tissue proline concentrations than the mock-treated plants (Figure 5A). As the water stress continued, all plants accumulated more proline, but the mock- and CACC119-treated plants showed significantly higher proline accumulation than the CACC109-treated plants (Figure 5B). This result indicated that the mock- and CACC119-treated plants experienced relatively stronger water stress intensity than the CACC109-treated plants.

We also investigated the time-course expression patterns of the proline biosynthesis genes *Delta 1-Pyrroline-5-carboxylate synthetase 1* and 2 (*P5CS1* and *P5CS2*) in the CACC109-, CACC119-, and mock-treated plants grown under well-watered and water-stressed conditions. As expected, no significant differences in the transcript levels of *P5CS1* and *P5CS2* were found among the treatment groups under well-watered conditions (Figures 5C,D). The expression of *P5CS1* and *P5CS2* was upregulated by water stress in all treated rice plants. Specifically, the expression levels of *P5CS1* and *P5CS2* were higher in the CACC109-treated plants than in the mock- and CACC119-treated plants at the early and middle stages of water stress but lower at the late stage of water stress (Figures 5C,D). The observed trends in the transcript levels of *P5CS1* and *P5CS2* were consistent with proline accumulation. These data suggested that rice produced proline in response to water stress and that inoculation with the PGPR further improved stress tolerance in rice plants by enhancing proline accumulation.

3.6 Effects of CACC109 and CACC119 on the expression of genes responsible for eliminating reactive oxygen species

Under drought, reactive oxygen species (ROS) trigger defense mechanisms associated with ABA and Ca^{2+} flux (Mahmood et al., 2019). However, excessive ROS accumulation leads to cell death through oxidative damage, emphasizing the necessity of ROS-detoxifying systems for plant resilience to drought.

We analyzed the time-course expression of antioxidant-related genes, such as *catalase* (*CAT*), *superoxide dismutase* (*SOD*), *peroxidase* (*POD*), *ascorbate peroxidase* (*APX*), and *glutathione peroxidase* (*GPX*), in the mock-, CACC109-, and CACC119-treated plants under well-watered and water-stressed conditions. Under non-stressed conditions, no significant differences in the expression of antioxidant-related genes were found among the treated plants (Figure 6). However, under water stress, the expression levels of these genes were significantly higher in the mock-, CACC109-, and CACC119-treated plants than in their respective non-stressed controls (Figure 6). Specifically, during drought, the levels of *OsCAT* and *OsPOD* were significantly higher in the CACC109- and CACC119-treated plants than in the mock-treated controls (Figures 6A,B). At the early stage of water stress, *OsAPX* expression was higher in the mock- and CACC109-treated plants than in the CACC119-treated plants. As the

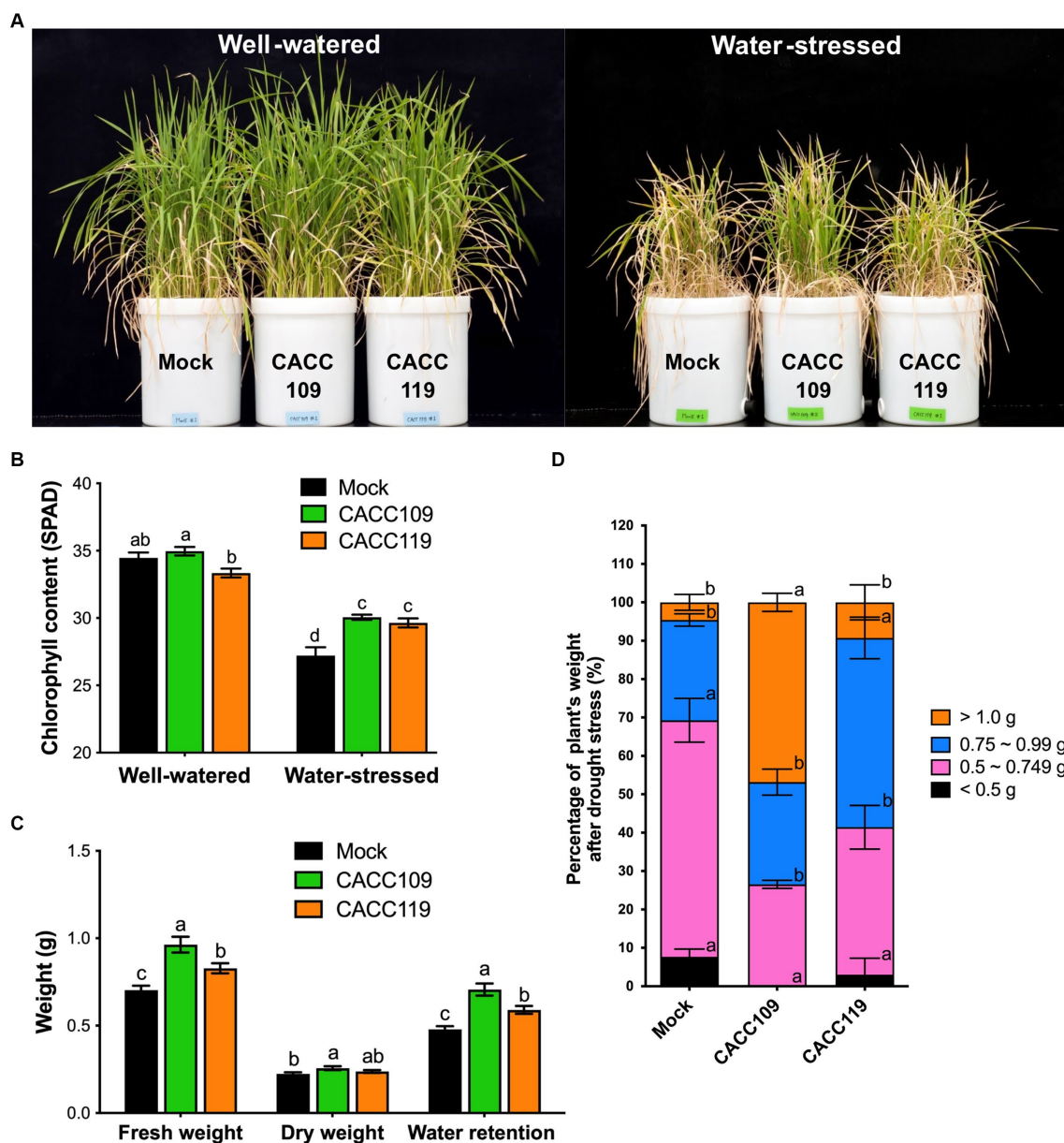


FIGURE 4

Enhanced drought tolerance in rice plants treated with CACC109 and CACC119. (A) Representative images of rice plants treated with mock, CACC109, or CACC119 under both well-watered and water-stressed conditions. (B) The chlorophyll content of rice plants grown under well-watered or water-stressed conditions. (C) Fresh weight, dry weight, and water retention (fresh weight-dry weight) of rice plants under drought. (D) Individual fresh weights of rice under water-stressed conditions. In (B–D), values are means $\pm$ SD ($n = 30$, individual plants). Different letters represent significant differences ($p < 0.05$, two-way ANOVA and Tukey's test in B; Student's t -test in C,D). Control plants (mock) were treated with equal volumes of saline solution as negative control. The soil water content in the water-stressed group throughout the drought stress period was determined using a moisture analyzer MA37 and calculated as a percentage of field capacity. The experiment was performed two times.

stress continued, *OsAPX* expression became higher in the CACC109-treated plants than in the mock- and CACC119-treated plants. However, in the late stages of stress, *OsAPX* expression was notably higher in the mock- and CACC119-treated plants than in the CACC109-treated plants (Figure 6C). The expression levels of *OsSOD* and *OsGPX* were higher in the CACC109-treated plants than in the mock- and CACC119-treated plants at the early and middle stages but lower at the late stage of drought (Figures 6D,E).

Antioxidant activity was significantly higher in the PGPR-treated plants under water stress than in those under control conditions.

Additionally, the expression of antioxidant-related genes was modulated below a certain threshold because of the enhanced stress tolerance mediated by the PGPR.

3.7 Effects of CACC109 and CACC119 on the expression of DRGs

The above results suggest that CACC109 and CACC119 positively affect plant responses to drought. Drought triggers a significant

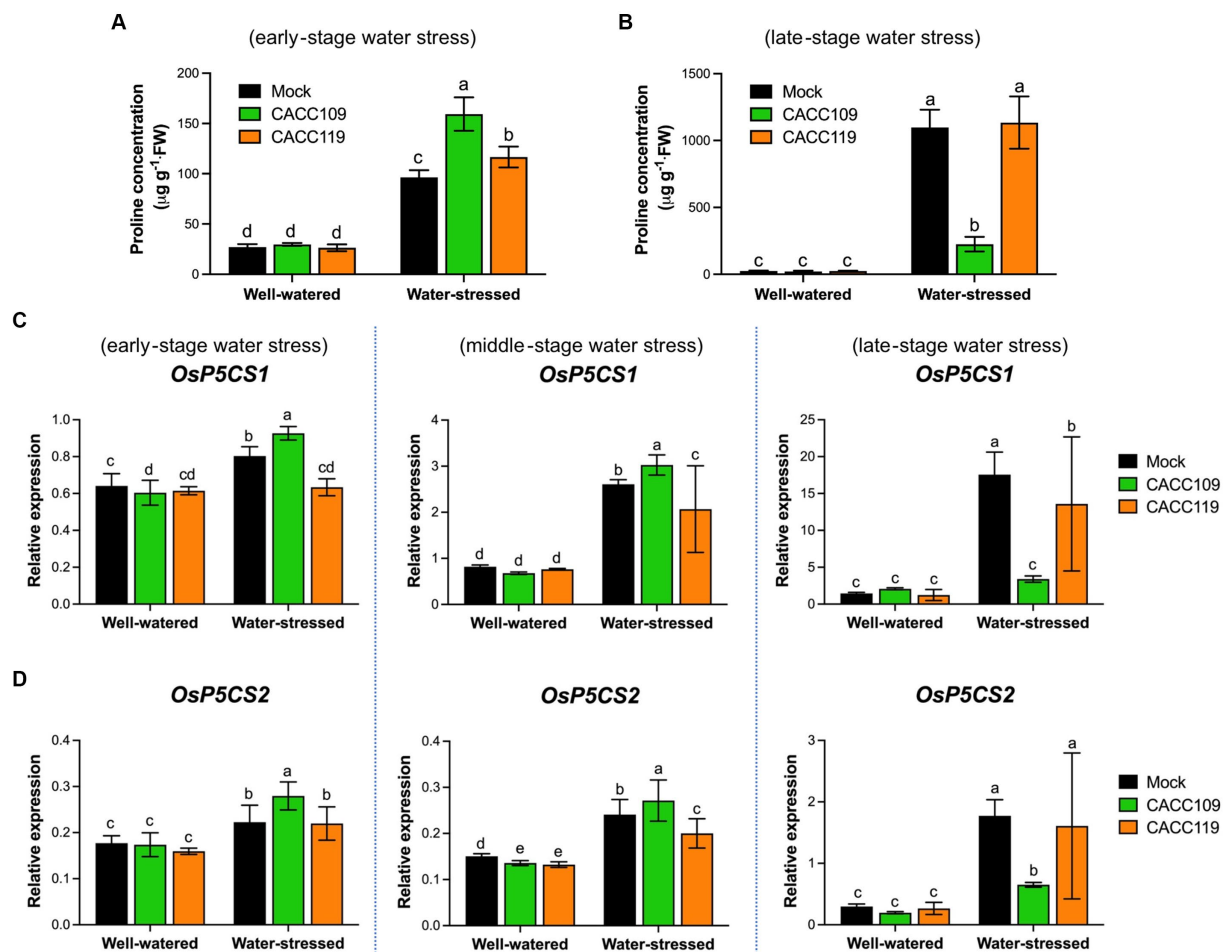


FIGURE 5

Effects of CACC109 and CACC119 on drought-induced proline production. (A,B) Proline content measured in the leaves of mock-, CACC109-, and CACC119-treated plants with or without drought stress in accordance with the method described by (Lugtenberg and Kamilova, 2009). Proline levels were quantified with four biological replicates at (A) 10 days and (B) 35 days post-drought. (C,D) Expression levels of *pyrroline-5-carboxylate synthetase 1* (*P5CS1*) and *P5CS2* in rice leaves grown with mock, CACC109, or CACC119 under well-watered and water-stressed conditions. *P5CS* catalyzes the first step of proline biosynthesis from glutamate. Six-week-old plants were subjected to drought by maintaining soil water potential at 20–40% for 37 days. Leaf tissues were collected from three independent plants treated with mock, CACC109, or CACC119 in each of the two pots under well-watered and water-stressed conditions at 10 (early), 20 (middle), and 35 (late) days after drought treatment. The expression levels of *OsP5CS1* and *OsP5CS2* were estimated by quantitative real-time PCR, and *OsActin* was used as an internal control for normalization. Values are mean $\pm$ SD of two independent experiments ($p < 0.05$, two-way ANOVA and Tukey's test).

reprogramming of gene expression (Shinozaki and Yamaguchi-Shinozaki, 2006). To gain insights into the molecular mechanisms underlying PGPR-induced drought tolerance, we analyzed the expression of ABA-dependent [e.g., *OsbZIP23* (basic region/leucine zipper motif), *OsNAC066* (NAM, ATAF1/2, and CUC2), *OsAREBs* (ABA-responsive element binding protein)] and ABA-independent DRGs [e.g., *OsWRKY47*, *OsDREB2s* (dehydration-responsive element binding protein), and *OsERD1* (early response to dehydration1)] in the mock-, CACC109-, and CACC119-treated plants with or without water stress. Under well-watered conditions, no significant differences in the expression of all DRGs were found between the leaf tissues from the mock- and PGPR-treated plants (Figures 7, 8). The expression levels of all DRGs were significantly higher in the mock-, CACC109-, and CACC119-treated plants under water stress than in their well-watered counterparts, indicating that all plants were affected by drought (Figures 7, 8).

Analysis of the expression of ABA-dependent DRGs showed that during water stress, *OsZIP23* expression peaked in the CACC109-treated plants, whereas the CACC119-treated plants exhibited notably higher *OsZIP23* expression than the mock-treated plants at the late stage (Figure 7A). Meanwhile, *OsNAC066* expression was highest in the CACC109-treated plants, followed by the CACC119-treated plants, and then the control plants at all time points after drought (Figure 7B). At the early stage of water stress, the expression levels of *OsAREB1* and *OsAREB2* were similar or lower in the PGPR-treated plants than in the mock-treated plants. However, as the stress progressed to the middle and late stages, the expression of these genes became significantly higher in the CACC109- and CACC119-treated plants than in the mock-treated plants (Figures 7C,D). The expression of *OsAREB8* showed no noticeable difference between the mock- and PGPR-treated plants under water stress (Figure 7E).

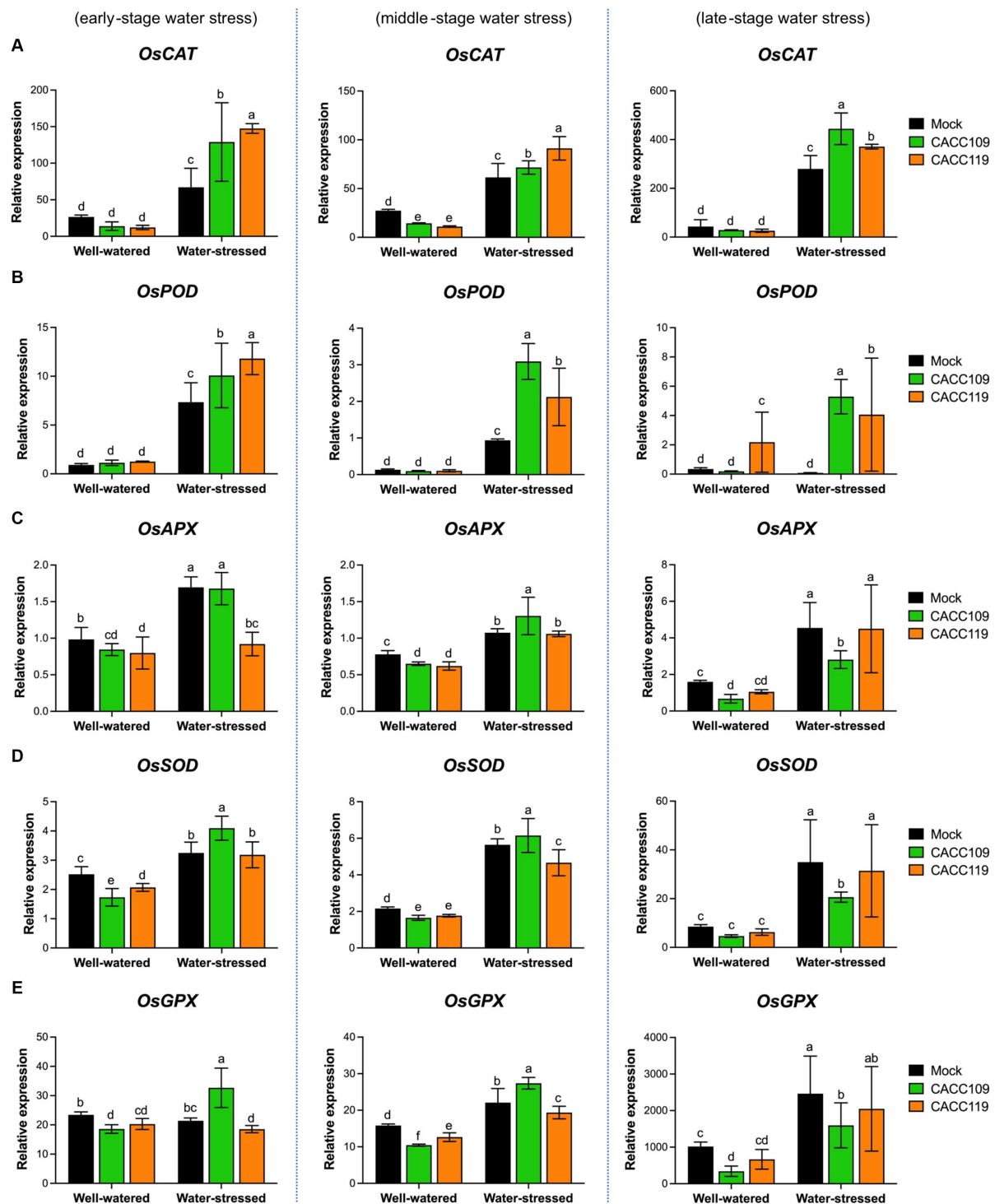


FIGURE 6

Effects of CACC109 and CACC119 on the expression of antioxidant-related genes in rice leaves. Expression of (A) *OsCAT* (catalase), (B) *OsPOD* (peroxidase), (C) *OsAPX* (ascorbate peroxidase), (D) *OsSOD* (superoxide dismutase), and (E) *OsGPX* (glutathione peroxidase) in mock-, CACC109-, and CACC119-treated plants grown under well-watered or water-stressed conditions. Six-week-old plants were subjected to drought stress by maintaining soil water potential at 20–40% for 37 days. Leaf tissue was collected from three independent plants treated with mock, CACC109, or CACC119 in each of the two pots under well-watered and water-stressed conditions at 10 (early), 20 (middle), and 35 (late) days after drought treatment. Data were normalized by the comparative cycle threshold method with *OsActin* as the internal control and presented as relative expression. Values are mean $\pm$ SD of two independent experiments. Different letters indicate significant differences ($p < 0.05$, two-way ANOVA and Tukey's test).

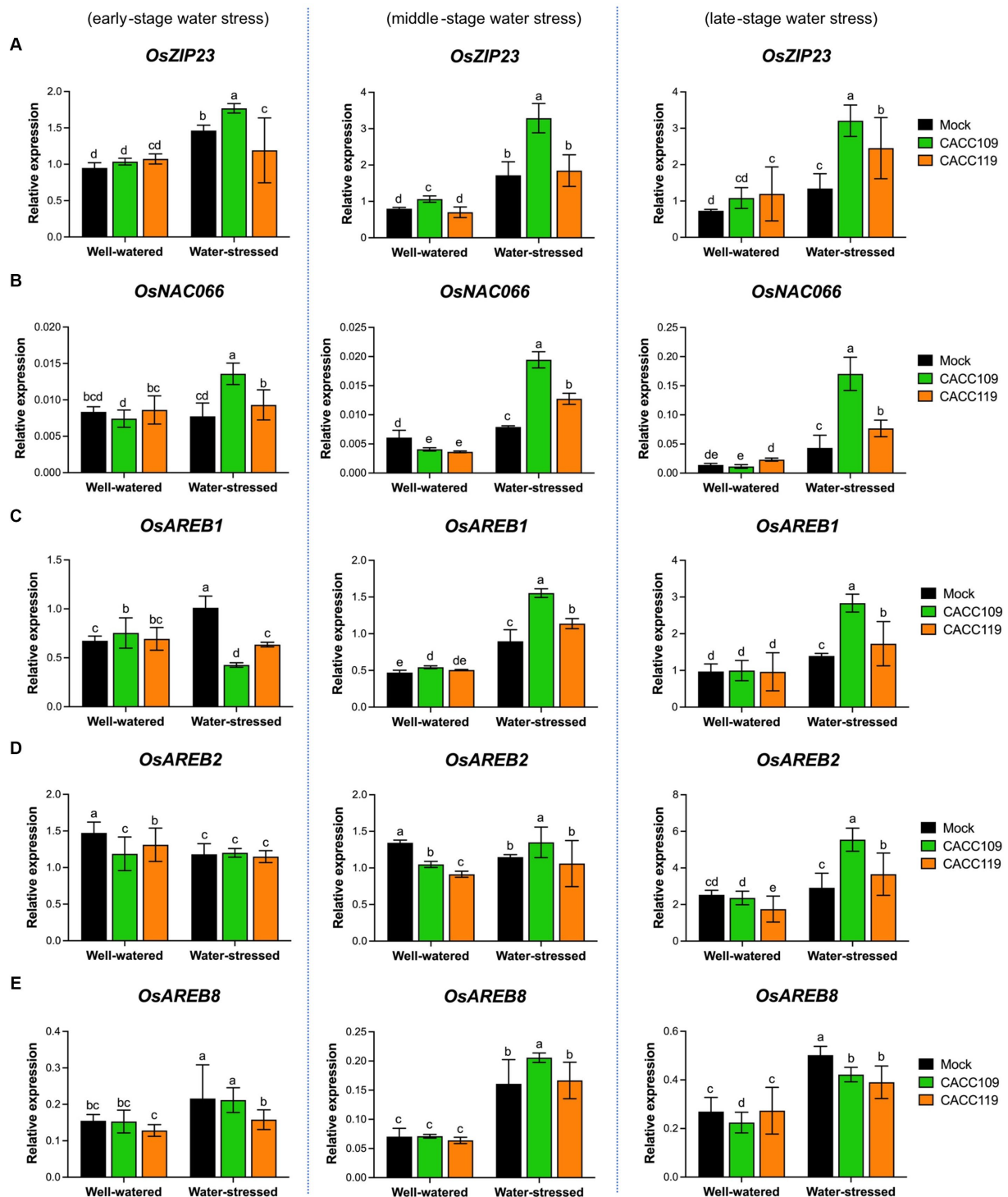


FIGURE 7

Effects of CACC109 and CACC119 on the expression of ABA-dependent drought-responsive genes in rice leaves. Expression of (A) *OsZIP23*, (B) *OsNAC066*, (C) *OsAREB1*, (D) *OsAREB2*, and (E) *OsAREB8* in mock-, CACC109-, and CACC119-treated plants grown under well-watered or water-stressed conditions. Six-week-old plants were subjected to drought stress by maintaining soil water potential at 20–40% for 37 days. Leaf tissues were collected from three independent plants treated with mock, CACC109, or CACC119 in each of the two pots under well-watered and water-stressed conditions at 10 (early), 20 (middle), and 35 (late) days after drought treatment. The expression levels of drought-responsive genes were estimated by quantitative real-time PCR, and *OsActin* was used as an internal control for normalization. Values are mean $\pm$ SD of two independent experiments. Different letters indicate significant differences ($p < 0.05$, two-way ANOVA and Tukey's test).

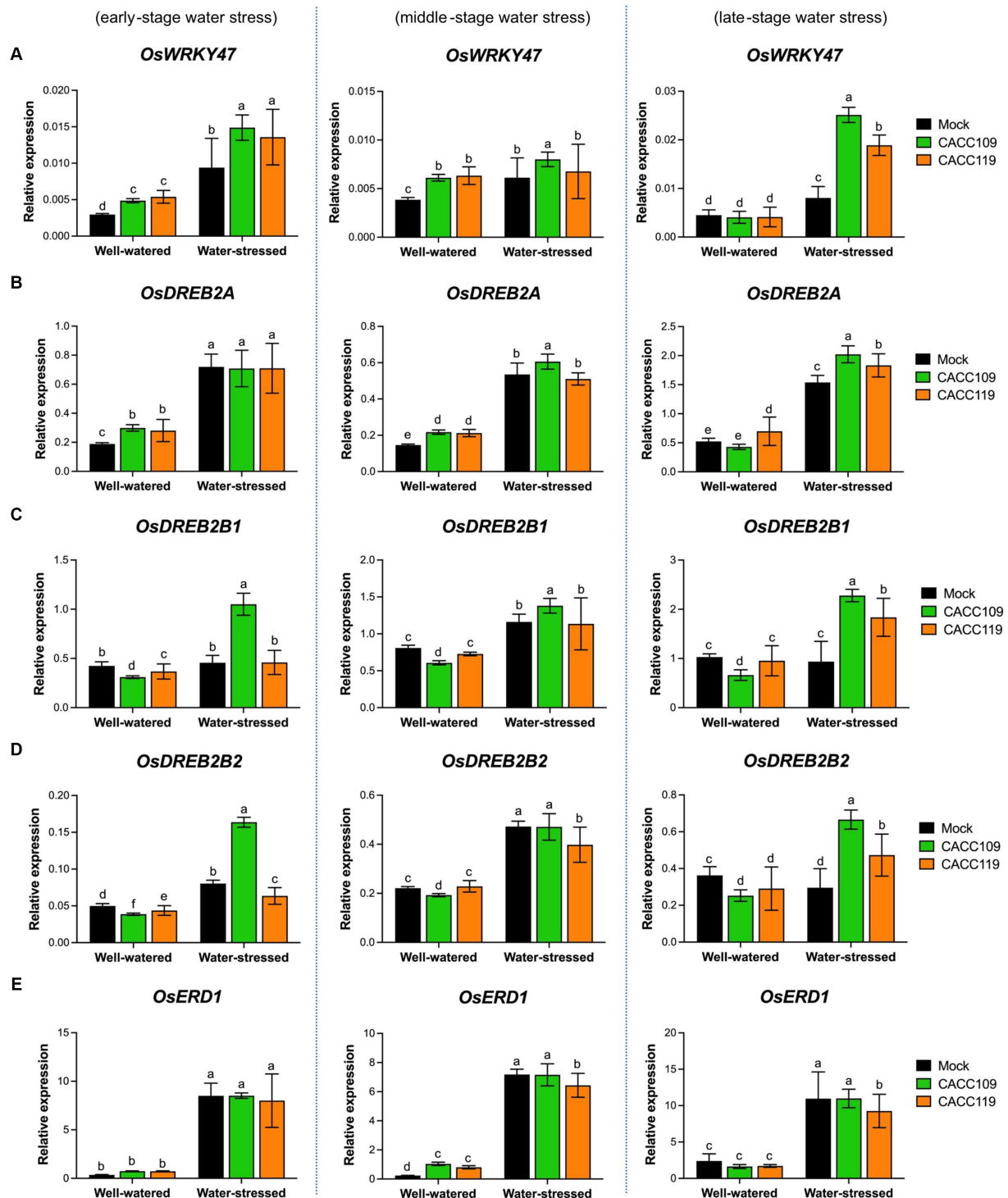


FIGURE 8

Effects of CACC109 and CACC119 on the expression of ABA-independent drought-responsive genes in rice leaves. Expression of (A) *OsWRKY47*, (B) *OsDREB2A*, (C) *OsDREB2B1*, (D) *OsDREB2B2*, and (E) *OsERD1* in mock-, CACC109-, and CACC119-treated plants grown under well-watered or water-stressed conditions. Six-week-old plants were subjected to drought by maintaining soil water potential at 20–40% for 37 days. Leaf tissues were collected from three independent plants treated with mock, CACC109, or CACC119 in each of the two pots under well-watered and water-stressed conditions at 10 (early), 20 (middle), and 35 (late) days after drought treatment. Expression levels of drought-responsive genes were estimated by quantitative real-time PCR, and *OsActin* was used as an internal control for normalization. Values are mean $\pm$ SD of two independent experiments. Different letters indicate significant differences ($p < 0.05$, two-way ANOVA and Tukey's test).

Analysis of the expression of ABA-independent DRGs displayed that at the early stage of water stress, the CACC109- and CACC119-treated plants showed significantly higher *OsWRKY47* expression than the mock-treated plants. However, at the middle stage of water stress, all plants exhibited similar expression levels of *OsWRKY47*. At the late stage, *OsWRKY47* expression was significantly upregulated in the PGPR-treated plants compared with the mock-treated plants (Figure 8A). At the early and middle stages of water stress, the expression of *OsDREB2A* was similar in both the PGPR-treated and mock-treated plants. However, in the late stage, the CACC109- and CACC119-treated plants showed significantly higher levels of *OsDREB2A* expression compared to the mock-treated plants (Figure 8B). The expression levels of both splice variants of *OsDREB2B*, *OsDREB2B1* and *OsDREB2B2*, were higher in the CACC109-treated plants than in the CACC119- and mock-treated plants at the early stage of water stress. At the middle stage, the PGPR- and mock-treated plants exhibited comparable expression levels of *OsDREB2B1* and *OsDREB2B2*. However, at the late stage, the expression levels of *OsDREB2B1* and *OsDREB2B2* were significantly higher in the PGPR-treated plants than in the mock-treated plants, which displayed similar levels to the non-stressed plants (Figures 8C,D). No significant difference in *OsERD1* expression was found between the mock- and PGPR-treated plants under water stress (Figure 8E).

These results indicated that CACC109 and CACC119 enhanced the drought tolerance in rice plants by upregulating the expression of DRGs via ABA-dependent and -independent pathways.

3.8 Genome analysis of CACC109 (PRJNA932602) and CACC119 (PRJNA1049983)

Whole-genome sequencing of *B. megaterium* CACC109 and CACC119 yielded 672 and 621 Mb of data with approximately 34× and 5× coverage, respectively. Sequencing reads with a Phred score greater than Q30 were used for *de novo* assembly using PacBio sequencing data. The Microbial Assembly resulted in draft genomes of ~5.3 and 5.7 Mb (5,309,578 and 5,703,136 bp) with 4 and 10 contigs with N₅₀ of 5,083,237 and 4,735,530 bp, respectively. The average GC contents of the CACC109 and CACC119 genomes were 38.1 and 37.8%, respectively. Annotation of the CACC109 and CACC119 draft genomes resulted in 5,423 and 5,787 CDSs with median lengths of 714 and 702 bp, 42 and 49 rRNAs, and 130 and 187 tRNAs, respectively, as predicted by Prodigal 2.6.2 (Supplementary Table S3). These genomic features were visualized in circular maps of the *B. megaterium* CACC109 and CACC119 genomes (Supplementary Figure S2).

We deciphered genes enhancing plant growth and abiotic stress resilience from the entire genomes of CACC109 and CACC119. The presence of genes associated with phosphate solubilization, nitrogen fixation, and IAA production highlighted their potential as PGPs. In addition, the detection of genes related to siderophore production and ACC deaminase activity underscores their ability to thrive under adverse environmental conditions and improve plant tolerance to abiotic stresses (Supplementary Table S4). The identification of genes associated with motility and chemotaxis further indicated that CACC109 and CACC119 are plant-associated bacteria capable of navigating to plant roots in response to plant-secreted substances and nutrients (Supplementary Table S5).

3.9 Effects of PGPR on soluble organic components in soil

To investigate whether the PGPR affect the balance of soluble organic components in soil, we compared the levels of P₂O₅, Ca²⁺, and K⁺ in soils treated with mock, CACC109, or CACC119. Under water stress, P₂O₅ content was significantly higher in the CACC109- and CACC119-treated soils than in the mock-treated soils. Furthermore, the application of both PGPR significantly increased the K⁺ and Ca²⁺ contents in the soils by 22.9 and 31.4%, respectively, compared with the mock treatment (Supplementary Figure S3). These results indicated that the PGPR CACC109 and CACC119 played a role in altering the balance of soluble organic components in soils under drought.

4 Discussion

Drought is an abiotic stress that can severely affect the growth and production of crops, including rice, and the agricultural demand for food security (Zhang et al., 2018). Many strategies have been developed to increase the resilience of plants to abiotic stresses, allowing them to thrive in challenging environments (Ashry et al., 2022). Among these, inoculation with PGPR is effective in increasing crop productivity in drought-stressed environments (Rodríguez-Salazar et al., 2009). The enhancement of plant growth by PGPR can be attributed to several mechanisms, including the synthesis of PGP hormones and other PGP activities (Glick, 1995). Over the past two decades, various soil microorganisms have been used to alleviate abiotic stresses in plants. PGPR mitigate the effects of drought on crops (Niu et al., 2018; Jochum et al., 2019; Batool et al., 2020). However, the selection of bacteria with abiotic stress tolerance and *in vitro* PGP properties is crucial to alleviate abiotic stress in plants (Jinal et al., 2021).

In the present study, 31 isolates were obtained from ginseng rhizosphere and field soils, of which 28 were identified as *Bacillus* spp. Among the isolates, *B. megaterium* CACC109 and CACC119 showed comparatively better osmotic stress tolerance (Figure 1A). Both strains exhibited EPS and siderophore production (Pallai, 2005; Naseem et al., 2018), nitrogen fixation (Cattelan et al., 1999), IAA synthesis (Loper and Schroth, 1986), and ACC deaminase activity (Glick, 2014) (Figures 1C–H). Notably, CACC109 showed additional advantages, including higher ACC deaminase activity, phosphate solubilization, and EPS production under osmotic stress, enhancing its ability to promote plant growth and alleviate abiotic stress. Phosphate-solubilizing microorganisms act as biofertilizers (Sivasakthi et al., 2013) that increase phosphorus availability and crop yield (Shi et al., 2014). Nitrogen-fixing *Bacillus* species are promising in agriculture because they facilitate plant nutrient uptake (Novo et al., 2018). The IAA produced by PGPR promotes plant growth and nutrient absorption (Loper and Schroth, 1986; Venturi and Keel, 2016; Gowtham et al., 2017). Microbial EPS production under stress conditions enhances bacterial survival, indicating drought tolerance (Sandhya et al., 2009). Specifically, PGPR produce EPS composed of complex organic macromolecules, including polysaccharides, proteins, and uronic acid. These compounds protect microbial cells by stabilizing their membrane structure against adverse environmental conditions such as drought, high salinity and extreme temperatures

(Ojuederie and Babalola, 2017). Siderophores are critical for microbial survival and facilitate iron binding and uptake, particularly under conditions of low iron availability. Higher siderophore levels correlate with improved drought tolerance in plants (Andrews et al., 2003; Arzanesh et al., 2011). ACC deaminase activity in many PGPR helps reduce ET levels in plants, mitigating growth inhibition under stress and thereby promoting plant growth (Glick, 2014).

In the present study, we evaluated the effects of the selected PGPR strains, CACC109 and CACC119, on seed germination, seedling growth, and drought stress in rice plants. Both strains improved rice seed germination and root development under osmotic stress induced by PEG 6000 and mannitol (Figure 2) possibly by enhancing EPS production, which mitigated damage and promoted metabolic processes in the seeds (Bakhshandeh et al., 2020). Similarly, a previous study reported a 50% increase in germination rate under stress conditions with the application of EPS-producing bacterial strains (Tewari and Arora, 2014). CACC109 and CACC119 also significantly improved the drought tolerance of rice plants (Figure 4). Notably, CACC109 showed superior EPS production to CACC119 under high osmotic stress (Figure 1C), which is consistent with the observed phenotype. Bacteria can survive under stressful conditions by producing EPS, which increases water retention, regulates the diffusion of organic carbon sources, and allows irreversible attachment and colonization of roots by forming a network of fibrillar material that binds bacteria to the root surface (Chenu and Roberson, 1996; Bashan and Holguin, 1997). EPS are hydrated compounds containing 97% water in the matrix, protecting both bacteria and plants from desiccation. Plants treated with EPS-producing bacteria exhibit enhanced water retention and drought stress tolerance (Bensalim et al., 1998). Bacterial EPS can form a biofilm on the root surface, ultimately enhancing soil structure and supporting plant development while mitigating the impact of drought stress (Sandhya et al., 2009). EPS produced by various bacterial strains enhance soil permeability by improving soil aggregation and maintaining a higher water potential and better nutrient uptake around roots, resulting in increased plant growth and drought tolerance (Upadhyay et al., 2012; Vejan et al., 2016). Niu et al. (2018) reported that several PGPR strains, including *Enterobacter hormaechei*, *Pseudomonas fluorescens*, and *Pseudomonas migulae*, produce EPS and enhance seed germination and seedling growth under drought conditions. The inoculation of sunflower rhizosphere with the EPS-producing rhizobial strain YAS34 under drought conditions significantly increases the ratio of root-adhering soil per root tissue (Alami et al., 2000). Vurukonda et al. (2016) found that inoculating maize seeds with EPS-producing bacterial strains *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, and *Proteus penneri* increases soil moisture and relative water contents, shoot and root length, leaf area, and plant biomass under drought conditions. The results of present study suggest that EPS-producing CACC109 and CACC119 are promising candidates for enhancing plant stress tolerance and promoting seedling establishment under adverse environmental conditions.

The CACC109- and CACC119-treated rice plants had significantly higher shoot and root lengths, leaf widths, plant weights, and chlorophyll contents than the control plants (Figures 3, 4). Notably, CACC109 treatment substantially increased root biomass due to axial and lateral root development, whereas CACC119 exerted a similar but less pronounced effect (Supplementary Figure S1). This result is consistent with the growth-promoting effects of PGPR reported in

previous studies. For example, *Bacillus aryabhattai* AB211 from maize (Bhattacharyya et al., 2017), *Bacillus aryabhattai* SRB02 from soybean roots (Park et al., 2017), *Bacillus* sp. BCLRB2 in saline soil (Hadi Brahim et al., 2019), *Bacillus subtilis* EA-CB0575 from banana roots (Franco-Sierra et al., 2020), and *Bacillus* sp. D5 from saffron corms (Magotra et al., 2021) significantly improve plant growth through various mechanisms, such as synthesis of hormones, facilitation of phosphate uptake, and production of beneficial compounds. Our results highlight the potential benefits of nutrient uptake and enhanced adaptability to adverse abiotic stress conditions in rice plants.

Drought decreases plant growth as essential nutrients and solutes are redirected toward stress-related processes (Brestic et al., 2018). Nonetheless, bacterial inoculation enhances plant growth, resulting in significant increases in root and shoot lengths and leaf area (Lin et al., 2020). Different PGPR strains enhance the growth of various plants under abiotic stresses, such as drought and salinity (Kumari et al., 2016; Creus et al., 2020; Mishra et al., 2020; Muhammad Usman Aslam et al., 2020). In the present study, the application of CACC109 and CACC119 significantly improved the shoot length, leaf width, plant weight, chlorophyll content, and proline production in the rice plants under drought, ultimately increasing the drought tolerance in the PGPR-treated plants compared with the mock-treated plants (Figures 3, 4).

Drought can affect the chlorophyll levels in plant leaves and consequently decrease photosynthesis (Liu et al., 2019; Khayatnezhad and Gholamin, 2021). It can significantly reduce photosynthetic efficiency by altering the leaf area, affecting chlorophyll synthesis, increasing lipid peroxidation, and promoting chlorophyll degradation (Khayatnezhad and Gholamin, 2021). Consequently, plants capable of maintaining higher chlorophyll levels under drought stress may exhibit greater tolerance to water shortage and sustain increased growth and biomass production. This hypothesis is supported by the higher total chlorophyll contents and water retention in the CACC109- and CACC119-treated plants than in the mock-treated plants under drought.

The accumulation of osmolytes such as proline, sugars, and proteins in plants is a key indicator of drought tolerance because it facilitates osmotic adjustment and prevents water loss (Vaishnav and Choudhary, 2019; Karimzadeh et al., 2021). In the present study, proline production and proline biosynthesis gene expression significantly increased in the plants treated with CACC109 and CACC119 under drought (Figure 5). One of the defense strategies of PGPR is to improve the tolerance of plants to drought stress, indicating the substantial contribution of CACC109 and CACC119 in alleviating drought in plants by enhancing their defense mechanisms.

Endogenous signaling molecules such as ROS play crucial roles in plant growth, development, and stress adaptation, acting as double-edged swords because of their dual nature as signaling molecules and potential toxins, especially under drought (Miller et al., 2010; Steffens, 2014). Although excessive ROS accumulation can be detrimental to plant cells, plants can enhance drought tolerance by modulating ROS levels through the activities of various antioxidant enzymes, such as SOD, CAT, POD, and GPX (Blokina, 2003; Miller et al., 2010; Fang et al., 2015; Nadarajah, 2020). Elevated expression of ROS detoxification genes improves plant drought tolerance by mitigating the deleterious effects of oxidative stress on plant metabolism (Ning et al., 2010). In the present study, the CACC109-treated plants had

significantly higher expression of antioxidant-related genes *OsSOD*, *OsAPX*, *OsCAT*, *OsPOD*, and *OsGPX* under drought, whereas the CACC119-treated plants had notably higher expression of *OsCAT* and *OsPOD* compared with the mock-treated plants (Figure 6). These findings suggest that CACC109 and CACC119 influence cellular ROS homeostasis and potentially improve drought tolerance in rice plants by regulating oxidative stress levels. In particular, the relative expression trend of these genes varies over time. Specifically, the relative expression of *OsCAT* and *OsPOD* was higher at the late stage of water stress, whereas that of *OsAPX*, *OsSOD*, and *OsGPX* was lower at the same stage in the plants inoculated with CACC109 (Figure 6). This differential expression of antioxidant genes can be attributed to several factors. The enzymes APX, GPX, CAT, and POD utilize different mechanisms to scavenge hydrogen peroxide (H_2O_2) and manage oxidative stress in plants. Ascorbate peroxidase requires an ascorbate and glutathione (GSH) regeneration system, known as the ascorbate-glutathione cycle. In the first reaction of this cycle, catalyzed by APX, H_2O_2 and ascorbate produce H_2O and monodehydroascorbate (Mittler, 2002). Similarly, GPX reduces H_2O_2 to H_2O but uses GSH directly as the reducing agent (Noctor et al., 2014). By contrast, CAT directly converts H_2O_2 to H_2O and $1/2 O_2$ and is primarily involved in detoxifying H_2O_2 rather than regulating it as a signaling molecule in plants (Apel and Hirt, 2004). Meanwhile, peroxidase can utilize various substrates to reduce H_2O_2 , often employing phenolic compounds as electron donors, and is involved in a wide range of physiological processes (Gill and Tuteja, 2010). Changes in the balance of these scavenging enzymes trigger compensatory mechanisms. For instance, a decrease in CAT activity upregulates APX and GPX to maintain ROS homeostasis. Similarly, POD activity may increase in response to stress conditions to compensate for changes in the levels or activity of other antioxidant enzymes, thus contributing to the overall ROS detoxification and stress adaptation (Apel and Hirt, 2004). Inoculation with CACC109 could influence the expression of ROS detoxification genes by modulating plant hormone balance, signaling pathways, or enhancing drought stress-specific response mechanisms. This phenomenon could result in a more targeted activation of *OsCAT* and *OsPOD* while downregulating others such as *OsAPX*, *OsSOD*, and *OsGPX* in the late stage. These observed differential expressions likely reflect the strategic and dynamic response of plants to effectively manage ROS levels. Further studies involving detailed temporal gene expression analysis and the role of CACC109 inoculation may provide deeper insights into these regulatory mechanisms.

Plants have evolved numerous defense mechanisms against drought, among which transcription factors (TFs) play a crucial role in regulating the expression of stress-related genes. Members of the basic leucine zipper (bZIP) family are important TFs responsible for ABA signaling and plant responses to drought (Joo et al., 2021). *OsZIP23* is a key regulator of drought and salinity tolerance in rice (Xiang et al., 2008). AREBs, which belong to the group A subfamily of bZIP TFs, play key roles in the ABA signaling pathway in rice. They regulate the expression of stress-responsive genes, especially under drought and salinity, thus contributing to the stress tolerance mechanisms in rice (Yoshida et al., 2010). NAC, one of the largest families of TFs in plants, is involved in the regulation of growth and stress responses (Nakashima et al., 2012; Shao et al., 2015). *OsNAC66* positively regulates oxidative and drought stress tolerance, as demonstrated by functional analyses of overexpression and RNAi transgenic rice plants (Yuan et al., 2019). WRKY TFs play an

important role in regulating transcriptional reprogramming associated with tolerance to multiple abiotic stresses in plants (Chen et al., 2012). *OsWRKY47* is a positive regulator of drought response, and *OsWRKY47*-overexpressing rice plants exhibit greater drought tolerance than wild-type plants (Raineri et al., 2015). DREB TFs play critical roles in improving abiotic stress tolerance, mostly in an ABA-independent manner (Dubouzet et al., 2003; Lata and Prasad, 2011). In rice, *OsDREB2A* expression is induced by water stress, resulting in increased drought tolerance (Cui et al., 2011). The *OsDREB2B* transcript exhibits functional and non-functional forms under drought, with alternative splicing induced by its pre-mRNA, thereby enhancing drought tolerance (Matsukura et al., 2010). These findings highlight the critical role of *OsDREB2* in regulating drought tolerance in rice plants.

In the present study, we investigated the molecular mechanisms underlying PGPR-induced drought tolerance by examining the expression of DRGs. CACC109 and CACC119 positively influenced the response of rice plants to drought. Under well-watered conditions, the mock- and PGPR-treated plants showed no significant difference in DRG expression (Figures 7, 8). However, under water stress, the PGPR- and mock-treated plants showed significantly higher expression of DRGs than the control plants. *OsZIP23* expression peaked in the CACC109-treated plants. *OsDREB2A* expression was significantly higher in both CACC-treated plants than in the mock-treated plants at the late drought stage. The expression of *OsDREB2B1* and *OsDREB2B2* peaked in the CACC109-treated plants during water stress. The highest expression levels of *OsNAC66* were observed in the CACC109-treated plants. The expression levels of *OsAREB1* and *OsAREB2* were significantly higher in both CACC-treated plants than in the mock-treated plants at the middle and late stages of water stress. However, *OsAREB8* expression was similar or significantly lower in the CACC-treated plants at these same stages. The AREB family members, though functionally related, can have different regulatory controls and promoter regions responding to specific signals. The inoculation with CACC could interact differently with the signaling pathways involving *OsAREB8* compared to *OsAREB1* and *OsAREB2*. This could result in a distinct modulation of *OsAREB8* expression, possibly due to specific hormonal changes or stress signals induced by CACC treatment. Both PGPR-treated plants exhibited significantly higher *OsWRKY47* expression at the early and late stages of water stress. Moreover, the expression of *OsDREB2B1* and *OsDREB2B2* was significantly upregulated in the CACC109-treated plants during the early stage and in both PGPR-treated plants at the late stage of drought. These results suggest that CACC109 and CACC119 enhance drought tolerance in rice via ABA-dependent and -independent pathways, as reflected by the altered expression of the abovementioned DRGs. Taken together, the results of our study highlight the beneficial effects of PGPR CACC109 and CACC119 on plant response to drought and provide novel insights into the molecular mechanisms underlying PGPR-induced drought tolerance in rice.

The whole genome analysis of CACC109 and CACC119 reveals a suite of genes that underscore their potential as effective PGPR, particularly under stress conditions. The genomes of these strains encode genes associated with key physiological traits that enhance plant growth and stress resilience. For instance, the presence of genes related to phosphate solubilization, nitrogen fixation, and IAA production

highlights their ability to improve nutrient availability and hormonal balance, which are crucial for plant growth and stress adaptation (Supplementary Table S4) (Lugtenberg and Kamilova, 2009). Moreover, the detection of genes involved in siderophore production and ACC deaminase activity points to their role in mitigating the adverse effects of abiotic stresses. Siderophores can help plants acquire iron under limited conditions, while ACC deaminase can reduce the levels of ET, a hormone that often exacerbates stress responses (Hayat et al., 2010; Singh et al., 2022). The presence of genes related to motility and chemotaxis further indicates that these bacteria can navigate toward plant roots, enhancing their colonization and interaction with plant roots in response to plant-secreted signals and nutrient gradients (Supplementary Table S5) (Feng et al., 2021). These genomic insights are instrumental in understanding how CACC109 and CACC119 can positively impact rice growth under drought stress. The array of beneficial traits encoded in their genomes supports their potential as effective PGPR, capable of not only promoting growth but also enhancing stress resilience. Future research should focus on validating these genomic predictions in practical field settings, particularly under varied stress conditions, to fully elucidate their roles and optimize their application for crop improvement.

The PGPR have long been recognized for their beneficial effects on plant growth under optimal conditions. However, their efficacy can be complex when plants face different types of stress. Recent studies suggest that while PGPR can significantly enhance plant growth, their benefits might be context-dependent, especially under stress conditions. For instance, under deficit stresses such as drought, the growth-promoting effects of PGPR might be counterproductive as they could divert the plant's energy away from crucial stress responses, leading to potential negative outcomes (Glick, 2012; Vurukonda et al., 2016). Conversely, under cumulative stresses, such as salinity, PGPR might help alleviate some of the stress impacts through mechanisms such as dilution effects, which can help reduce the severity of stress impacts on plant growth (Kumar et al., 2023). In the present study, the strains CACC109 and CACC119 demonstrated positive effects on rice plants under drought stress (Figures 3, 4). This finding is consistent with the notion that some PGPR strains can enhance plant resilience to specific stress conditions. However, the response of these strains to cumulative stresses, including combinations of drought and salinity, remains unexplored. Further research is warranted to understand how these PGPR strains perform under such combined stresses. This would provide a comprehensive view of their functional capabilities and help differentiate between mere growth promotion and genuine alleviation of stress-induced growth inhibition, offering deeper insights into the interplay between PGPR and environmental stressors.

While this study offers valuable insights into the potential of CACC109 and CACC119 as PGPR for promoting plant growth and enhancing drought tolerance in rice plants, the limitations of the present study should be acknowledged. Firstly, the study focused only on rice plants, and the efficacy of these PGPR strains may vary across different plant species or even across cultivars of the same species. Additionally, the experimental conditions may not accurately replicate real agricultural environments, potentially affecting the generalizability of the findings. Moreover, the mechanisms underlying the observed effects of the PGPR strains have not been fully elucidated, indicating the need for further research to comprehensively understand their mode of action. Furthermore, the long-term effects of PGPR inoculation on soil

health and ecosystem dynamics were not investigated in this study, underscoring the necessity for future studies to assess the broader ecological implications of PGPR use in agriculture.

5 Conclusion

This study aimed to identify novel PGPR strains that can promote plant growth and alleviate drought stress. *B. megaterium* strains CACC109 and CACC119 displayed various PGP activities. They significantly enhanced rice seed germination under osmotic stress and promoted root growth under stressed and non-stressed conditions. Additionally, these strains improved overall plant growth parameters, such as shoot and root lengths and weights, and leaf widths, while also enhancing plant physiological characteristics, such as chlorophyll levels and osmolyte production. Notably, the rice plants treated with CACC109 and CACC119 exhibited enhanced drought tolerance, as evidenced by their higher survival rates, increased chlorophyll and proline contents, reduced water loss rates, and upregulated expression of antioxidant-related and drought-responsive genes. Overall, this study provides valuable insights into the beneficial effects of PGPR on plant growth and their response to environmental stressors, offering promising avenues for increasing crop productivity and mitigating the impacts of climate change on agriculture.

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

SL: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing, Resources. J-AK: Formal analysis, Investigation, Methodology, Validation, Resources, Writing – review & editing. JS: Investigation, Writing – review & editing. SC: Investigation, Writing – review & editing. GJ: Writing – review & editing. YK: Funding acquisition, Supervision, Validation, Writing – review & editing, Conceptualization, Resources.

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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/fmicb.2024.1430546/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Effects of CACC109 and CACC119 on rice shoot growth and root development. (A) Comparison of shoot growth and root development in

9-week-old mock-, CACC109-, and CACC119-treated rice plants grown under well-watered conditions. (B) Magnification of the root parts.

SUPPLEMENTARY FIGURE S2

Circular map of the genome and genome features circles of *B. megaterium* CACC109 (A) and CACC119 (B). From the outside to the inside, the outer black circle represents the scale line in Mbps; the second gray band represents contigs; the third and fourth circles represent the annotated reference genes (specifically, CDSs) found in the forward and reverse strands, respectively; the fifth circle displays only rRNA and tRNA found in the genome; the sixth circle displays the GC skew metric. The genomic mean GC-skew value is used as the baseline, relative to which higher-than-average values are displayed in green, whereas lower-than-average values are displayed in red; the seventh circle displays the GC ratio metric. GC ratio also uses the genomic mean GC ratio value as its baseline, with higher-than-average values in blue and lower-than-average values in yellow.

SUPPLEMENTARY FIGURE S3

Effects of CACC109 and CACC119 on soluble organic components. Comparison of soil nutrients (P_2O_5 , K^+ , and Ca^{2+}) between mock and PGPR (CACC109 and CACC119)-treated soils in pots. Data are presented as the mean $\pm$ SD of at least three independent samples. Different letters indicate significant differences ($P < 0.05$, Student's *t*-test). The experiment was performed three times.

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Relative multi-beneficial effect of MOs on plant health of chickpea (*Cicer arietinum* L. var. PG-186)

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The phosphate solubilizing properties of *Lysinibacillus macroides* ST-30, *Pseudomonas pelleroniana* N-26, and *Bacillus cereus* ST-6 were tested for the chickpea crop of the Tarai region of Uttarakhand. These microbially inoculated plants have shown significant ($p > 0.05$) improvement in the plant health and crop health parameters, viz., root length, shoot length, fresh weight, dry weight, nodule number, nodule fresh weight, nodule dry weight, chlorophyll content, and nitrate reductase. The highest shoot length (46.10 cm) and chlorophyll content (0.57 mg g^{-1} fresh weight) were observed in ST-30 at 75 DAS with $20 \text{ kg P}_2\text{O}_5/\text{ha}$. Similarly, for plant P content, an increase of 90.12% over control was recorded in the same treatment. Treatments consisting of *Lysinibacillus macroides* ST-30 along with $20 \text{ kg/ha P}_2\text{O}_5$ were found to be most suitable as phosphatic fertilizer. Conclusively, sustainable agriculture practices in the Tarai as well as the field region may be developed based on a strategy of exploring microbial inoculants from the pristine region of the Western Himalayas. The presence and abundance of bacterial inoculants were confirmed through qRT-PCR. We conclude that the effective plant growth-promoting bacterium *Lysinibacillus macroides* ST-30 broadens the spectrum of phosphate solubilizers available for field applications and might be used together with $20 \text{ Kg/ha P}_2\text{O}_5$.

KEYWORDS

chickpea, filed trial, phosphate solubilizers, RT-PCR, principal component analysis, Agricultural practices, Sustainable land management

1 Introduction

Chickpea (*Cicer arietinum*) is a significant staple crop, providing a valuable source of protein and essential nutrients for millions of people worldwide. Chickpea contains 18–22% protein, 52–70% carbohydrate, 4–10% fat, and a sufficient quantity of minerals, calcium, phosphorus, iron, and vitamins. In addition, it is also important for sustainable agriculture as it improves the physico-chemical and biological properties of the soil. It improves the soil structure due to deep roots and fixes approximately $25\text{--}30 \text{ kg N ha}^{-1}$ through symbiosis (Reddy and Reddy, 2005), minimizing dependency on chemical fertilizers. A sufficient supply of phosphorus to the plant increases the nodulation and fastens its maturity. Phosphorus as a

TABLE 1 Treatment detail in field trial.

No	Treatment	Details
1	T1	Uninoculated control (without PSB and P ₂ O ₅)
2	T2	Uninoculated +20 kg ha ⁻¹ of P ₂ O ₅
3	T3	Uninoculated +40 kg P ₂ O ₅ ha ⁻¹
4	T4	PSB ST-30 + 0 P ₂ O ₅
5	T5	PSB ST-30 + 20 kg ha ⁻¹ of P ₂ O
6	T6	PSB ST-30 + 40 kg ha ⁻¹ of P ₂ O
7	T7	PSB N-26 + 0 P ₂ O ₅
8	T8	PSB N-26 + 20 kg ha ⁻¹ P ₂ O ₅
9	T9	PSB N-26 + 40 kg ha ⁻¹ P ₂ O ₅
10	T10	PSB ST-06 + 0 P ₂ O ₅
11	T11	PSB ST-06 + 20 kg ha ⁻¹ P ₂ O ₅
12	T12	PSB ST-06 + 40 kg ha ⁻¹ P ₂ O ₅

micronutrient also helps in root and shoot development and improves photosynthesis and other physico-biochemical processes (Singh et al., 2018).

However, a substantial proportion of soil phosphorus remains inaccessible to plants due to its insoluble form. Phosphate-solubilizing bacteria (PSB) possess the unique ability to convert insoluble phosphorus into a soluble form, thus enhancing its bioavailability to plants. The most significant phosphate-solubilizing bacterial genera are reported, such as *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Microbacterium*, *Serratia*, *Rhizobium*, *Bradyrhizobium*, *Salmonella*, *Alcaligenes*, *Chromobacterium*, *Arthrobacter*, *Streptomyces*, *Thiobacillus*, and *Escherichia* (Zhu et al., 2011). As per acid production theory, phosphate solubilization by phosphate-solubilizing microorganisms (PSMs) takes place through the synthesis of various organic acids such as malic, succinic, glyoxalic, fumaric, α -keto butyric, tartaric, citric, oxalic, 2-keto gluconic, and gluconic acids. The release of organic acid acidifies the medium (Puente et al., 2004; Singh et al., 2012; Rani et al., 2013). Different microorganisms produce different types of organic acid. The organic acid that is released in culture filtrate reacts with the insoluble phosphate (Ahmed and Kibret, 2014).

Gluconic acid is the major acid secreted by P solubilizers and synthesized by a mechanism involving direct oxidation of glucose through two key proteins, namely membrane-bound quinoprotein and glucose dehydrogenase (GDH) (Kim et al., 1997; Patel et al., 2008). GDH requires pyrroloquinoline quinone (PQQ) as a cofactor, which is the product of a *pqq* operon comprised of six genes (*pqqA*, B, C, D, E, and F) in *Klebsiella pneumonia*, *Enterobacter intermedium* 60-2G, and *Rahnella aquatilis* (Kim et al., 1998, 2003). PQQ is essential for the formation of holoenzymes, which lead to the production of gluconic acid from glucose. Han et al. (2008) have revealed a lack of 2-keto gluconic acid production through GA oxidation by the enzyme gluconic acid dehydrogenase due to the inactivation of *pqq* genes in *Enterobacter intermedium* 60-2G. Polymerase chain reaction (PCR) studies were conducted in the *S. marcescens* CTM 50650 strain (Farhat et al., 2009) to check the presence of genes involved in the expression of mucopolysaccharides (MPS) via the activation of the direct oxidation pathway of glucose (GDH encoded by the *gdh* gene and pyrroloquinoline quinone (*pqq*) genes involved in the biosynthesis of the required PQQ

cofactor). This capacity makes PSB a promising candidate for improving nutrient uptake efficiency and subsequently influencing the overall productivity of crops such as chickpeas. Looking into the importance of legume crops for soil health management and their requirement for phosphate, an experiment was designed to optimize the level of P fertilizers along with PSB. This research aims to provide insight into the impact of P solubilizers on the health and nodulation efficiency of chickpeas, which are responsible for nitrogen fixation by the plant. The nitrogen-fixing potential was also measured through the nitrate reductase test of the plant. The dominance of the inoculated bacterium was also confirmed throughout the experiment through quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

2 Materials and methods

2.1 Strain and culture condition

Three strains, namely ST-30 (*Lysinibacillus macrolides*; accession No. KX396054), ST-06 (*Bacillus cereus*; accession No. MF496242), and N-26 (*P. pelleroniana*; accession No. JN055435), were used for the field study. Phosphate solubilizer and diazotrophic isolate, strain N-26, originally isolated from the soil of the Nainital region (2,084 m, 29.38°N 79.45°E) of Uttarakhand, was obtained from departmental culture collection and used in this study (Tomer et al., 2017).

2.2 Plant growth promotion studies under field conditions on chickpea (*Cicer arietinum*, PG-186)

The field trial was performed in Pantnagar (altitude of 343.84 m above mean sea level, 29°N latitude and 79.3°E longitudes), which falls under the foothills of “Shivalik” ranges of “Himalaya” a narrow belt called “Tarai.” Field Trial The experiments on chickpea (*Cicer arietinum*, PG-186) were conducted between November and April (2015–2016) using a randomized block design (RBD) at the Norman E. Borlough Crop Research Centre of G.B. Pant University of Agriculture and Technology, Pantnagar, Dist. Udham Singh Nagar (Uttarakhand). The growth promotory efficiency and P solubilization potential of these selected bacterial strains were checked under natural field conditions. Seeds of chickpea (PG-186) were surface sterilized prior to sowing (Goel et al., 2002). The seeds were sown on 26 November 2015, and the crop was harvested on 13 April 2016. There were a total of 12 treatments (T1–T12) (Table 1).

Thirty-six plots of 8.4 m² were made. All analyses were carried out in triplicate. After sowing, agronomical plant parameters (root length, shoot length, fresh weight, dry weight, nodule number, nodule fresh weight, and nodule dry weight), leaf nitrate reductase activity (Hageman and Hucklesby, 1971), total leaf chlorophyll content (Hiscox and Israelstam, 1979), and total plant P content (Jackson, 1962) were checked at 40, 60, and 75 DAS. Shoot length and root length were measured by fully expanding the leaves and roots. The fresh weight of the plant, nodule number, and nodule fresh weight were weighed immediately after harvesting, while dry weight was measured after oven drying the samples at 50°C for 10 days to get a

constant weight. Simultaneously, rhizospheric soil samples were collected for bacterial community analysis by sterile spatula in sterile polythene bags and transported to the laboratory aseptically in cold conditions. Each soil sample was collected in triplicates, which were later mixed to make a single bacterial composition per treatment.

2.3 Statistical analysis

All the data from the field trial study was statistically analyzed using the statistical program analysis of variance (ANOVA) and general linear model procedure (SPSS, ver. 16.0) to reveal the significant effect of different treatments. Duncan's multiple range test (DMRT) was applied to determine the difference between individual events at a significant difference ($p \leq 0.05$). Principal component analysis was performed with the help of JMP 9 software.

2.4 Bacterial community analysis through real-time PCR (q PCR) analysis

The effect of PSBs on native microflora was assessed using qRT-PCR. The rhizospheric soil sample (≤ 15 cm) was collected from the rhizosphere of chickpea from each plot and pooled for DNA isolation, as previously described in the article.

The copy number of 16S rDNA from the collected soil samples was quantified using the primer set 16S F/R (5'-CCTACGGGAGG CAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3') with the iCycler iQ™ Multicolor (Bio-Rad Lab, Hercules, CA, USA) instrument, employing SYBR green chemistry (Kumar et al., 2014).

3 Results

3.1 Impact of P solubilizers on shoot and root growth

A significant increase in shoot length was observed in bio-inoculant-treated plants over the control. The highest shoot length was observed in ST-30 (46.10 cm) at 75 DAS with 20 Kg P_2O_5 /ha which was even greater than ST-30 with 20 Kg P_2O_5 /ha and almost similar to ST-30. Similarly, in the case of P-06 and N-26 treatments, no significant effect of phosphate fertilizer was observed (Figures 1A,C, 2).

Similarly, maximum root length was observed in ST-30 along with 20 Kg P_2O_5 /ha at different sampling stages, which was approximately 50% higher than the control. The treatment of S-06 and N-26 almost nullified the need for P fertilizers in terms of root length, as similar results were obtained in treatments that were inoculated without any chemical fertilizers and another treatment which was inoculated along with 20 and 40 kg ha^{-1} of chemical fertilizers (Figures 1B–D, 2).

3.2. Fresh weight and dry weight

A significant increase in fresh weight was observed in bio-inoculant-treated plants over their control. The highest fresh weight of 8.83 g was recorded in a treatment of ST-30 with 20 Kg P_2O_5 /ha. The highest recorded fresh weight growth is 65.73% in terms of percentage increase over control at 40 DAS (Figure 1C). A similar pattern was observed in 60 and 75 DAS, respectively. The pattern of

fresh weight was followed in dry weight, where maximum dry weight was again obtained in ST-30 with 20 Kg P_2O_5 /ha at 40 (1.85 g), 60(3.81 g), and 75 (5.21 g) DAS (Figure 1D).

3.2 Effect of P solubilizers on nodules of chickpea

Maximum numbers of nodules were observed in ST-30 along with 20 Kg P_2O_5 /ha at 60DAS which decreased at 75DAS which shows the degeneration of nodules after 60 days. The increase in nodulation was about more than 100% with respect to control. A significant increase in nodulation was observed after the inoculation of different PSBs. This shows the role of phosphate metabolism in the nodulation efficiency of legumes. A similar trend was also observed in nodule fresh weight and dry weight. A significant decline in nodule count, fresh weight, and dry weight was recorded at 75 DAS, showing degeneration of nodules after a certain time. The results also correlated with shoot length and root length (Figures 2A–C). The three bacteria, individually and in consortium, showed more vigor in terms of nodulation and nitrogen content than non-inoculated plants (Figure 2D).

3.3 Leaf chlorophyll content and nitrate reductase activity

Plant chlorophyll and nitrate reductase activity were studied to show plant growth promotion by bio-inoculants. At 40 DAS, the chlorophyll content of absolute control was measured as 2.12 mg g^{-1} fresh weight, while the highest chlorophyll content of 2.57 mg g^{-1} fresh weight was recorded in ST-30 along with 20 Kg P_2O_5 /ha. This is 38.76% in terms of percentage increase over control. This is followed by treatment having ST-30 with 40 Kg P_2O_5 /ha (33.55% increase over control), which was at par with ST-06 with 40 Kg P_2O_5 /ha (28.77% increase over control). Chlorophyll content increased when chemical fertilizer over the bio-inoculum was provided up to 20 kg ha^{-1} but did not show a significant increase and even sometimes decreased at 40 kg ha^{-1} (Figure 3). A decrease in chlorophyll content was also recorded at 75 DAS, which shows the maturation phase of the crop.

Nitrate reductase, the first in a series of enzymes that reduce nitrate to ammonia, is sensitive to a number of environmental factors (McAllister et al., 2012). Activity varies under the light intensity influence, carbon dioxide levels, temperature variation, H_2O availability, and nitrate availability (Fageria and Baligar, 2005). Croy and Hageman (1970) took it as a biochemical marker for prediction yield and protein concentration, which was later followed by Zhang et al. (2015) and others. Further nitrogen (N) uptake by crops can be controlled by their growth rate and can vary according to the requirement of nitrogen incorporation in plants at a particular growth period (Barret et al., 2000). Maximum activity was recorded in PSB N-26 + 40 kg P_2O_5 ha^{-1} (0.75 μ mol NO_2 g^{-1} fresh weight) at all the sampling intervals, followed by PSB N-26 + 20 kg P_2O_5 ha^{-1} (0.73 μ mol NO_2 g^{-1} fresh weight) at 40 DAS, which was 1.56 and 1.55 at 60DAS and 1.47 and 1.46 at 75DAS, respectively, for PSB N-26 + 40 kg P_2O_5 ha^{-1} and PSB N-26 + 20 kg P_2O_5 ha^{-1} , which was more than 100% increase w.r.t control (Figure 3).

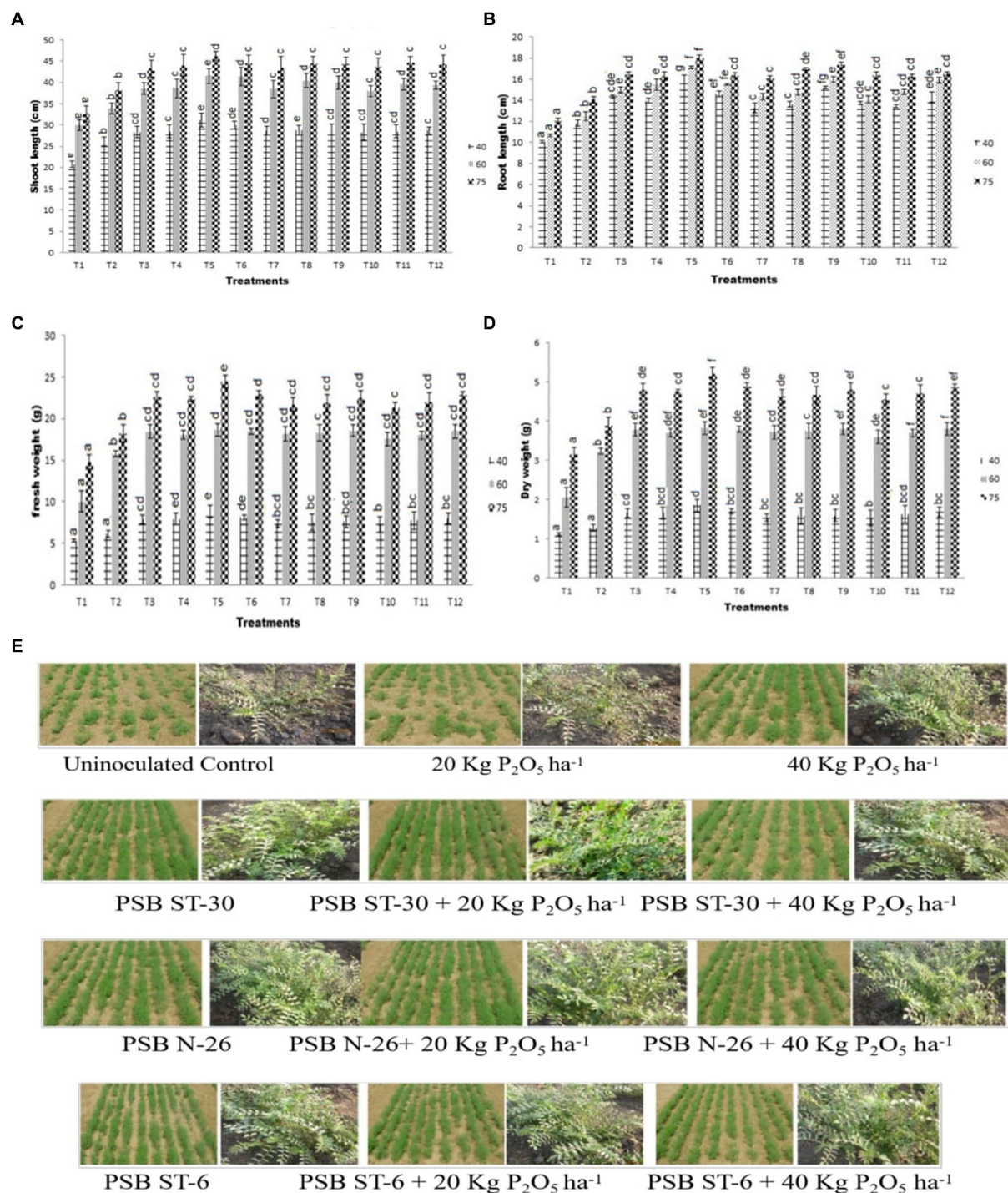


FIGURE 1

Effect of PSB (ST-30, N-26, and ST-6) on (A) shoot length (cm) and (B) root length (cm) at 40, 60, and 75 DAS, respectively, (C) effect of PSB (ST-30, N-26, and ST-6) on (A) fresh weight (g) and (D) dry weight at 40, 60, and 75 DAS, respectively, and (E) comparative effect of PSB ST-30, N-26, and ST-6 on plant health of *Cicer arietinum* L. var. PG-186 at 40 DAS.

Nitrate reductase assay was performed to study the assimilation of nitrate in plants, whereas chlorophyll content was measured to monitor plant health. Plant nitrate reductase activity was found to have a positive correlation with soil nitrate concentration. Leaf nitrate reductase and chlorophyll content both increased in the plants treated with bio-inoculums (ST-30, N-26, and ST-6) when compared to

uninoculated absolute control and treatments containing only chemical fertilizers. The temporal pattern of increase in leaf nitrate reductase and chlorophyll content in treatment plants at different intervals was 40 < 60 > 75 (DAS) in all crops with maximum nitrate reductase and chlorophyll content at 60 DAS. Control plants also followed the same trend.

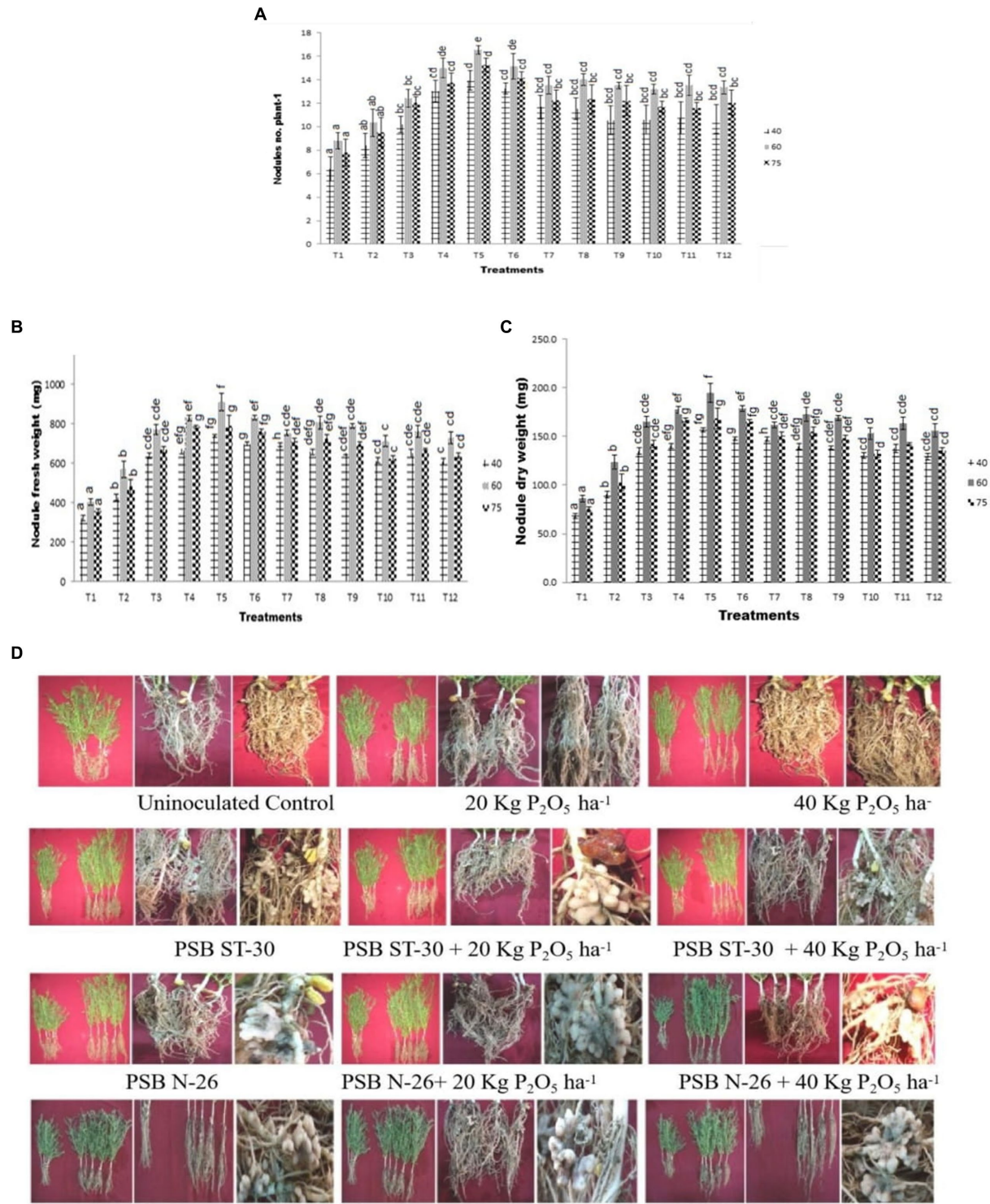


FIGURE 2
Effect of PSB (ST-30, N-26, and ST-6) on (A) nodule count plant⁻¹, (B) nodule fresh weight (g), and (C) nodule dry weight (g) at 40, 60, and 75 DAS, respectively, (D) comparative effect of PSB viz. ST-30, N-26, and ST-6 on shoot length, root length, and nodulation of *Cicer arietinum* L. var. PG-186 under different treatments at 60 DAS.

3.4 Principal component analysis

The PCA analysis shows a positive correlation of all the treatments with different parameters (chlorophyll content, nitrate reductase

activity, fresh weight and dry weight of the plant, nodule fresh and dry weight, nodule number, and shoot and root length) except T1 [uninoculated control (without PSB and P₂O₅)] and T2 (uninoculated +20 kg ha⁻¹ of P₂O₅), which did not show any correlation with

treatments (Figure 4). This trend was observed in different time intervals, i.e., 40 days, 60 days, and 75 days. All the other treatments had a positive correlation with different parameters, showing the positive impact of all other treatments on different parameters. Similarly, Rhodes et al. (2014) also evaluated green manure crops for the management of nematodes in soil through PCA analysis.

3.5 Plant P content

Significantly, the highest plant P content of 5.77 mg g^{-1} dry weight was recorded in ST-30 along with $20 \text{ Kg P}_2\text{O}_5/\text{ha}$ which was a 90.12% increase over control. This is followed by treatment having ST-30 and $40 \text{ Kg P}_2\text{O}_5/\text{ha}$ with 87.92% (5.70 mg g^{-1} dry weight) increase over absolute control, which is at par with treatment having ST-06 with $40 \text{ Kg P}_2\text{O}_5/\text{ha}$ with 69.24% increase over control at 40DAS (Figure 5). Treatments containing N-26 with a blend of $20 \text{ Kg P}_2\text{O}_5/\text{ha}$ and $40 \text{ Kg P}_2\text{O}_5/\text{ha}$ have shown a 56.05 and 62.65% increase over control,

respectively. Similarly, treatments consisting of ST-06 with the blend of $20 \text{ Kg P}_2\text{O}_5/\text{ha}$ showed a 65.94% increase over control.

A similar trend was observed at 60 DAS, where the highest plant P content of 7.10 mg g^{-1} dry weight was recorded in ST-30 along with $20 \text{ Kg P}_2\text{O}_5/\text{ha}$. This is 111.73% in terms of percentage increase over control, followed by treatment having ST-30 and $40 \text{ Kg P}_2\text{O}_5/\text{ha}$ with 106.76% increase over absolute control, which is at par with treatment having N-26 with $40 \text{ Kg P}_2\text{O}_5/\text{ha}$ with 85.89% increase over control with recorded plant P content 6.23 mg g^{-1} dry weight. The trend was followed even at 75 DAS.

3.6 Monitoring of bacterial community for the diversity and occurrence of inoculated strain

Quantification of soil samples showed that soil treated with ST-30 had a maximum of 16S rRNA gene copy no. at every stage of

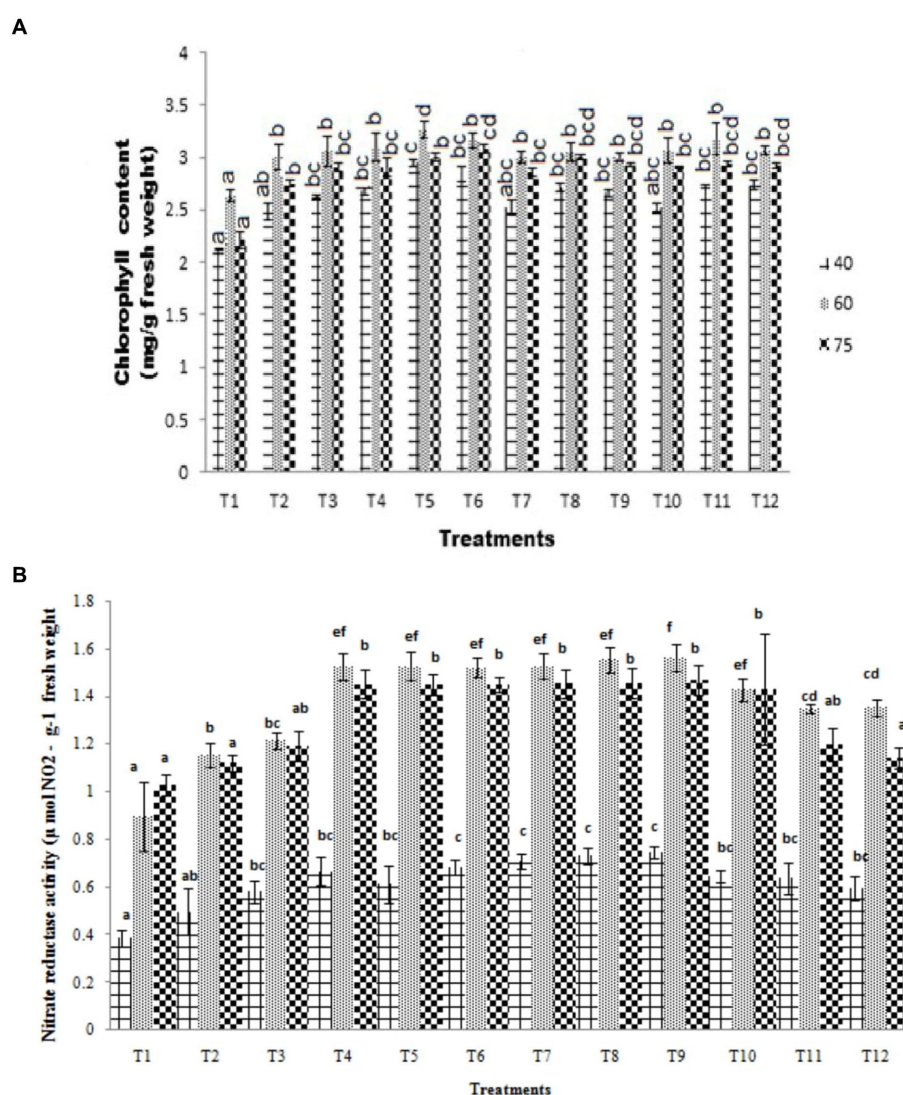


FIGURE 3

(A) Effect of PSB (ST-30, N-26, and ST-6) on the chlorophyll content (mg g^{-1} fresh weight) at 40, 60, and 75 DAS, respectively. (B) Effect of PSB (ST-30, N-26, and ST-6) on nitrate reductase activity ($\mu \text{mol NO}_2^- \text{ g}^{-1}$ fresh weight) at 40, 60, and 75 DAS, respectively.

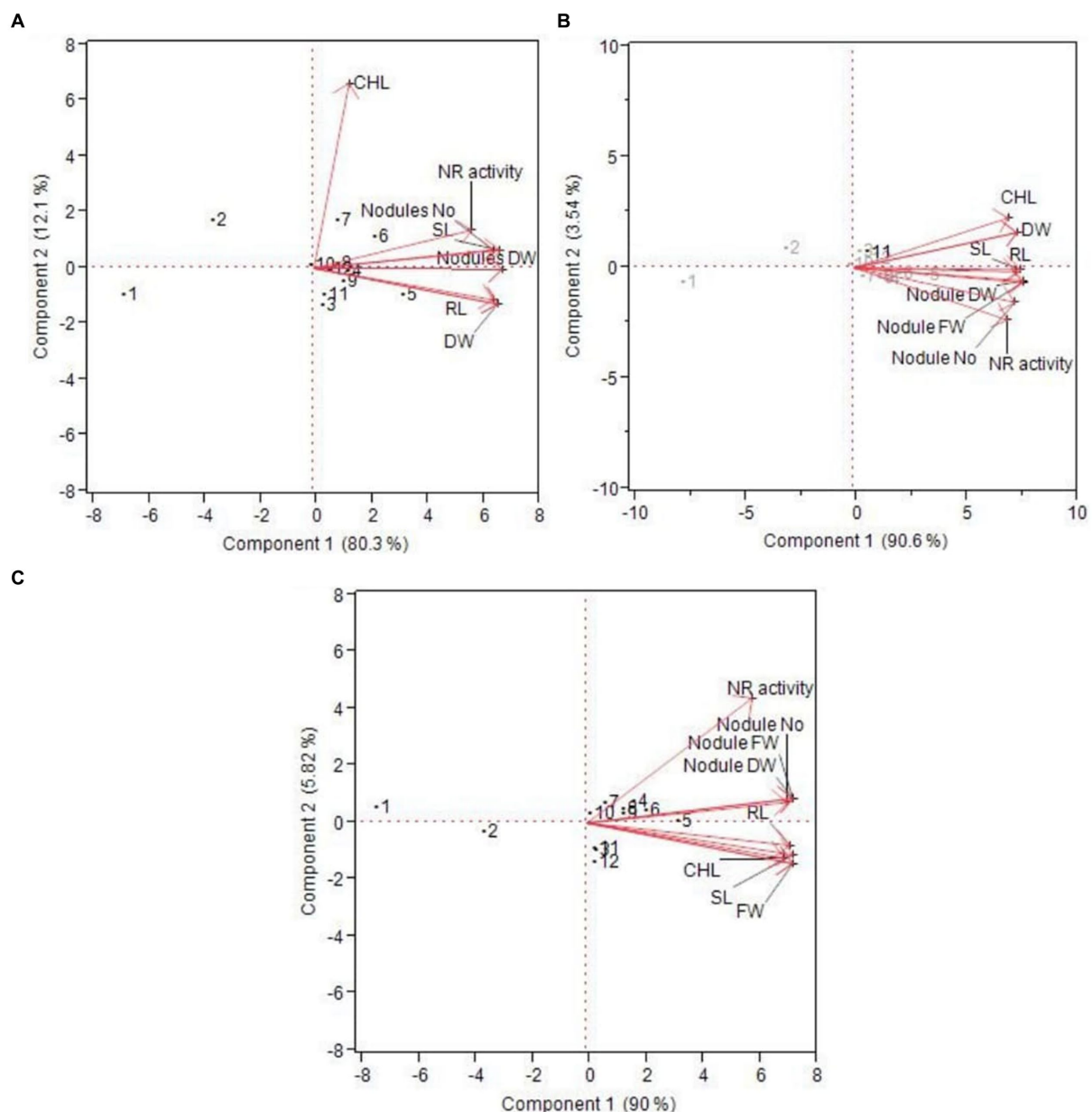


FIGURE 4
Principal component analysis of different plant health parameters at (A) 40 days, (B) 60 days, and (C) 75 days of treatments.

sampling. Treatment having ST-30 as PSB has 6.26×10^9 (Croy and Hageman, 1970), 9.41×10^9 , and 4.77×10^{10} at 40, 60, and 75 DAS, respectively (Table 2). The results of total bacterial abundance in soil at different times are shown in Figure 6. Quantification showed a linear relation ($R^2 = 0.993$) between the log value of bacterial genomic DNA and real-time PCR threshold cycles over the range of examined DNA concentrations. Soil samples of treatment consisting of 20 Kg P_2O_5 ha $^{-1}$ and 40 Kg P_2O_5 ha $^{-1}$ had the lowest 16S ribosomal gene copy no. at all stages of sampling. Furthermore, all PSB-treated treatments had remarkably higher 16S rRNA gene copy numbers than uninoculated control and only chemical fertilizer-treated treatments.

4 Discussion

The results show the positive implications of microbial inoculants on plant health, which is ultimately related to its vigor and productivity. A similar study was also conducted by Rajwar et al. (2018) using psychrotolerant *Pseudomonas jesenii* MP1 and *Acinetobacter* sp. ST02 against chickpeas. They obtained promising results in terms of plants' agronomical and biochemical properties. The positive applications of PGPR on plant fresh and dry weights are multifaceted, encompassing improved nutrient uptake, induced systemic resistance, auxin production, enhanced water use efficiency, stress tolerance, and increased photosynthetic efficiency. The effect of

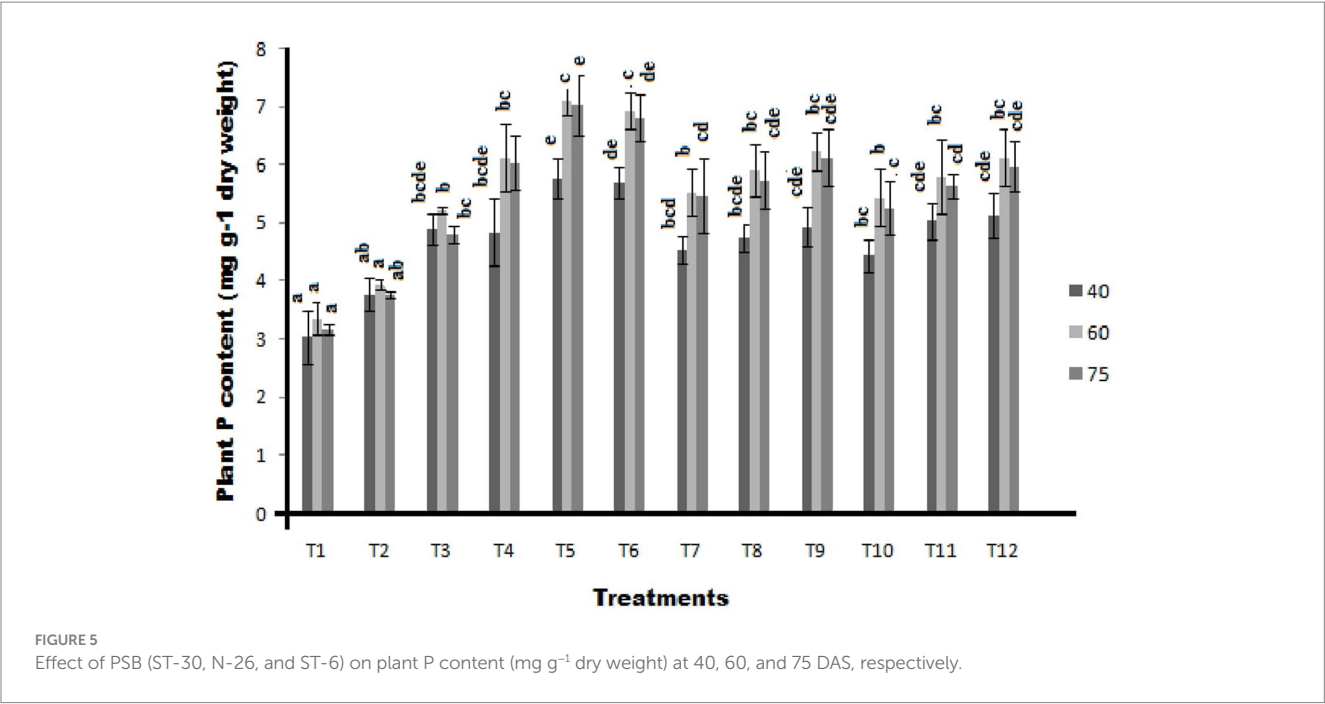


TABLE 2 Quantification of the 16 S r RNA gene from different treatment soils at different time intervals using real-time PCR.

	Copy no. of ribosomal genes at		
	40 DAS ^a	60 DAS ^a	75DAS ^a
Presowing soil	2.21 × 10 ⁹		
Uninoculated control	3.41 × 10 ⁹	3.90 × 10 ⁹	5.51 × 10 ⁹
20Kg P ₂ O ₅ ha ⁻¹	2.20 × 10 ⁸	7.10 × 10 ⁸	4.25 × 10 ⁹
40Kg P ₂ O ₅ ha ⁻¹	1.21 × 10 ⁸	7.41 × 10 ⁸	9.51 × 10 ⁸
ST-30	6.26 × 10 ⁹	9.41 × 10 ⁹	4.77 × 10 ¹⁰
ST-30 + 20Kg P ₂ O ₅ ha ⁻¹	4.10 × 10 ⁹	8.27 × 10 ⁹	2.78 × 10 ¹⁰
ST-30 + 40Kg P ₂ O ₅ ha ⁻¹	9.71 × 10 ⁸	3.53 × 10 ⁹	1.58 × 10 ¹⁰
N-26	4.20 × 10 ⁹	7.59 × 10 ⁹	2.51 × 10 ¹⁰
N-26 + 20Kg P ₂ O ₅ ha ⁻¹	4.21 × 10 ⁹	7.417 × 10 ⁹	2.54 × 10 ¹⁰
N-26 + 40Kg P ₂ O ₅ ha ⁻¹	3.24 × 10 ⁹	4.48 × 10 ⁹	9.54 × 10 ⁹
ST-6	5.91 × 10 ⁹	6.40 × 10 ⁹	9.51 × 10 ⁹
ST-6 + 20Kg P ₂ O ₅ ha ⁻¹	3.45 × 10 ⁹	5.45 × 10 ⁹	8.54 × 10 ⁹
ST-6 + 40Kg P ₂ O ₅ ha ⁻¹	9.67 × 10 ⁸	2.40 × 10 ⁹	7.54 × 10 ⁹

^aThe formula used for copy no. calculation is: number of copies = (amount × N/length × 1 × 10⁹/m).
Where m = average weight of a base pair (bp) is 650 Daltons, N = Avogadro's number (6.022 × 10²³ molecules/mole).

PGPR on growth, nodulation, and nutrient accumulation in lentils was also studied by Zafar et al. (2012). They proposed the application of PGPR for the growth and nutrient accumulation of crops as an alternative to chemical fertilizers. The nodulation efficiency was improved after the application of P solubilizers, as pulses are observed to be heavy P feeders, which are required for nodulation and hence

the growth of pulses. Flores-Duarte et al. (2022) also studied the impact of nodulation-enhancing rhizobacteria on *Medicago sativa*. They studied the impact of three strains, viz., *Pseudomonas* sp. L1, *Chryseobacterium soli* L2, and *Priestia megaterium* L3, on *Medicago sativa* under greenhouse conditions.

The highest chlorophyll content was observed when chemical fertilizer (up to 20 kg ha⁻¹) over the bio-inoculum was provided but did not show a significant increase at 40 kg ha⁻¹. Chlorophyll content is a measure of plant health as it is involved in photosynthesis (Shukla et al., 2015). Chlorophyll content in leaves also relies on phosphorous concentration, as it helps the plant survive under adverse conditions. Furthermore, its deficiency reduces plant protein and chlorophyll content. Phosphorous scarcity is also responsible for alternations in chlorophyll components, thus affecting absorption. However, the biochemical characteristics and biosynthesis of chlorophyll depend on the uptake of optimal phosphorous (Razaq et al., 2017). A noteworthy upsurge in the chlorophyll content was noticed in lettuces planted in soil introduced with superphosphate (Azzi et al., 2017). Similarly, NR activity was highest in 60 days, along with bacterial inoculation. These results revealed that the inoculation of bio-inoculums (ST-30, N-26, and ST-6) in soil facilitates plant nutrient uptake from the soil, which leads to better plant growth. Recently, Azzi et al. (2017) reported that phosphate fertilizer application in alkaline soil contaminated by cadmium boosted the NR activity in *Lactuca sativa*. Furthermore, diazotrophs are capable of altering nitrate and ammonium in plants, resulting in the induction of enzymes required for nitrogen metabolism (Dos Santos et al., 2017). The highest activity in the case of treatments having N-26 can be the reason for it, as it is a diazotroph too, besides PSB (Agarwal, 2013). Nevertheless, NR activity can be used as a parameter for nitrogen use efficiency in the selection of genotypes responsive to nitrogen fertilization or adapted to nitrate restriction (Sinha et al., 2015). Chen et al. (2016) revealed that phosphate-solubilizing bacteria help enrich the soil microbial communities by accelerating litter decay and nitrate release, thus increasing the available nitrate content in the soil, which is directly proportional to plant nitrate

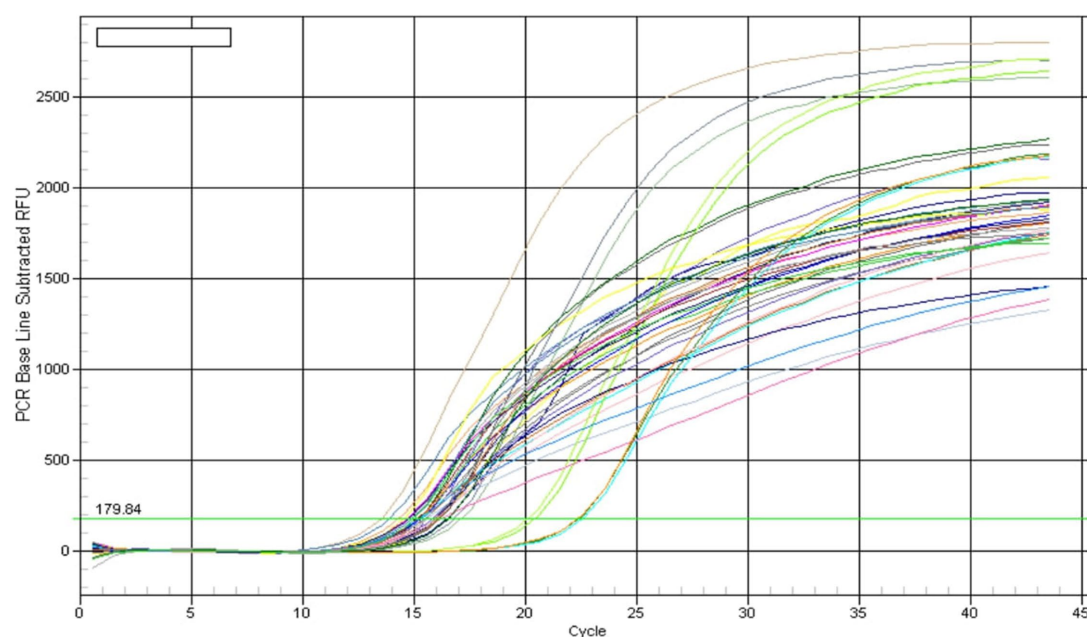


FIGURE 6
Amplification curve of real-time PCR of soil samples collected at 0, 40, 60, and 75 days after sowing, respectively.

reductase activity. Furthermore, NR activity can be used as a biochemical marker for predicting grain yield and grain protein production (Boulter, 2012; Krishnamoorthy et al., 2013).

The application of phosphorus seemed to have a direct effect on the P content of plant samples. In all plants, plant P content was higher than the uninoculated control. Bargaz et al. (2021) also studied the impact of PSB on the below ground performance of crops for their health and improved P acquisition from the soil. Stephan et al. (2015) also proved that PSB increases the soil phosphorus content, making it available for plant uptake. In all bioagents (ST-30, N-26, and ST-6) treated crops, plant P content was increasing with time up to 60 DAS but there after at 75 DAS it showed a slight declination. It revealed that plants have the highest 'P' demand in the young stage, at the time of flowering and nodule formation (Divito and Sadras, 2014). In a similar study, Bechtaoui et al. (2020) studied the impact of PGPR co-inoculation on bean production under varying phosphorous availability. They observed that regardless of the applied phosphorus source, co-inoculation of PGPR significantly increased biomass and phosphorus concentrations in plants as well as in pods. In another study by Zineb et al. (2019), inoculation of PSB was observed to improve the uptake and effectiveness of rock phosphate as a fertilizer.

The qPCR analysis also confirmed the dominance of microbial inoculants up to 60DAS, which is a good indicator of their activity in the field. The impact of bio-inoculant on soil microbial communities has been studied earlier using DGGE (Herrmann et al., 2012), qPCR (Babic et al., 2008), ribosomal intergenic spacer analysis "RISA" (Schumpp and Deakin, 2010), and other techniques.

5 Conclusion

Microorganisms play a pivotal role in the functioning of crops by influencing their physiology and development. Rhizosphere

microorganisms promote plant growth and protect plants from pathogen attack by different mechanisms. In this perspective, an exploration of cold-adaptive bacteria as representative candidates for plant growth promotion and nutrient acquisition was conducted and could be a better alternative for improved crop sustainability. The inoculated bacterial strains proved their potential as a better alternative to chemical phosphatic fertilizers in terms of plant growth, nodulation efficiency, and plant nutrient uptake. The plant biochemical parameters (chlorophyll and nitrate reductase tests) also correlate with agronomic data, which shows that bio-inoculants not only help growth promotion but also have properties that improve stress tolerance in host plants. The dominance of inoculated P solubilizers throughout the experiment shows they have the potential to fight the competition with native microflora and have a good shelf life in field conditions, advocating their application as commercial fertilizer for the future.

Data availability statement

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

Author contributions

ST: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. PK: Formal analysis, Software, Writing – original draft. DS: Formal analysis, Writing – review & editing. KP: Writing – review & editing. FK: Writing – review & editing. JB: Formal analysis, Writing – review & editing.

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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/fmicb.2024.1452553/full#supplementary-material>

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Isolation, identification, and mechanism analysis of plant growth-promoting rhizobacteria in tobacco

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Plant growth, crop yield, and pest and disease control are enhanced by PGPR (Plant growth promoting rhizobacteria), which are beneficial microorganisms found in a close symbiosis with plant roots. Phytohormones are secreted, nutrient uptake is improved, and soil properties along with the microbiological environment are regulated by these microorganisms, making them a significant focus in agricultural research. In this study, the efficient PGPR strain T1 was isolated and screened from tobacco inter-root soil, and identified and confirmed by ITS sequencing technology. Tobacco growth indicators and soil property changes were observed and recorded through potting experiments. The activities of key enzymes (e.g., sucrase, catalase, urease) in soil were further determined. High-throughput sequencing technology was utilized to sequence the soil microbial community, and combined with macro-genomics analysis, the effects of T1 strain on soil microbial diversity and metabolic pathways were explored. Following the application of T1, significant improvements were observed in the height, leaf length, and width of tobacco plants. Furthermore, the physical and chemical properties of the soil were notably enhanced, including a 26.26% increase in phosphorus availability. Additionally, the activities of key soil enzymes such as sucrase, catalase, and urease were significantly increased, indicating improved soil health and fertility. Comprehensive joint microbiomics and macrogenomics analyses revealed a substantial rise in the populations of beneficial soil microorganisms and an enhancement in metabolic pathways, including amino acid metabolism, synthesis, and production of secondary metabolites. These increase in beneficial microorganisms and the enhancement of their metabolic functions are crucial for plant growth and soil fertility. This study provides valuable references for the development of innovative microbial fertilizers and offers programs for the sustainable development of modern agriculture.

KEYWORDS

growth and development, microbiome and metagenomic analysis, plant growth-promoting rhizobacteria, tobacco, soil improvement

1 Introduction

Tobacco (*Nicotiana tabacum* L.), belonging to the *Solanaceae* family and *Nicotiana* genus, is an annual herb. As a crucial pillar industry for China's economic development, tobacco has increased overall productivity. In recent years, large quantities of chemical fertilizers and pesticides have been applied to pursue economic benefits (Durham and Mizik, 2021). Tobacco growth and soil physical and chemical properties have been significantly deteriorated. Recently, the enhancement of tobacco productivity primarily relies on the extensive use of chemical fertilizers and pesticides. This dependence on chemical inputs has damaged the soil's physical and chemical properties and microenvironment. Researchers have found that plant growth-promoting rhizobacteria (PGPR) can effectively promote plant growth and improve soil quality, thereby reducing the dependence of tobacco agriculture on chemical fertilizers and pesticides (Khatoun et al., 2020). The rhizosphere refers to a minute region around plant roots distinct in their physical, chemical, and biological properties due to the influence of root activities. Microorganisms colonizing this area are known as rhizosphere microorganisms (Odelade and Babalola, 2019). PGPR are microorganisms that can extensively colonize or survive in the soil at the plant root surface (Martínez-Viveros et al., 2010). Unlike phylloplane microbes, which are located on the surface of plant leaves and are directly exposed to the external environment, endophytes reside within the internal tissues of the plant. Rhizosphere microorganisms are primarily found in the soil surrounding plant roots, with their interactions with the plant being mediated through substances secreted by the roots (Pii et al., 2015).

Researchers have found that PGPR directly or indirectly promotes plant growth, suppressing plant diseases and enhancing stress resistance (Basu et al., 2021). Additionally, soil physicochemical properties and microbial structure improvements were observed (Vocciante et al., 2022). Previous research indicates that PGPR synthesizes and releases phytohormones, including IAA, cytokinin, and gibberellins, which enhance root secretion and bolster plant resistance to abiotic stresses (Deng et al., 2023; Ulrich et al., 2021).

Through the metabolism and physiological activities of PGPR, chelated minerals in the soil are activated, making previously inaccessible nutrients available for reuse and creating conditions conducive to plant growth and development (Ha et al., 2014). For example, *Ochrobactrum haematophilum*, isolated from the inter-root of sweet potato, was found through metabolome and transcriptome analyses to provide phosphorus to plants under phosphorus-deficient conditions by secreting significant quantities of organic acids, thus participating in the phosphorus solubilization process (Ding et al., 2021).

The resistance of wheat to arid and semi-arid environments was enhanced through the release of potassium by *Bacillus megaterium*, which facilitated the production of extracellular polysaccharides and phytohormones (Rashid et al., 2022). Additionally, the growth and metabolism of pathogenic bacteria in the soil inter-root were inhibited by PGPR by producing antimicrobial substances, leading to intracellular lysis (Hammami et al., 2013). Concurrently, beneficial bacteria in PGPR compete for environmental resources

such as nutrients, occupy favorable conditions for microbial growth and reproduction, and suppress the survival of harmful bacteria (Bhattacharyya and Jha, 2012).

Following the inter-root application of PGPG to plants, crop yields are increased, and modifications to the structure and function of the soil microbiota are induced (Ai et al., 2022). After applying PGPG, interactions occur among soil nutrients, enzymes, and microorganisms, enhancing the microecological conditions of the soil (Grover et al., 2021; Fiorentino et al., 2018). The conversion of soil nutrients is influenced, and as a result, microbial decomposition of soil organic matter (OM) leads to alterations in the content of effective N, P, and K in the soil and changes in soil enzymes. The effective soil nutrients and enzyme activities reflect the presence and activity of corresponding functional microorganisms, through which the microbial response mechanisms to environmental changes are evaluated (Nannipieri et al., 2002). Previous studies have shown that the community structure of indigenous soil microorganisms was altered to some extent by PGPR, and beneficial microorganisms capable of transforming soil nutrients were recruited and induced in the soil (Li et al., 2021). The co-application of PGPR and biochar enhanced soil sucrase activity, electrical conductivity (EC), and TK concentration. Significant reductions in catalase and superoxide dismutase activity and malondialdehyde levels were observed following the application of *Acinetobacter johnsonii*. Improvements in soil physiochemistry and the uptake of essential nutrients by maize were observed, and salt tolerance was conferred (Ren et al., 2021).

However, research on tobacco PGPR has been limited. Additionally, the effects of the interaction between tobacco and PGPR on the community and the composition of soil microorganisms are largely unknown. The microbiome includes microorganisms (bacteria, archaea, lower and higher eukaryotes, viruses), genomes, and surrounding environmental conditions (Marchesi and Ravel, 2015). The plant microbiome includes all microorganisms inhabiting the plant host, encompassing symbiotic, mutualistic symbioses, and harmful pathogens (Müller et al., 2016). From plant germination to maturation, the microbiome plays a critical role, interacting closely with the host (Berg et al., 2016). Potential diseases are warded off from plants by microorganisms that promote plant development (Pérez-García et al., 2011). Colonization sites for plant microorganisms have been classified into interleaf, inter-root, and endophytic microorganisms, with the diversity of inter-root microorganisms contributing to the stability of the soil ecosystem (Trivedi et al., 2020; Carrión et al., 2019). Therefore, studying the microbiome is crucial for understanding the mechanisms of action of PGPR on tobacco.

In addition, the market is dominated by bacterial bioagents, with fewer fungal bioagents available. Although significantly effective, bacteria are less stable and more susceptible to environmental influences (de Vries et al., 2018). Fungal bioagents are noted for their stronger environmental adaptability than bacterial bioagents, surviving under a broader range of conditions (Anwar and Shahnaz, 2022). Multiple biological control mechanisms, including the production of antibiotics, parasitic effects, and induction of plant resistance, are possessed by fungal

TABLE 1 Soil and strain tested.

S. No	Parameters	Experimental result
Soil samples	Organic matter	42.54 g/kg
	Alkaline hydrolyzable nitrogen	53.97 mg/kg
	Available phosphorus	45.71 mg/kg
	Available potassium	178.5 mg/kg
	pH	6.40
Strain		
	T1	<i>Trichoderma harzianum</i>

bioagents, which are less likely to develop resistance (Admassie et al., 2019). Additionally, forming spores or other survival structures in the soil by fungal formulations provides a long-term effect. Consequently, the study of fungi-centered bioagents is particularly important.

This study successfully screened the highly effective PGPR *Trichoderma harzianum* from inter-root soil and applied it to tobacco soil. The effect of PGPR on tobacco growth was evaluated by measuring tobacco plants' growth indexes and root vigor. Further, soil enzyme activity and effective nutrients were measured by PGPR to evaluate the improvement of tobacco soil. The mechanism of PGPR on tobacco growth and development and soil improvement was deeply investigated by combining the joint analysis of the microbiome and macro genome. It provides a foundation and useful reference for developing new microbial fertilizers.

2 Materials and methods

2.1 Experimental materials

Variety for test: Use of baking tobacco variety K326.

Test soil: Continuous cropping soil, sourced from the tobacco station in Linqu, Weifang, with 4 years of continuous cropping, characterized by the following physicochemical properties: organic matter at 42.54 g/kg, alkaline hydrolyzable nitrogen at 53.97 mg/kg, available phosphorus at 45.71 mg/kg, available potassium at 178.5 mg/kg, and a pH of 6.40 (Table 1).

Experimental strains: The strains were screened from high-quality and healthy fields of soil and have probiotic properties such as phosphorus solubilization, potassium solubilization, IAA production, and cellulose reduction (Supplementary Table S2). The GenBank accession number(s) of nucleotide sequence(s) was PQ135054. The strains include: T1 was *Trichoderma harzianum*. Distilled water and commercial microbial preparation are designated as negative (CK1) and positive controls (CK2), respectively. The finished bacterial agent CK2 used the liquid microbial fertilizer "Infusion of Golden Liquid" provided by Fujian Sanmu Biotechnology Co., Ltd., the main component of which is the jelly-like *Bacillus* (Table 1).

2.2 Experimental design

The experiment was conducted in pots within the greenhouse of Dai Zong Campus, Shandong Agricultural University, featuring two control groups: negative control CK1 and positive control CK2. Bacterial suspensions were prepared by inoculating strains into LB (Luria-Bertani) broth. They were incubated in a shaker at 37°C and 180 rpm for 16 h to obtain the seed liquid, which was then adjusted to an OD600 of ~2.5. The diluted seed liquid was inoculated into 50 ml LB broth using a 2% inoculum rate for each 50 ml LB liquid medium. When tobacco seedlings in floating trays develop four cotyledons, select uniformly healthy plants for transplantation into pots. Each treatment receives the same dose of different microbial suspensions: dilute 1.5 ml of each suspension to 300 ml and irrigate the root zone of the seedlings. CK1 was added with an equal amount of water, CK2 was added with an equal amount of finished bacterial agent, and the experimental group was added with an equal amount of bacterial liquid. All the treatments were set up in six parallels.

2.3 Isolation and screening of functional bacteria in tobacco

Ten grams of healthy soil are taken and placed in a sterile Erlenmeyer flask, to which 90 mL of sterile water is added. The flask is placed in a constant temperature shaker and oscillates at 25°C and 200 rpm for 4 h. The resulting solution is diluted in a 10-fold serial dilution, and 100 µL of the diluted solution is spread evenly onto a PDA (Potato Dextrose Agar) plate. The inoculated PDA plates are incubated at 25°C in a constant temperature incubator for 48 h, and the growth of colonies is observed. Colonies with distinct morphologies are picked from the original plate and transferred to new PDA plates for further purification. The purified fungal colonies are suspended in sterile water containing 15% glycerol and stored at -80°C in an ultra-low temperature freezer.

2.4 Analysis of physiological indicators

According to "Tobacco Agronomic Traits Survey and Measurement Methods" (YC/T142-2010) (Dai et al., 2024), plant height, stem circumference, number of effective leaves, maximum leaf length, and leaf width of tobacco plants were measured at 5 d intervals after transplanting, and the maximum leaf area was calculated (leaf area = leaf length × leaf width × 0.6345). After 30 days, three representative plants per treatment were analyzed for dry matter accumulation by separating them into roots, stems, and leaves, measuring fresh weight before drying at 105°C for 30 min and then at 80°C until constant weight for dry weight recording. Root system metrics such as total length, surface area, diameter, and number of tips and branches were measured using a Microtek Phantom 9980XL scanner. Chlorophyll content was assessed every 5 days in the 3rd–4th cotyledons using a SPAD-502 meter.

2.5 Analysis of soil physical and chemical properties

After 30 d of growth, representative plants were selected for sampling, and three soil samples per treatment were mixed well to test the soil pH, alkaline hydrolyzable nitrogen (AN), available phosphorus (AP), available potassium (AK), and organic matter (OM) content. The pH meter was used to determine the soil pH, sodium bicarbonate extraction-molybdenum-antimony anti-spectrophotometric was used to determine AP (He et al., 2018), ammonium acetate-flame photometer was used to determine AK (Zhou et al., 2021), and alkaline dissolved nitrogen was used to determine AN (Nebbioso and Piccolo, 2011). The potassium dichromate volumetric method was used to determine OM (Zeyede, 2020).

2.6 Analysis of soil enzyme activity

Representative plants were selected for sampling after 30 d of tobacco plant growth, and three soil samples were taken from each treatment and mixed homogeneously using a kit (purchased from Beijing Prime Biological Co., Ltd.) to determine sucrase, urease, and catalase in the soil.

2.7 Extraction and functional analysis of soil microorganisms

2.7.1 Sequencing of 16SrRNA and ITS gene amplicons of inter-root soil microorganisms

Genomic DNA was extracted from rhizosphere soil samples using the Omega E.Z.N.ATM Mag-Bind Soil DNA Kit. PCR amplification targeted the V3–V4 regions of the 16S rRNA gene using the universal primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC), as well as the ITS1-ITS2 regions using the universal primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) (Coenye et al., 1999). The library size was verified by 2% agarose gel electrophoresis, and the library concentration was determined using a Qubit 4.0 fluorescence quantifier to ensure consistent long cluster results and high-quality sequencing data. Sequencing was conducted on the Illumina MiSeq platform.

2.7.2 Analysis of amplicon sequence processing and bioinformatics

Sequencing data were processed on the BioSignal Cloud Platform (<https://ngs.sangon.com/>). The process involved removing primer sequences, filtering out low-quality sequences, and splicing short pairs of reads from double-end sequencing into single sequences using Flash. These spliced reads were further refined to obtain clean reads. Sequences were restricted to $\geq 97\%$ similarity, and non-repetitive sequences were clustered into operational taxonomic units (OTUs) using UPARSE (version 7.0.1090). To assess α -diversity, indices such as Chao 1, Ace, Simpson, Shannon, Shannoneven, and Sobs were calculated using

Mothur software (Nossa et al., 2010). To visualize changes in microbial composition, principal component analysis (PCA) was conducted on treated samples using QIIME and R software (v. 3.5.3) (Abdi and Williams, 2010). Additionally, redundancy analysis (RDA) was conducted to identify environmental factors, with variance inflation factor (VIF) analysis used for screening (Chen et al., 2019).

2.7.3 Soil macro-genome sequencing

Genomic DNA was extracted from the inter-root soil samples using the OMEGA kit E.Z.N.ATM Mag-Bind Soil DNA Kit, and the DNA was quantitatively analyzed using a Qubit 4.0 fluorescence quantifier Ltd. for sequencing on the Illumina MiSeq sequencing platform to obtain a large amount of raw data. Megahit software was used to perform multi-sample hybrid splicing of clean reads; SPAdes software was used to perform hybrid splicing of unmatched reads, and fragments ≤ 500 pb were filtered for downstream analyses, such as statistics and subsequent gene prediction. DIAMOND was used to compare the gene set protein sequences with the KEGG database, to obtain the corresponding KO numbers of the sequences, and to count the abundance of each functional level of KEGG in each sample. The sequencing results were compared with the KEGG (<http://www.kegg.jp/kegg/>) database using DIAMOND software at default values to predict microbial metabolic functions.

2.8 Data analysis

The experimental data were counted and graphed using Microsoft Excel 2021 and Origin 2023 and analyzed for the significance of differences using Duncan's test with SPSS Statistics 25.0 software.

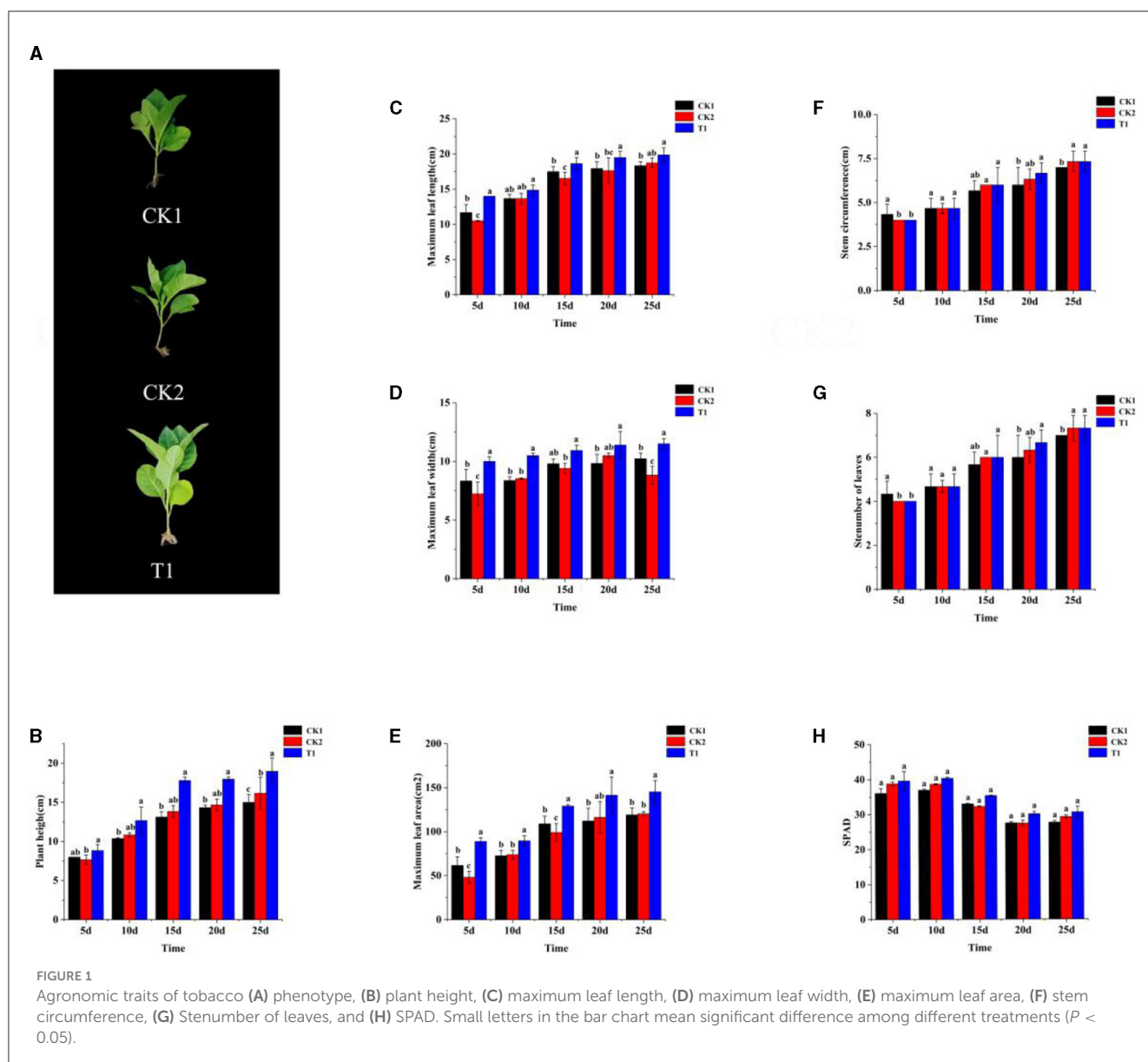
3 Results

3.1 Genetic characterization of the PGPR

Sequence comparison was conducted using the Blast tool in the NCBI database, and a phylogenetic tree was constructed using Mega11 software (Supplementary Figure S1). The analysis revealed that strain T1 resides on the same minimal branch as *Trichoderma harzianum*, indicating the closest evolutionary distance. Based on ITS genome sequence analysis and physiological and biochemical assessments, the strains were preliminarily identified as *Trichoderma harzianum*.

3.2 Effect of PGPR on agronomic traits of tobacco

To verify the growth-promoting effect of the PGPR on tobacco, the screened fungi were tested in pots. All treatments with microbial inoculum exhibited a pro-vigorant effect under continuous soil conditions compared to the CK. At 30 days after transplanting, tobacco phenotypes were assessed under four different application conditions, and all treatments with



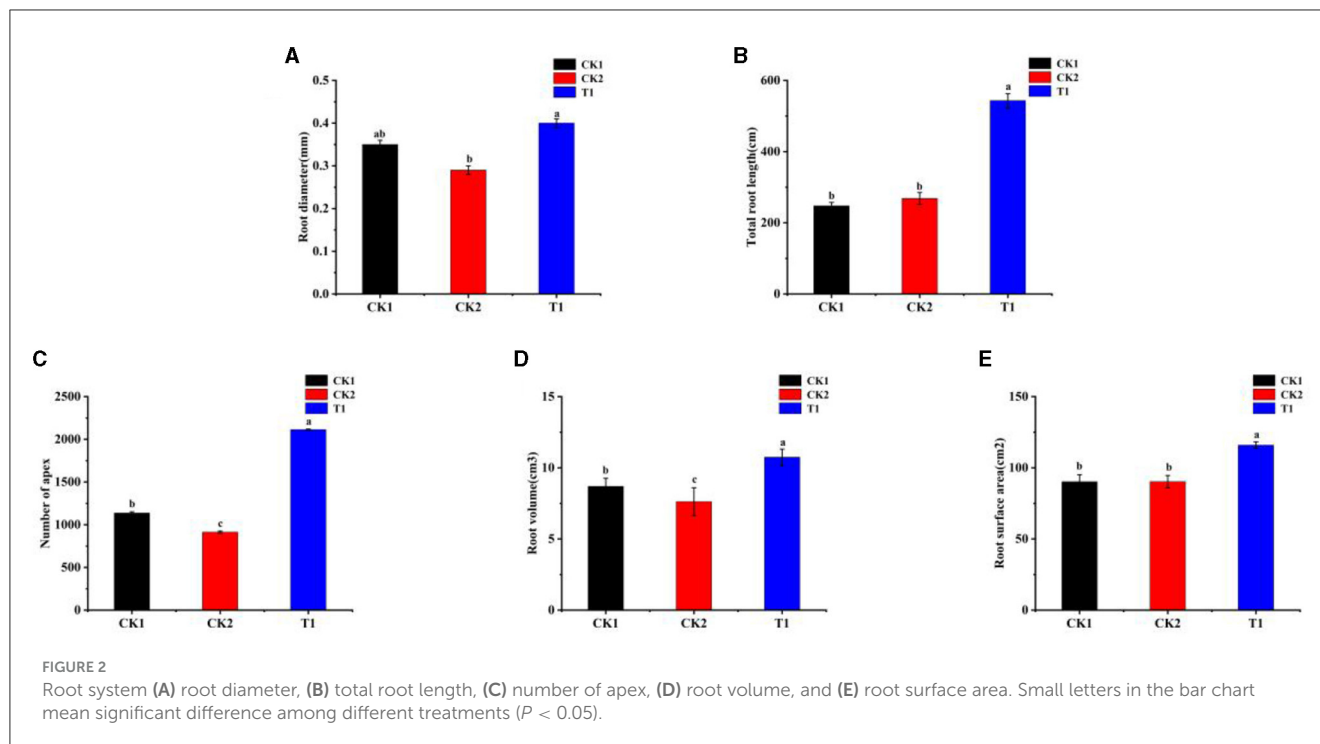
mycorrhizal fungi showed better growth (i.e., bigger and more leaves, taller plants) than CK1 (Figure 1A). Regarding leaf length, all microbial inoculum treatments promoted tobacco leaf length, with the T1 treatment significantly outperforming the other treatments throughout the growing period (Figure 1B). Moreover, 10 days after transplanting, plant height, leaf length, width, and maximum leaf area were significantly higher in the T1 treatment compared to the CK (Figures 1B–E). However, there were no significant differences between T1 and CK in terms of stem circumference and the number of effective leaves. While there were observable differences between treatments in stem circumference and the number of leaves, these differences did not reach a significant level (Figures 1F, G).

The effects of different microbial inoculums on SPAD values of baked tobacco leaves showed significant differences (Figure 1H). At 5 days after transplanting, SPAD values for all treatments with microbial inoculums were significantly higher than those for CK1, with the T1 treatment showing a particularly significant

difference compared to CK1. At 10 days after transplanting, the SPAD values for CK2 and T1 treatments showed a significant advantage, increasing by 5.62 and 7.96%, respectively, compared to CK1. Although the chlorophyll content in each treatment decreased 15 days after transplanting, the SPAD values for the T1 treatments remained significantly higher than those of CK1. By 20 days after transplanting, the SPAD value of T1 was significantly higher than all other treatments, with an increase of 6.15% compared to CK1, and there was no significant difference between CK1 and CK2. At 25 days after transplanting, the difference between T1 and CK1 remained significant.

3.3 Effect of PGPG on the root system of tobacco

During the seedling stage, the role of roots is considered far more significant than that of leaves. Initially, the survival and



early growth of the plant rely heavily on a robust root system, especially during the seedling stage, where roots form the basis for the absorption of water and nutrients (Jackson et al., 2007). A healthy root system effectively absorbs water and nutrients from the soil, providing the foundation for rapid plant growth (Fageria and Moreira, 2011). In contrast, although leaves are the primary sites for photosynthesis and the production of economic value in tobacco, their function is not fully utilized during the seedling stage. Therefore, the root parameters of tobacco were measured. There were significant differences in the effects of different treatments of PGPR on the growth and development status of tobacco roots. The difference between CK2 and CK1 was not significant. The results showed that the root diameter of T1 treatment was higher than that of CK1, which was 14.2% higher than that of CK1 (Figure 2A).

Regarding the total root length of the root system, the T1 treatments showed a significant advantage, which increased by 83.31% compared with CK1 (Figure 2B). For the number of root tips in the root system, T1 was increased by 67.20% compared with CK1 (Figure 2C). In addition, the T1 treatment had a significant advantage of 29.69% compared with CK1 (Figure 2D). Meanwhile, CK2 treatments were smaller than CK1 and reduced by 12.31%. Compared with CK1, root surface area was significantly increased in all treatments, and T1 treatment was significantly higher than other treatments and increased by 18.65% compared with CK1 (Figure 2E).

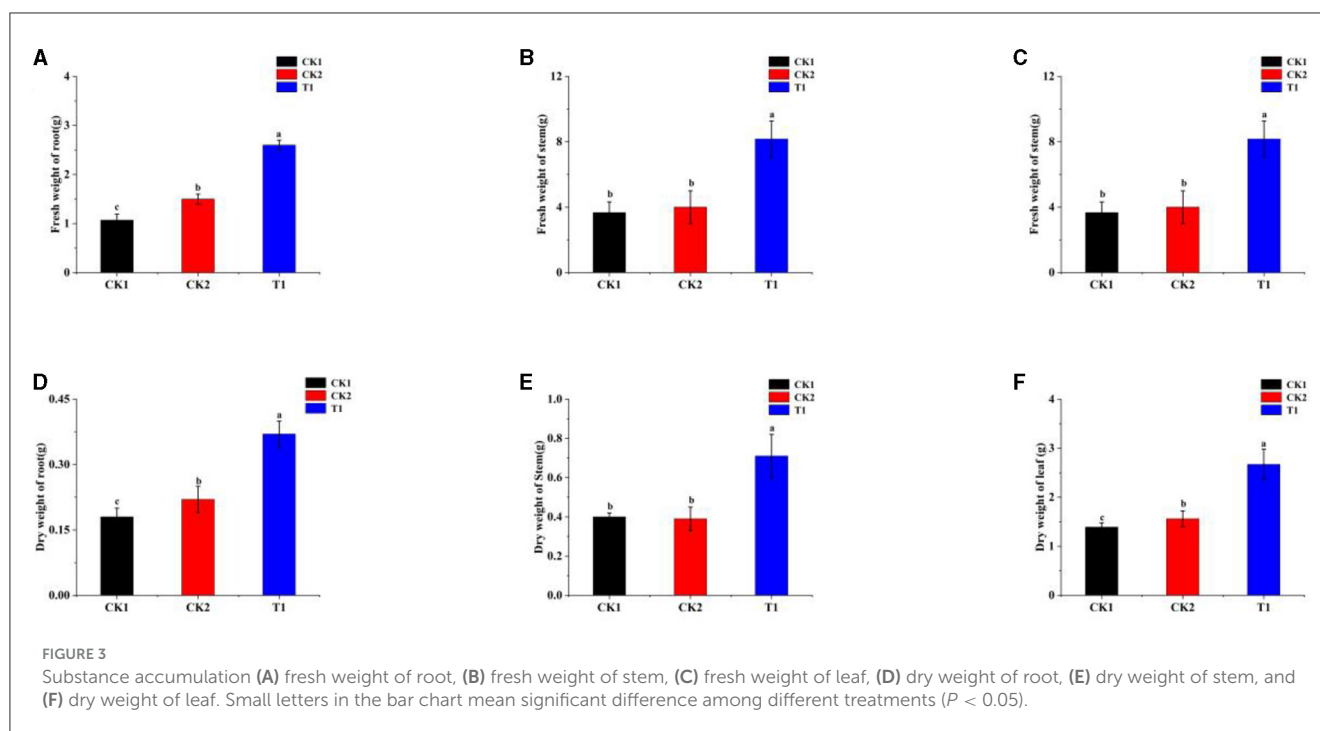
3.4 Effect of PGPR on tobacco biomass

The effects of different treatments on the accumulation of roasted tobacco substances showed significant differences. The T1 treatment was significantly superior to the other treatments for root fresh weight, with a 142.99% increase over CK1 (Figure 3A). T1

treatment showed a significant advantage for stem fresh weight, and all stem fresh weights of microbial-agent applied treatments were higher than that of control CK1 (Figure 3B). Regarding leaf fresh weight, the T1 treatment was particularly outstanding, with a 77.04% increase compared with CK1, which was significantly better than the other treatments (Figure 3C). In addition, the root dry weight of the T1 treatment also demonstrated a significant advantage with a 105.56% increase over CK1, significantly different from other treatments (Figure 3D). For stem dry weight, all treatments except CK2 treatment were higher than CK1, especially T1, which was 77.50% higher than CK1 (Figure 3E). T1 treatment also showed a significant difference in leaf dry weight compared with other treatments, increasing 92.09% compared with CK1, and all treatments had higher leaf dry weight than CK1 (Figure 3F).

3.5 Effect of PGPR on soil physicochemical properties and enzyme activities

Carbon, nitrogen, phosphorus levels, and soil enzyme activities are key indicators of soil fertility. To assess these factors, we investigated changes in soil properties and the activities of essential soil enzymes following the application of microbial inoculums. The measurements included dissolved alkaline nitrogen (AN), organic matter (OM), quick-acting phosphorus (AP), quick-acting potassium (AK), and soil pH. The results showed no significant difference in AN content between treatments (Figure 4A). However, the AP content in the T1 treatment was significantly higher than in other treatments, showing a 26.26% increase compared to the CK1 treatment (Figure 4B). While there was no significant difference in AK content across treatments, all treatments exhibited higher AK levels than CK1 (Figure 4C). The T1 treatment also significantly increased OM content in the soil by



14.48% compared to CK1, with a significant difference (Figure 4D). Regarding soil pH, all treatments led to a decrease in pH levels (Figure 4E).

Second, the activities of SU, CAT, and UR in the test soils were measured, revealing significant differences in enzyme activities among the treatments. All treatments involving the applied strain showed higher SU activity than CK1 (Figure 4F). Specifically, the SU activity in the T1 treatment was significantly higher than in other treatments, showing a remarkable increase of 140.13% compared to CK1. Although the effect of different treatments on CAT activity in the soil was not significantly different, the highest CAT activity was recorded in the T1 treatment, with an increase of 5.59% compared to CK1 (Figure 4G). UR activity in the soil was also significantly affected by the different inter-root biotrophic bacteria. Both CK2 and T1 showed higher UR activity content than CK1, with T1 significantly boosting UR activity by 40.25% compared to CK1 (Figure 4H).

3.6 Effect of PGPR on microbial diversity

3.6.1 Analysis of high-throughput sequencing results and out clustering

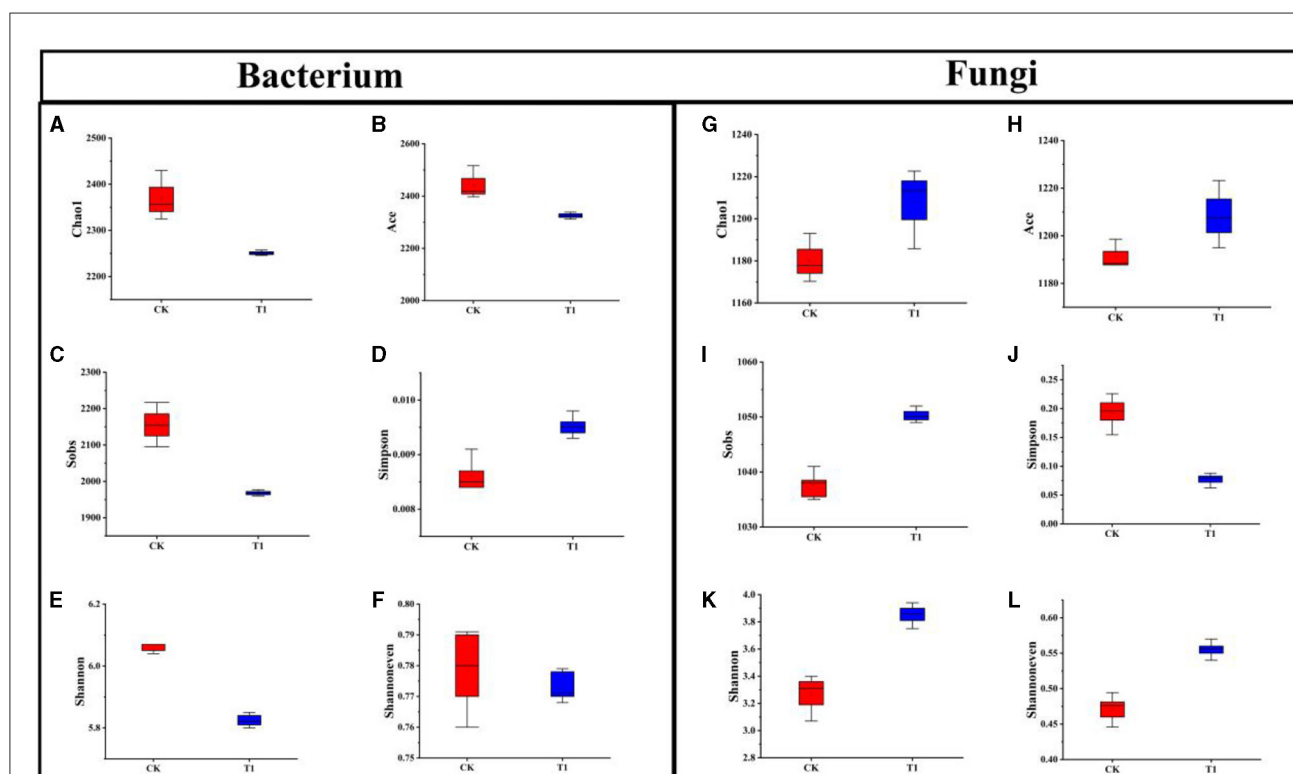
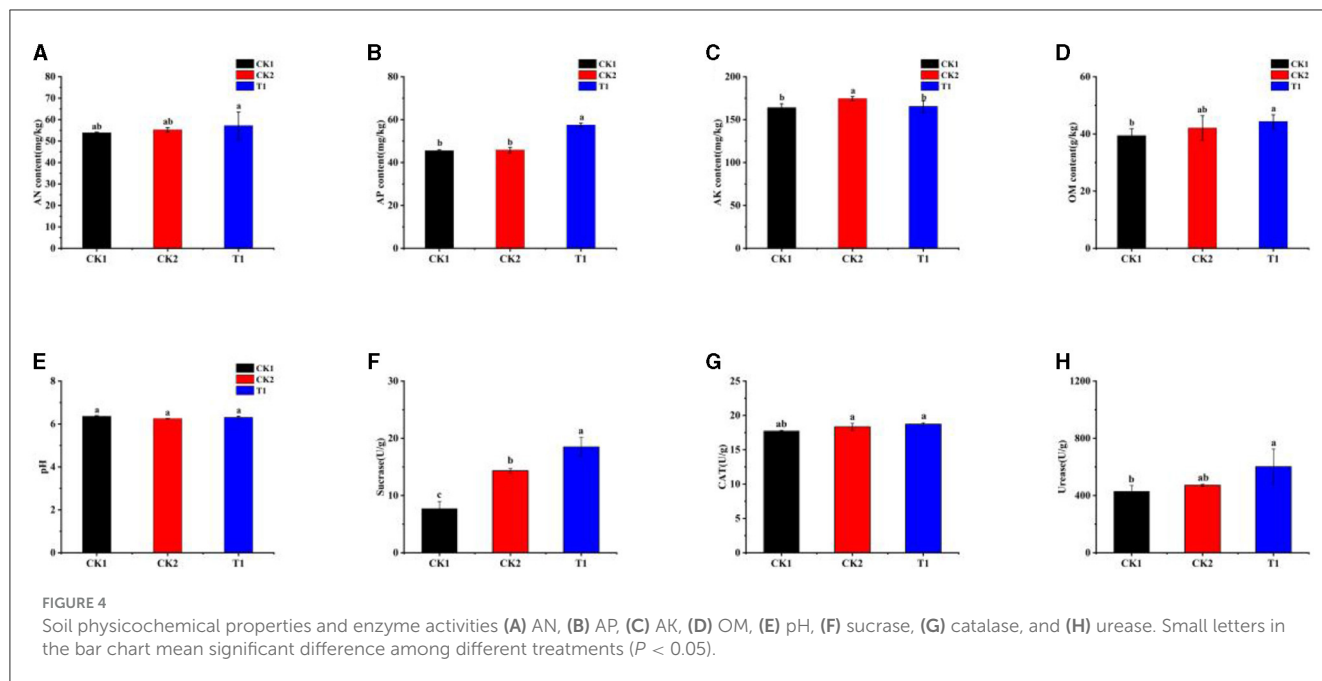
To verify the sequencing quality and sequencing depth, the reliability of the sequencing data volume was assessed by the dilution curve. As the number of sequencings increased, the dilution curve gradually smoothed out, and the value became higher and higher and tended to be 1 (Supplementary Figures S2A, B). This indicated that most of the species in the soil samples were detected, that more data volume would only generate a small number of new OTUs, and that the sequencing data was sufficient. The results were representative of the real situation of the samples. They could be used for the subsequent analysis of the diversity of the soil community and the composition of the species.

The distribution of species community-specific and shared OTUs is shown by the Venn diagram. The inter-root bacterial community had 3,634 OTUs in the two treatments, whereas 599 specific OTUs were specific to T1, and 796 were specific to the CK. The fungal community had 2,082 OTUs in the two treatments, while 481 and 463 specific OTUs were specific to CK and T1, respectively (Supplementary Figures S2C, D).

To explore the differences and distances in the composition of bacterial and fungal communities in different treatments, PCA analysis of bacterial and fungal communities in different treatments was performed based on OTU level. The PC1 and PC2 axes of the bacterial community were segregated at 0.087 and 0.76%, respectively. The T1 treatment was dispersed individually compared to CK, indicating differences in species composition among the treatments. The fungal community had 0.055 and 0.901% PC1 and PC2 axes segregation rates, respectively, and were relatively dispersed among treatments with differences in species composition structure (Supplementary Figures S2E, F).

3.6.2 Effect of alpha and beta diversity of soil microorganisms

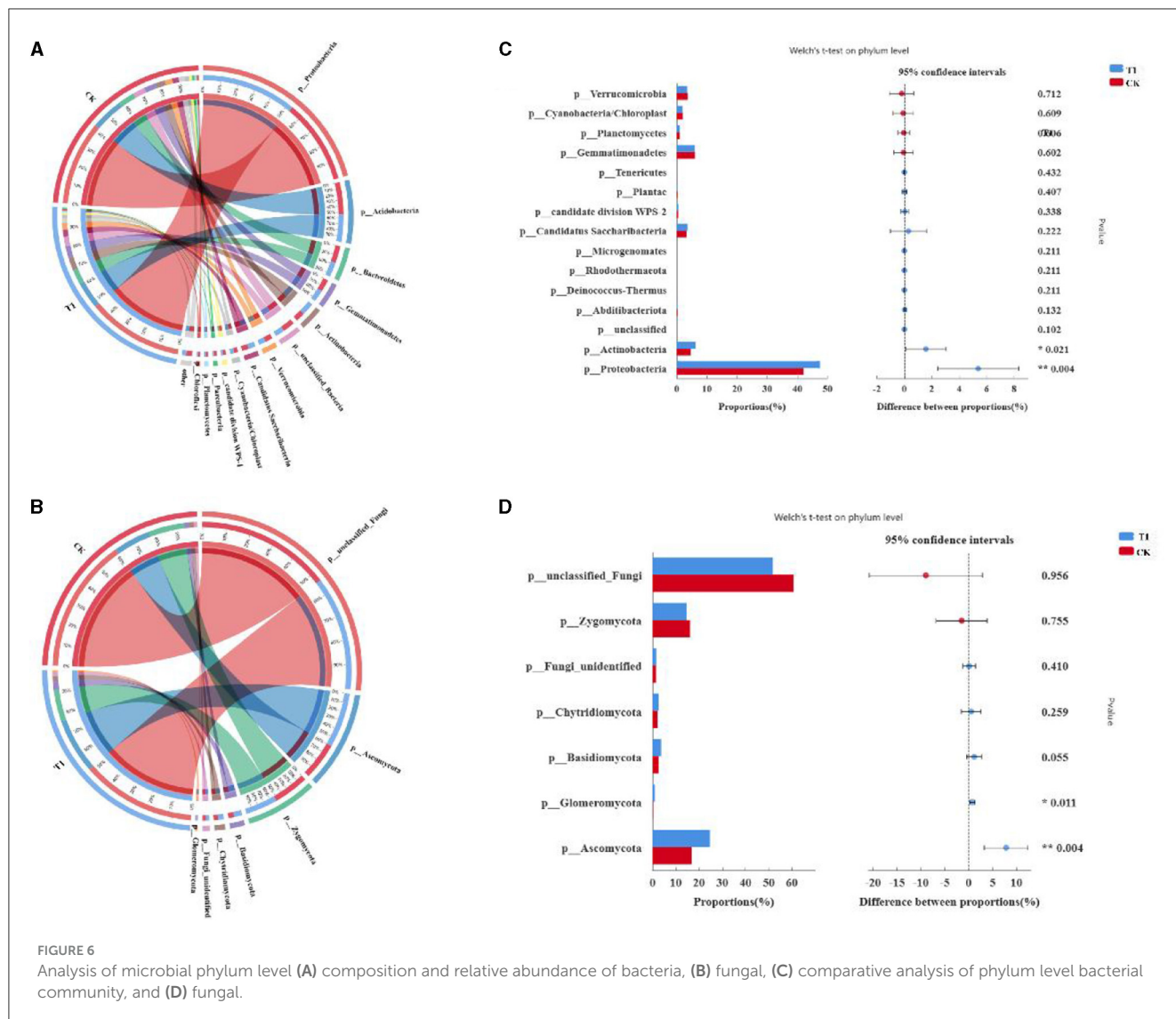
The effect of different treatments on the α -diversity of inter-root bacteria, Chao1, and Ace indices decreased in the T1 treatment compared to CK, indicating that the T1 treatment decreased the relative abundance of inter-root bacteria in the microbial community (Figures 5A, B). Sobs index was higher in CK than in T1 treatments, suggesting that the actual number of OTUs was the highest in the CK treatment (Figure 5C). The increase in Simpson's index and decrease in Shannon's index in the T1 treatment compared to CK indicated that the community diversity of inter-root bacteria was reduced in the T1 treatment (Figures 5D, E). The Shannoneven index was higher in CK than in the T1 treatment, indicating that the uniformity of the inter-root bacterial



community was higher in the CK treatment than in the T1 treatment (Figure 5F).

The effects of different treatments on the α -diversity of inter-root fungi were significant. The Chao1 and Ace indices of T1 increased compared to CK, indicating that the T1 treatment increased the number of OUTs of inter-root fungi in the

community and improved the relative abundance of fungal flora in the microorganisms (Figures 5G, H). The Sobs index of T1 was greater than that of the CK control treatment, suggesting that the number of OUTs observed in the CK treatment was less than that of T1 (Figure 5I). Whereas, the Simpson's index of T1 declined compared to that of CK, the Shannon index increased,



indicating that the T1 treatment increased the community's diversity (Figures 5J, K). The Shannoneven index of the CK treatment was smaller than that of the T1 treatment, indicating that the T1 treatment had a high uniformity in the distribution of the inter-root fungal flora (Figure 5L).

3.6.3 Effect of PGPR on microbial composition and structure

To clarify the effect of different treatments on the species composition of microbial communities, changes in dominant species of bacteria and fungi were comparatively analyzed at the phylum level (Figures 6A, B), and the sum of species with relative abundance <0.01 was defined as Others. The dominant species at the phylum level of bacteria were *Proteobacteria*, *Acidobacteria*, *Sphingomonas*, *Gemmatimonadetes*, *unclassified_Bacteria*, *Candidatus*, *Gp3*, *Saccharibacteria*, *Chitinophagaceae*, *Subdivision3*, and *Rhizobiales*. The most dominant phyla are *Rhizobiales*, *Gp1*, *Betaproteobacteria*, *Streptophyta*, *Bradyrhizobium*, *Alphaproteobacteria*, and *Acidobacterium*. *Proteobacteria*,

Acidobacteria, and *unclassified_Bacteria* are the most dominant phyla. The average relative abundance was 7.55, 5.48, and 5.44%, respectively. The highest relative abundance of dominant species at the level of fungal phyla was *unclassified_Fungi*, *Mortierella*, *Ascomycota*, *Sordariomycetes*, *Alternaria*, *Trichoderma*, *Fungi_unidentified*, *Ascomycota*, *Ascomycota*. Among them, *unclassified_Fungi*, *Mortierella*, are the most dominant phyla. The average relative abundance was 63.2, 15.8, and 5.44%, respectively.

Comparative analyses of bacteria and fungi were performed to further explore species composition differences between treatments at the phylum level, comparing bacterial and fungal species with *P*-values in the top 15 and fungal species with *P*-values in the top 7. Compared with CK (Figures 6C, D), the relative abundance of *Proteobacteria*, *Candidatus Saccharibacteria*, and *Actinobacteria* was increased, and *Verrucomicrobia*, *Gemmatimonadetes*, and *Cyanobacteria/Chloroplast* were decreased by T1 in the bacterial community. Species composition of the fungal community differed at the phylum level; the relative divisions of *Chytridiomycota*, *Ascomycota*, and *Basidiomycota* were increased, and the relative

divisions of *unclassified_Fungi*, *Zygomycota*, were decreased by T1 compared to CK.

The species composition of bacteria and fungi in different treatments at the genus level was analyzed. The dominant genera in the bacterial community were mainly *Sphingomonas*, *Gemmatimonadaceae*, *unclassified_Bacteria*, *Chujaibacter*, *Gp3*, and *Saccharibacteria* (Figure 7A). The dominant genera in the fungal community at the genus level were mainly *unclassified_Fungi*, *Mortierella*, *Sordariomycetes*, *Ascomycota*, *Alternaria*, and *Trichoderma* (Figure 7B). Analysis of the differences in species composition of bacterial and fungal communities between treatments at the genus level showed that at the bacterial genus level, the relative abundance of the genera *Devosia*, *Mycobacterium*, *GPL4*, *Bradyrhizobium*, *Alcaligenaceae*, *Trinickia*, *Chujaibacter*, *Rhodanobacteraceae*, and *Micropepsaceae* was significantly increased by T1 (Figure 7C). For the fungal genus level, T1 increased the relative abundance of *Hypocreaceae*, *Trichoderma*, and *Chytridiomycetes* (Figure 7D).

To investigate further the differences in the composition of bacterial and fungal communities in the inter-root soil of tobacco under different treatments, linear discriminant analysis and influence factor (LEfSe) were used to discover the species that best explained the differences between groups under different treatments. Data from inter-root soil samples of tobacco plants were analyzed and counted at five levels from phylum to genus. A total of 55 different levels of differential taxa were identified in the bacterial community. A total of 32 differential species were enriched in the CK treatment group and 21 in the T1 treatment group (Figure 7E). In the bacterial community, for the phylum level, the five enriched taxa in CK are *p_Acidobacteria*, *p_Bacteroidetes*, *p_Chloroflexi*, *p_Parcubacteria*, and *p_unclassified_Bacteria*. The two enriched taxa in T1 are *p_Actinobacteria* and *p-Proteobacteria*. For the class level, the seven enriched taxa in CK are *c_Acidobacteria_Gp3*, *c_Acidobacteria_Gp4*, *c_Acidobacteria_Gp6*, *c_Chitinophagia*, *c_norank_Parcubacteria*, *c_Deltaproteobacteria*, and *c_Deltaproteobacteria*. The seven enriched taxa in T1 are *c_Actinobacteria*, *c_Betaproteobacteria*, and *c_Gammaproteobacteria*. For the order level, the eleven enriched taxa in CK are *o_Acidobacteria_Gp3*, *o_Acidobacteria_Gp4*, *o_Acidobacteria_Gp6*, *o_Chitinophagales*, *o_Parcubacteria*, *o_Sphingomonadales*, *o_Alphaproteobacteria*, *o_Nitrosomonadales*, *o_Betaproteobacteria*, *o_Myxococcales*, and *o_unclassified_Bacteria*. The six enriched taxa in T1 are *o_Micrococcales*, *o_Micropepsales*, *o_Rhizobiales*, *o_Burkholderiales*, *o_Gammaproteobacteria*, and *o_Xanthomonadales*. For the family level, the eight enriched taxa in CK are *f_Acidobacteria_Gp3*, *f_Acidobacteria_Gp4*, *f_Acidobacteria_Gp6*, *f_Chitinophagaceae*, *f_Sphingomonadaceae*, *f_Alphaproteobacteria*, *f_Betaproteobacteria*, and *f_unclassified_Bacteria*. The seven enriched taxa in T1 are *f_Micropepsaceae*, *f_Bradyrhizobiaceae*, *f_Devosiaceae*, *f_Burkholderiaceae*, *f_Gammaproteobacteria*, *f_Rhodanobacteraceae*, and *f_Xanthomonadaceae*. For the genus level, the six enriched taxa in CK are *g_Gp1*, *g_Gp3*, *g_Gp4*, *g_Flavisolibacter*, *g_Parcubacteria*, and *g_unclassified_Bacteria*. The six enriched taxa in T1 are *g_Micropepsaceae*, *g_Bradyrhizobium*,

g_Devosia, *g_Trinickia*, *g_Acidibacter*, *g_Rhodanobacter*, and *g_Chujaibacter*.

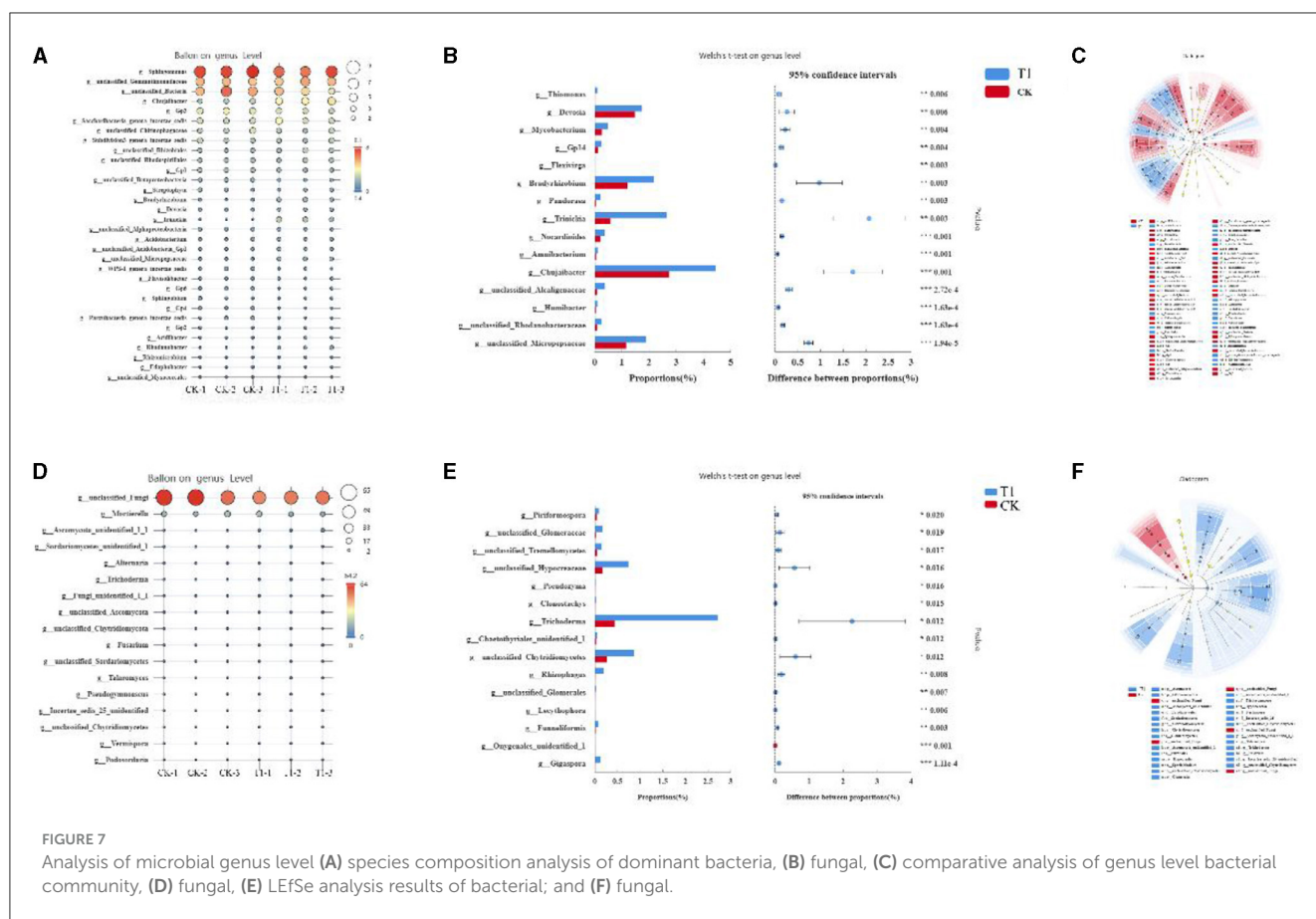
A total of 31 taxa with different levels of differentiation were identified in the fungal community. There were five and 26 differential species in CK and T1, respectively. The enriched taxa of CK phylum, order, family, and genus are *unclassified_Fungi* (Figure 7F). For the phylum level, there were two enriched taxa in T1, namely *Ascomycota* *Glomeromycetes*. For the order level, there are six enriched taxa in T1, which are *p_Ascomycota*, *p_Eurotiales*, *p_Sordariomycetes*, *p_Microbotryomycetes*, *p_Chytridiomycetes*, and *p_Glomeromycetes*. For the order level, there are four enriched taxa in T1, which are *o_Ascomycota*, *o_Eurotiales*, *o_Hypocreaceae*, *o_Sporidiobolales*, *o_Chytridiomycetes*, *o_Glomerales*. For the family level, the six enriched taxa in T1 are *f_Ascomycota*, *f_Trichocomaceae*, *f_Hypocreaceae*, *f_Nectriaceae*, *f_Incertae_sedis_25*, and *f_Chytridiomycetes*. For the genus level, the four enriched taxa in T1 are *g_Ascomycota*, *g_Talaromyces*, *g_Trichoderma*, *g_Fusarium*, *g_Incertae_sedis_25*, and *g_Chytridiomycetes*.

3.6.4 Correlation analysis of soil physicochemical and microbial diversity

The addition of different inter-root biotrophic bacteria affects nutrient transformations in the soil and alters soil enzyme activities, contributing to changes in the microbial community in the soil. Therefore, the relationship between soil bacterial and fungal communities and environmental factors was investigated. Redundancy (RDA) analysis was used to correlate the physicochemical properties such as pH, AN, OM, AP, AK, and soil enzyme activities such as SC, CAT, and UR with the microbial communities in the soil (Figures 8A, B). The results showed that soil physicochemical property indexes, AN AP, and OM in soil were most affected by bacterial community, followed by OM. The effect of T1 treatment on AN, AP, and OM in soil was significantly positively correlated, and the correlation with AP was more significant.

Meanwhile, pH was significantly and negatively correlated with the T1 treatment. T1 treatment was significantly and positively correlated with SC and CAT in soil and not significantly with UR. By analyzing the effect of the fungal community on soil nutrients and enzyme activities, we found that AN, OM, and pH soil nutrients had the greatest effect on the soil, and the least correlation was with OM. The T1 treatment became significantly positively correlated with AN and AP and significantly negatively correlated with pH. Regarding soil enzyme activities, UR was most affected by fungal communities. T1 treatment was positively correlated with SC, CAT, and UR in soil, and UR was significantly correlated.

To further investigate the relationship between microbial community composition and soil nutrients and enzyme activities, a correlation heat map analysis was conducted at the genus level for dominant bacterial and fungal communities in relation to soil environmental factors (Figures 8C, D). In the bacterial community, genera such as *Devosia*, *Rhodanobacter*, *Chujaibacter*, *Bradyrhizobium*, *Trinickia*, *Gp6*, *Gp3*, *Gp4*, *Gp1*, and *Parcubacteria* played significant roles. These genera were positively correlated with soil physicochemical properties such as alkaline nitrogen



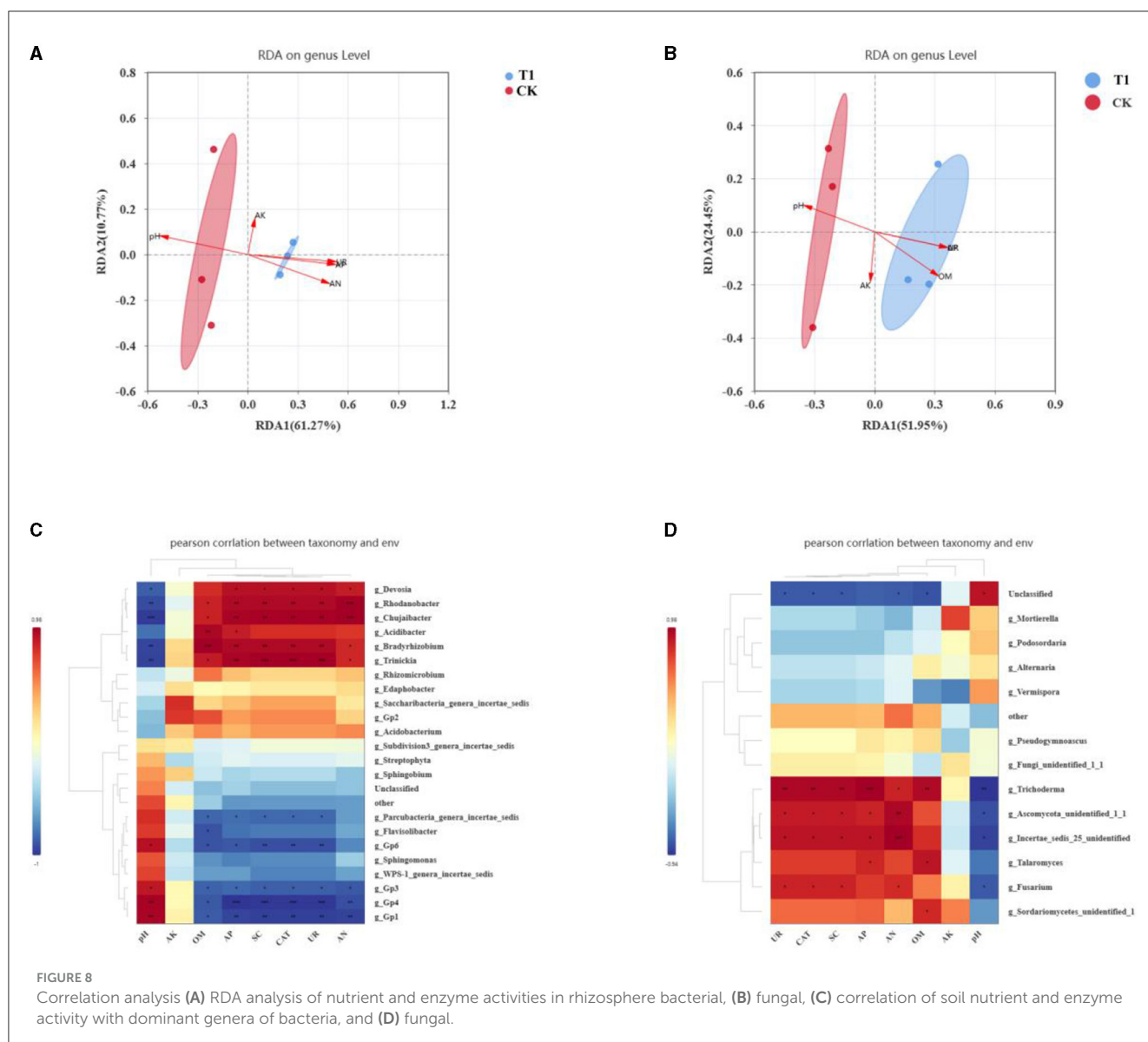
(AN), available phosphorus (AP), organic matter (OM), sucrose (SC), and catalase (CAT) activities while being negatively correlated with soil pH. Conversely, Gp6, Gp3, Gp4, Gp1, and *Parcubacteria* showed significant negative correlations with AN, AP, OM, SC, and CAT and positive correlations with soil pH. There was no significant correlation between available potassium (AK) and the dominant bacterial genera. In the fungal community, genera such as *Trichoderma*, *Ascomycota*, and *Fusarium* were significantly positively correlated with AN, AP, AK, SC, and CAT activities. Specifically, *Trichoderma* showed a positive correlation with OM in the soil, while *Ascomycota* and *Fusarium* were negatively correlated with AN, AP, SC, UR, and CAT. Soil pH was negatively correlated with *Trichoderma*. Similar to the bacterial community, no significant correlation was found between AK and the dominant fungal genera.

3.6.5 Effects of functional properties in soil microbial communities

Soil microbial metabolic functions, analyzed at both Pathway Level 1 and Level 2, reveal that metabolism is the most dominant activity among the six KEGG metabolic functions, highlighting its critical role in the life processes of microorganisms (Figure 9A). Within the KEGG Pathway database, the “global and overview maps” category, which encompasses a special set of metabolic pathway maps, contained the largest number of genes related to metabolic functions. The most prominent metabolic pathways

identified were amino acid, carbohydrate, energy, cofactors, and microbial and nucleotide metabolism. In the domain of cellular processes, the “cell communities – prokaryotes” pathway had the highest gene count, while “transmembrane transport” and “signaling” pathways topped the environmental information processing category. Within gene information processing, the pathway with the most genes was related to signaling. Furthermore, the highest number of genes associated with bacterial infectious diseases were found in disease pathways. In the organismal systems pathways, “aging,” “endoanalytic systems,” and “environmental adaptation” were the functional expressions with the highest gene counts.

To further analyze the mechanism of action of the inter-root biotrophic bacteria, the differences in different metabolic functions of microorganisms at KEGG tertiary metabolic levels induced by the inter-root biotrophic bacteria were annotated (Figure 9B). Compared to CK, soil microorganisms in tobacco plants significantly increased fatty acid degradation and metabolic functions, glycine, serine, and threonine metabolic functions, lysine degradation functions, tryptophan metabolic functions, and tryptophan metabolic functions in the treatment of *Bacillus paracolor* in the T1 treatment. Arginine and proline metabolic functions, benzoic acid metabolic function, phenylalanine, tyrosine, and tryptophan biosynthetic functions were higher in the T1 treatment than in the control CK treatment. Amino acids are nutrients that ensure plant growth and development and maintain the life activities of soil microorganisms, and T1 promoted the



biosynthesis function of amino acids in the soil microorganisms in the inter-root of the tobacco plant. Moreover, the synthesis of secondary metabolites of soil microorganisms was higher in the T1 treatment than in the CK treatment. T1 promoted the metabolic function of organic selenium compounds and the biosynthesis of cofactors compared with CK treatment.

Soil microorganisms are involved in material cycling and capacity flow in the soil, and to understand the metabolic functional properties of soil microorganisms involved in the dominant genera, a correlation analysis was conducted between the dominant bacteria in the soil and KEGG functions (Figure 9C). The correlation heat map revealed significant positive correlations between *Gibberella*, *Chujaibacter*, *Trichoderma*, and several functional categories, including membrane transport, drug resistance: antimicrobial, cellular community-prokaryotes, glycan biosynthesis, and metabolism, metabolism of other amino acids, metabolism of terpenoids and polyketides, and development and regeneration. *Chujaibacter* and *Trichoderma* also significantly and

positively correlated with signal transduction and cell motility. Conversely, significant negative correlations were observed between *Gibberella*, *Chujaibacter*, and *Trichoderma* and cell growth and death, energy metabolism, translation, and nucleotide metabolism. Additionally, *Chujaibacter* and *Trichoderma* were significantly negatively correlated with carbohydrate metabolism, neurodegenerative diseases, the circulatory system, infectious diseases, and parasitic diseases.

4 Discussion

In this study, *Trichoderma harzianum*, a PGPB, was isolated from tobacco inter-root soils, and its mechanisms of action on continuously cropped soils were investigated. The results indicated that tobacco growth and development were significantly enhanced, and notable improvements in soil physicochemical properties were observed. Soil enzyme activities, microbial species, and functional

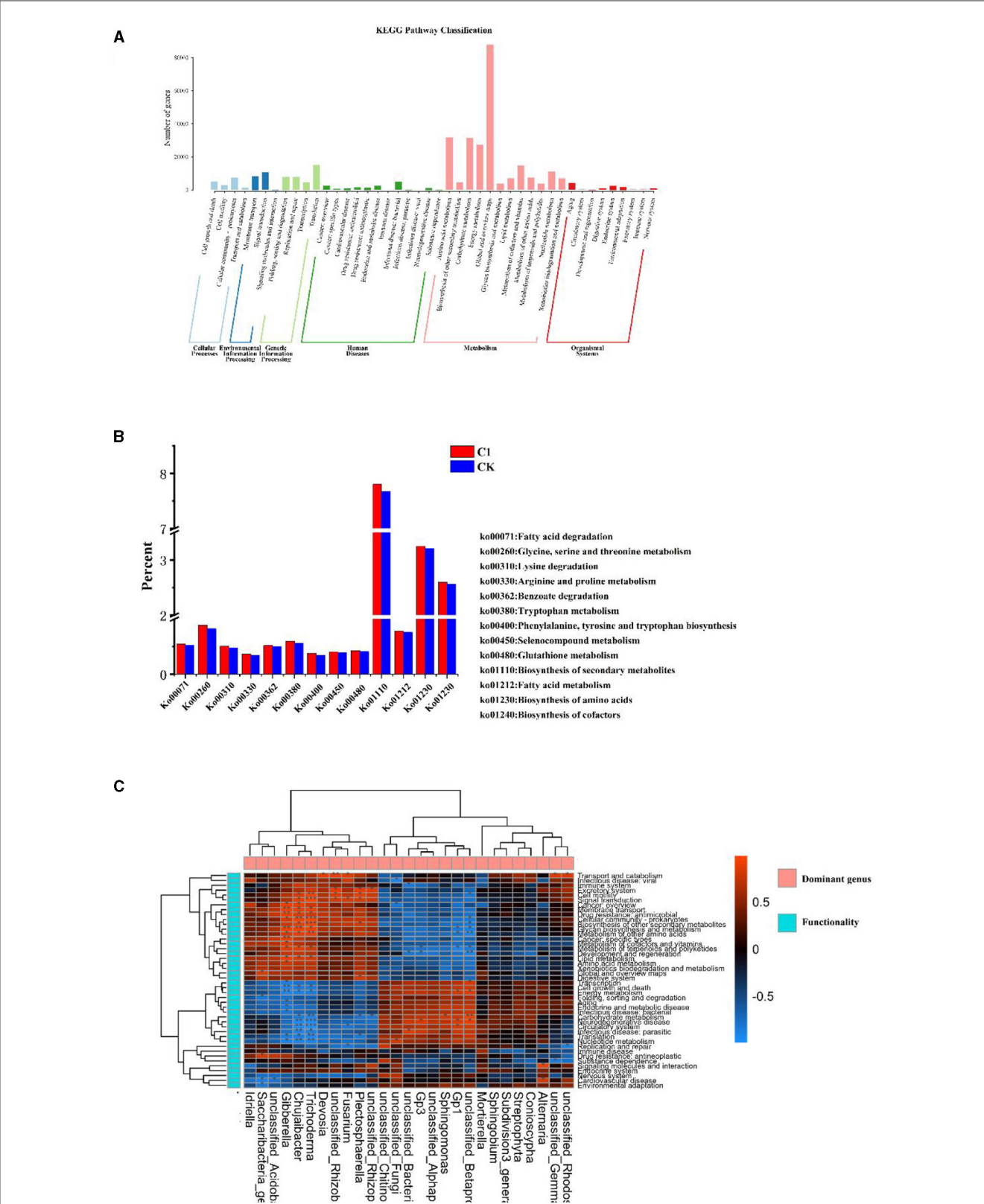


FIGURE 9 Analysis of metagenome (A) histogram of the number of genes at KEGG Pathway1 and Pathway2 levels, (B) influence of rhizosphere growth-promoting bacteria on the metabolic level of soil microorganisms KEGG Pathway3, and (C) heat map analysis of the correlation between dominant genera and KEGG function in soil.

diversity were further determined for the T1 treatment and CK. Sucrase, catalase, and urease levels were found to be significantly higher in the T1 compared to the CK. The T1 treatment increased beneficial microflora and promoted functions such as metabolism, amino acid synthesis, and secondary metabolite synthesis in soil microorganisms.

Agronomic traits are crucial prerequisites for expressing plant growth and quality, with plant growth and development directly indicated by these traits (Abdi and Williams, 2010). Agronomic traits and plant growth and development were found to be effectively enhanced by PGPR (Liu et al., 2023). For blueberries treated with the inter-root biotrophic bacteria *Pseudomonas* spp. and *Brucella* spp., significant increases were observed in the number of branches, leaves, chlorophyll content, and plant height compared to the control group (Lobato-Ureche et al., 2023). The growth of pepper seedlings was promoted by the inoculation of four indigenous strains of PGPR during their growth (Harahap et al., 2023). Significant increases in the number of grains, spikes, and root length were observed in rice following the application of PGPR (Abd El-Mageed et al., 2022). Studies have shown that adding *Bacillus mucilaginosus* to tobacco plants in the field positively affects plant height, maximum leaf length, maximum leaf width, and stem thickness. In the moderate addition group, the four indicators were 26.76, 15.24, 19.37, and 18.08% higher, respectively, compared to the control group (Zhao et al., 2024). In this study, treatment T1 significantly increased the height, maximum leaf length, maximum leaf width, and maximum leaf area of tobacco, consistent with the results of previous research.

Root development is directly linked to root vigor, which subsequently influences plant uptake and utilization of nitrogen and dry matter accumulation (Wang et al., 2019). The effects of inter-root biotrophic bacteria on crops are primarily manifested through their impact on the root system (Li et al., 2016). The morphology and structure of crop root systems influence their ability to absorb soil nutrients and water resources, subsequently affecting the growth of the above-ground parts (Lynch, 2013). A better root conformation increases plant access to water and nutrients in the growth substrate, improves plant water and fertilizer use efficiency, and enhances plant resistance (Mu et al., 2015; Bengough et al., 2011). Research shows that under salt stress, the application of PGPR significantly improves the root structure of oats, with root length increasing by 20.55 and 21.94% under 0 and 100 mM NaCl treatments, respectively (Zhang et al., 2023).

Additionally, PGPR from the *Bacillus* and *Enterobacter* genera has improved root architecture in wheat and maize, significantly enhancing root surface area, branching, length, and tips (Jochum et al., 2019). *Trichoderma harzianum*, which contains the cysteine-rich cell wall protein QID74, enhances root hair formation and elongation, increasing the absorption surface area and the efficiency of nutrient translocation to branches, thereby increasing plant biomass (Samolski et al., 2012). In this study, the root system was enhanced by T1 treatment. The total root length and the number of root tips of the root system were significantly increased by T1.

An increase in chlorophyll content was found to enhance the ability of plants to carry out photosynthesis significantly (Plus et al., 2005). Chlorophyll maintenance by *Bacillus* sp. was found to promote soybean growth (Mun et al., 2024). In barley, total

chlorophyll was significantly increased by 126% by applying four different PGPRs (Slimani et al., 2023). The tomato was inoculated with *Bacillus subtilis* + *Bacillus amyloliquefaciens* and *Bacillus amyloliquefaciens*; increases in chlorophyll content were 30 and 27%, respectively. Chlorophyll b was 20 and 16%, respectively; total chlorophyll was 54 and 43%, respectively, and carotenoids were 52 and 42%, respectively (Gashash et al., 2022). In this study, treatment T1 increased the chlorophyll content in the leaves, enhancing photosynthesis and supporting tobacco growth. This is consistent with previous research. Biomass is considered an important parameter of vegetation status and is crucial for plant production, carbon cycling, and nutrient allocation studies. Previous studies using a PGPR isolate as an inoculum positively affected different saffron plant parameters. Enhancements were observed in the number of leaves and chlorophyll content. The yield of the underground part of cotyledons was significantly increased, approximately by 1.91 times, compared to the CK (Chamkhi et al., 2023). This study observed significant increases in root stems and leaves' fresh and dry weights following T1 treatment.

Soil enzyme activity plays a crucial role in the global cycling of key elements such as C, P, and N and serves as a vital indicator for assessing soil health and fertility levels (Daunoras et al., 2024). These activities, mainly produced through the physiological metabolism of various microbial groups, reflect the presence and activity of corresponding functional microorganisms. By studying these enzyme activities, researchers can assess microbial response mechanisms to environmental changes (Baldrian, 2009; Cusack et al., 2011).

For instance, the enzyme activity of soil SC is indicative of the soil's maturity and fertility levels, influencing the transformation of organic matter (OM) within the soil (Li et al., 2023). Soil catalase (CAT) activity, associated with soil microbial activity, aids plants in resisting oxidative damage (Khanna et al., 2019). Additionally, the degradation of nitrogen-containing compounds is effectively catalyzed by soil urease (UR), thereby maintaining soil health (Dutta and Neog, 2015). Previous studies have found that soil sucrase and urease activities, among others, were enhanced by the mixed inoculation of pastures with PGPR strains (Chai et al., 2023).

Significant improvements in soil sucrase, urease, and catalase activities were also observed following the application of *Bacillus subtilis* bacterial fertilizer to cigar soils (Shang et al., 2023). In this study, the effect of inter-root facultative bacteria on soil enzyme activities was analyzed, revealing that SC, CAT, and UR activities were significantly increased in the T1 treatments compared to the CK1 and CK2 controls, with the most pronounced differences observed in the T1 treatments. The T1 treatments not only enhanced the activities of SC, CAT, and UR in the soil, thereby boosting, but also corresponded with an increase in the OM and AN content in the inter-root soils. This suggests that the acceleration of microbial redox reactions and the metabolism of carbon and nitrogen in the soil are likely due to the T1 treatment.

Additionally, plant defenses are systemically activated by co-inoculation with *Trichoderma* and bacteria, involving both salicylic acid (SA)-related responses and jasmonic acid (JA)-related defense responses. For instance, a synergistic reduction in *Erysiphe pisi* conidial development on pea leaves was observed following root

inoculation with *T. asperellum*–*P. fluorescens*, which was due to the systemic activation of JA-related defenses (Patel et al., 2016). This co-inoculation mechanism also enhances the activity of soil-related enzymes in plants, including phenylalanine ammonia-lyase (PAL), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and catalase (CAT) (Poveda and Eugui, 2022). Furthermore, urease (UR) plays a crucial role in characterizing the efficiency of urea utilization and its decomposition rate in the nitrogen cycle (Baligar et al., 1991). The addition of beneficial rhizosphere microorganisms has been shown to significantly increase the content of readily available nitrogen, thereby enhancing urea's conversion efficiency (Cookson and Lepiece, 1996).

The application of PGPR has been shown to alter the community structure of indigenous soil microorganisms to some extent. This alteration may be due to competition for limited soil nutrients and spatial distribution among indigenous microorganisms, or it may result from the promotion of characteristic secretions by plant roots, which recruit and induce beneficial microorganisms in the soil to transform soil nutrients (Li et al., 2021). Previous research has shown that applying *Trichoderma* significantly alters the number and diversity of native microbial populations, especially among bacteria. For example, in carrot crops, the application of *T. harzianum* significantly increased the populations of the *Bacillus* and *Pseudomonas* genera (Patkowska et al., 2020). Additionally, *T. harzianum* has shown high antagonism against pathogens, which is associated with the secretion of various proteins, including chitinase, mutanase, α -1,3-glucanase, α -1,2-mannosidase, carboxylic hydrolase ester, carbohydrate-binding module family 13, glucan 1,3- β -glucosidase, α -galactosidase, and neutral protease (de Lima et al., 2016).

After fumigation with *Trichoderma*, there was a significant increase in the abundance of beneficial microorganisms, particularly *Bacillus* and *Gemmatimonadaceae*. Both *Bacillus* and *Gemmatimonadaceae* are considered spectrum-beneficial bacteria and are recognized as spectrum-beneficial bacteria that can effectively enhance crop growth (Wu et al., 2022).

In this study, the application of T1 reduced the number of operational taxonomic units (OTUs) of bacteria and fungi in the soil. Regarding bacterial diversity, there was a reduction in species diversity and richness, while the relative abundance of dominant species increased with T1 treatment. Specifically, the relative abundance of dominant phyla, such as *Proteobacteria*, *Actinobacteria*, and *Candidatus Saccharibacteria*, was increased by T1 treatment. The phylum *Proteobacteria* is known for its complex physiological and metabolic capabilities, which play a crucial role in the carbon cycle (Liu, 2022). *Actinobacteria* participate in the metabolism of nitrogen and phosphorus in the soil, contributing to the decomposition of readily available phosphorus and nitrogen (Zeng et al., 2024). In addition, the relative abundance of *Ascomycota* and *Basidiomycota* in the fungal community was increased by T1 treatment.

As the main fungal decomposers in the soil, the fungal general within the *Ascomycota* phylum are predominantly saprophytes, which play a crucial role in the transformation of organic matter. Notably, the stammers within this group are particularly important in the decomposition of lignocellulose, a key structural component of plant cell walls (Yelle et al., 2008).

At the microbial genus level, the application of T1 treatment led to an increased relative abundance of dominant genera, including *Alcaligenaceae*, *Pandoraea*, and *Thiomonas*. The genus *Pandoraea* is recognized for its ability to mitigate drought stress and enhance growth traits in soybeans (Gonçalves et al., 2023). *Alcaligenaceae* has been shown to effectively promote growth and enhance salt resistance in canola (*Brassica napus* L.) (Abdel Latef et al., 2021). Additionally, the genes involved in the urea degradation process in *Thiomonas* strains have been reported to increase the rate of urea degradation in the soil, thereby enhancing soil fertility.

Urea degradation helps precipitate toxic metals such as iron, aluminum, and arsenic (Farasin et al., 2015). The T1 treatment also led to an increase in the relative abundance of *Hypocreaceae* and *Trichoderma* within the fungal community. *Trichoderma*, a member of *Hypocreaceae*, is known for its biocontrol and plant growth-promoting effects, including antimicrobial, antioxidant, and insecticidal activities, as well as growth enhancement (Lodi et al., 2023).

Soil microorganisms play an important role in cycling and transforming soil nutrients, root secretions, apoplastic materials, and other substances (Das et al., 2022). These functions, which help maintain a dynamic balance in the soil, are closely related to organic matter, mineral nutrients, and the life activities of microorganisms themselves (Fazeli-Nasab et al., 2022; Bhattacharyya et al., 2022). In this study, the application of T1 enhanced the metabolic functions of inter-root microorganisms in tobacco plants. This enhancement was observed in processes such as including amino acid biosynthesis and secondary metabolite synthesis. An increase was observed in metabolic activities such as membrane transport, drug resistance (antimicrobial), cellular community (prokaryotes), glycan biosynthesis and metabolism, other amino acid metabolism, terpenoids and polyketides, and development and regeneration induced by T1 treatment. Notably, the incidence of parasitic infectious diseases was reduced, indicating that the population of pathogenic bacteria in the soil was suppressed, leading to fewer diseases.

These changes in metabolic activities enhance the metabolism and energy flow of microorganisms in the soil (Khan et al., 2020; Yoo et al., 2021). In summary, T1 treatment can effectively improve soil properties and promote tobacco growth, providing a foundation for developing microbial fertilizers to alleviate continuous cropping barriers.

In this study, an efficient PGPR, *Trichoderma harzianum*, was isolated from the soil between tobacco roots, highlighting its potential in agriculture. This research provides a valuable reference for further exploration of fungal bioformulations and establishes a theoretical foundation for developing new fungal-centered microbial fertilizers, which can be integrated with existing agricultural management practices. However, this study has some limitations, including its confinements to a single type of soil, a lack of long-term research, and a focus on the application of a single strain. Therefore, future research should expand to include a broader range of crops and environments and conduct in-depth analyses of the economics and environmental impacts of long-term use to ensure the sustainable application of such bioformulation in modern agriculture.

5 Conclusion

This study successfully isolated *Trichoderma harzianum*, a PGPR strain with effective growth capabilities, from the soil between tobacco roots. The influence of this strain on the properties of soils subjected to continuous cropping was thoroughly examined. The results showed significant enhancements in the growth and development of tobacco plants, alongside notable improvements in the soil's physicochemical characteristics. A comparative analysis of soil enzyme activity, microbiome, and metagenome between the T1 treatment group and the CK revealed substantial increases in the activities of key enzymes such as sucrase, catalase, and urease in the T1 group. Furthermore, the T1 strain fostered an increase in beneficial microorganisms, which enhanced soil microbial functions related to amino acid metabolism, synthesis, and the production of secondary metabolites. These findings provide a strong foundation for the development of efficient microbial fertilizers with fungi at their core and contribute to the broader study of effective PGPR.

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 here: the isolated strains genome can be found in the NCBI repository (<https://www.ncbi.nlm.nih.gov/>), accession number: PQ135054.

Author contributions

CJ: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. FP: Data curation, Writing – original draft. LZ: Data curation, Writing – original draft. YZ: Data curation, Methodology, Validation, Formal analysis, Writing – original draft. JW: Data curation, Methodology, Validation, Formal analysis, Writing – original draft. JL: Data curation, Methodology, Investigation, Writing – original draft. BC: Data curation, Methodology, Investigation, Writing – original draft. CC: Data curation, Methodology, Validation, Formal analysis, Writing – original draft. CW: Resources, Supervision, Writing – original draft. YQ: Visualization, Investigation, Writing – original draft. RW: Visualization, Investigation, Writing – original draft. ZZ: Visualization, Investigation, Writing – original draft. JJ: Software, Validation, Writing – original draft. MY: Software, Validation, Writing – original draft. MS: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review &

editing. LY: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. QZ: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

FP, JJ, MY, and MS were employed by Shandong China Tobacco Industry Co., Ltd. YZ, JW, JL, BC, and CC were employed by Shandong Rizhao Tobacco Co., Ltd.

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1457624/full#supplementary-material>

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Next-generation sequencing-based comparative mapping and culture-based screening of bacterial rhizobiome in *Phytophthora capsici*-resistant and susceptible *Piper* species

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Black pepper (*Piper nigrum* L.), a highly valued spice crop, is economically significant as one of the most widely traded spices in the world. The global yield and quality of black pepper (*Piper nigrum* L.) are affected by foot rot-causing soil-borne oomycete pathogen *Phytophthora capsici*. To gain initial insights toward developing an approach that utilizes microbial genetic resources for controlling foot rot disease in black pepper, we mapped the rhizobiome communities in susceptible *Piper nigrum* L. and wild-resistant *Piper colubrinum*. The analysis showed compositional differences in the rhizobiome of two *Piper* species, which revealed higher diversity and the presence of more differentially abundant genera in *P. colubrinum*. Furthermore, *P. colubrinum* rhizobiome had a significantly higher abundance of known anti-oomycete genera, such as *Pseudomonas*, and a higher differential abundance of *Janthinobacterium*, *Variovorax*, and *Comamonas*, indicating their probable contribution to pathogen resistance. Predictive functional profiling in *P. colubrinum* rhizobiome showed highly enriched functional gene orthologs (KOs), particularly chemotaxis proteins, osmoprotectants, and other transport systems that aid in pathogen resistance. Similarly, pathways such as phenylpropanoid biosynthesis and other antimicrobial synthesis were enriched in *P. colubrinum* rhizobiome. The culturable diversity of the resistant root endosphere, which harbors efficient biocontrol agents such as *Pseudomonas*, strengthens the possible role of root microbiome in conferring resistance against soil-borne pathogens. Our results depicted a clear distinction in the rhizobiome architecture of resistant and susceptible *Piper* spp., suggesting its influence in recruiting bacterial communities that probably contribute to pathogen resistance.

KEYWORDS

Piper nigrum, *Piper colubrinum*, Rhizosphere microbiome, *Phytophthora* foot rot resistance, *Pseudomonas*, root endophytes

1 Introduction

Plants have the potential to support specific microbial communities associated with their habitat and requirements. These microbial communities are either specific to the host plant species or its genotype showing temporal as well as geospatial variation (Barraza et al., 2020). The rhizosphere is the part of the soil that is strongly attached to the root system of the host

plant, under its direct influence. The rhizobiome, i.e., the microbiome within the root endosphere along with the rhizosphere microbiome, significantly contributes to host health by promoting growth, providing essential nutrients, and inducing stress tolerance as well as pathogen resistance (Poudel et al., 2019; Trivedi et al., 2020). Essentially rhizobiome microbial community could be formed in three ways wherein there is initial colonization of a subset of bulk soil community in the rhizosphere followed by adherence and colonization of a part of this community onto the root surface to form so-called rhizoplane communities, finally, some of these microbes can also enter inside the root and colonize to become endophytic microbial community (Vukanti, 2020; Santoyo et al., 2021; Escudero-Martinez et al., 2022). Several studies have demonstrated the symbiotic association of bacteria in the rhizobiome of plant hosts, complementing to uptake of nutrients from the soil and providing resistance to biotic and abiotic stressors, and in turn, getting nutritional benefits (Vukanti, 2020; Santoyo et al., 2021). Although there is an interplay of multiple factors that control the assembly of bacterial rhizobiome, in general, the type of plant species is the key element that influences the community structure of bacteria in rhizobiome (Berg and Smalla, 2009; Wu et al., 2018). Bacterial rhizobiome establishes complex ecological network associations based on the selective effects exerted by the host plant, which might be independent of other environmental factors. This leads to clear differences in the community composition of the bulk soil when compared to the rhizosphere environment (Bulgarelli et al., 2015; Walters et al., 2018; Rodriguez et al., 2019). Understanding the bacterial community structure and function in rhizobiome specific to the host plant species encompasses the microbial component in sustainable agriculture, which is critical for amending farming practices that lead to optimized plant growth and yield. The disease resistance studies reveal that the integration of meta-omics techniques such as metagenomics can help to initiate further deeper research utilizing advanced methodologies such as microbiome engineering, metabolic engineering, and genome editing (Wille et al., 2021).

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae, dried fruits of which are used as spices and seasoning. In addition to this, they also contain antioxidant and antimicrobial terpenoids making them an ineluctable component in medicine, healthcare, and food processing (Salehi et al., 2019; Takooree et al., 2019). The cultivation of black pepper is greatly challenged by foot rot disease caused by the oomycete pathogen *Phytophthora capsici* Leonian (*P. capsici*), which persists in soil as resilient zoospores and spreads via irrigation water (Lamour and Hausbeck, 2003; Bhai et al., 2007). The wild *Piper* species, *Piper colubrinum* L. exhibits resistance against *P. capsici* foot rot disease and houses multiple resistance traits, therefore presumes high significance in biotechnological and nanotechnological interventions (Vindran and Remashree, 1998; Varma et al., 2009; Santhoshkumar et al., 2021). Belowground microbial communities are predominantly shaped by plant species, with a stronger influence observed in the rhizosphere bacterial communities and recent studies suggest that bacterial communities may protect host plants from pathogen infection by exerting positive effects on the rhizobiome compartments favoring beneficial microbes (Wu et al., 2018). The growing body of research shows that *Phytophthora* disease resistance is conferred by the microbiome which resides within and in the root vicinity. This indicates that there might be differences in the rhizobiome community composition of wild and

cultivated *Piper* species that may influence the disease resistance. Prior studies have demonstrated that this influence exists in other crops plants such as cotton (Qiao QingHua et al., 2017), *Phaseolus vulgaris* (Pérez-Jaramillo et al., 2017), wheat (Quiza et al., 2023). However, the bacterial rhizobiome structure of the *P. colubrinum* remains unknown. Moreover, none of the studies have compared bacterial community composition in wild and cultivated *Piper* in specific locations, which can reveal significant information regarding bacterial communities that may contribute to pathogen resistance. Hence, in this study, we mapped and compared the bacterial community structure and predicted function associated with the rhizobiome of black pepper *P. nigrum* which is susceptible to *P. capsici* infection and *P. colubrinum*, which is known for its resistance to this pathogen using high-throughput 16S ribosomal RNA (16S rRNA) gene-based amplicon sequencing. Both were grown under uniform conditions at the field level and bulk soil was also included for comparative analysis. This study is the first of its kind on black pepper, where the taxonomic composition, abundance, and diversity along with predicted functions of bacterial rhizobiome in wild and cultivated *Piper* are compared to bulk soil. Our study will provide primary insights into the influence of plant species on bacterial rhizobiome diversity in wild (disease resistant) and cultivated (disease susceptible) *Piper* species, which will significantly contribute to pathogen management and sustainable black pepper production.

Along with 16S rRNA-based screening, the study also reports on the culture-based isolation of the abundant bacterial endophytes from the rhizobiome of resistant *Piper colubrinum*, its phylogenetic classification, plant growth promotion, and biocontrol potential assays. We were able to isolate the abundant genus *Pseudomonas* from the root endosphere of *P. colubrinum*, which was able to inhibit the growth of *Phytophthora capsici* *in vitro*. The 16S rRNA-based metagenomic sequencing and the culturable data shed light on the fact that the resistant rhizobiome harbors bacterial genera with biocontrol potential which may confer disease resistance to the resistant plant species.

2 Materials and methods

2.1 Study site and sample collection

The soil samples were collected from two sites in the major black pepper growing tracts of the Western Ghats in Idukki district of Kerala, India (9°58'55.2"N 76°52'26.4" E, 9° 58' 54.1524" N 76° 52' 25.356" E, 600 m above mean sea level) during the south-west monsoon period in July 2022, when there is a higher chance of quick wilt incidence in black pepper. The average annual precipitation and temperature are 2345.90 mm and 23°C, respectively. The sampling site soil is classified as lateritic (Supplementary Figure S1) (Kumar et al., 2021). At each site, nine plants from the wild (*Piper colubrinum*) and cultivated (*Piper nigrum*) black pepper were selected for the rhizobiome collection. Each replicate was obtained by pooling together a set of 3 plants, totaling 18 soil samples including bulk soil (BS=3 replicates X 2 sites; rhizosphere soil =3 replicatesX2 plant speciesX2sites) (Supplementary Table S1). Furthermore, 12 samples (3X2X2) belonging to the rhizobiome were processed in the laboratory to obtain root endosphere samples. Bulk soil was collected in soil cores approximately 23–30 cm away from the base of the plant. For

rhizobiome soil collection, approximately 4–6 roots of 9–12 cm in length were procured from each plant using sterile pruning shears (Supplementary Figure S2). The roots along with the rhizosphere soil adhered to it were kept in a 50 mL tube with autoclaved phosphate buffer (6.35 g/L of NaH_2PO_4 , 8.5 g/L of Na_2HPO_4 , pH=6.5) and trimmed as necessary (McPherson et al., 2018). All samples were transported to the laboratory in iceboxes (4°C) and were sorted for downstream processing immediately to retain sample integrity and prevent any changes in microbial composition.

2.2 Processing of rhizosphere and root samples

The rhizosphere soil was separated by vortexing, followed by the sequential sonication of the roots with a set of predetermined parameters (60 s, 60 s, and 10 min) with an output power of 70 W and frequency of 42 kHz (White et al., 2015). This technique gently removes the rhizosphere soil and bacterial biofilms without damaging the plant roots and allows the isolation of microbial communities that exhibit different degrees of association with the root system. The roots, devoid of any soil particles after the sonication step, were segregated and transferred to tubes with fresh autoclaved phosphate buffer (pH=6.5). Surface sterilization was carried out by treating with 50% sodium hypochlorite for 2 min with shaking, followed by 70% ethanol for an additional 30–60 s. To remove any lingering chemicals, the samples were rinsed three times with sterile ultrapure water. Later, the roots were macerated and used for deoxyribonucleic acid (DNA) isolation (McPherson et al., 2018).

2.3 DNA extraction, nested PCR, and Illumina sequencing

Metagenomic DNA extraction from the samples was carried out using the DNeasy Power Soil Pro Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA quality was assessed using the Qubit assay (Invitrogen, ds High sensitivity Assay Cat# Q32854) and agarose gel electrophoresis. Using a nested polymerase chain reaction (PCR) approach, the metagenomic DNA was first amplified using primers 16SF: 5'-AGAGTTTGATCCTGGCTCAG'-3, 16SR: 5'-GGTTACCTTGTACGACTT'-3 targeting the 16S region (1500 bp). Later, the V3–V4 region (460 bp) was amplified using the primers V3–V4F: 5'-CCTACGGGNGGCWGCAG'-3 and V3–V4R: 5'-GACTACHVGGGTATCTAATCC'-3. The NEBNext Ultra Library Prep Kit for Illumina (NEB, Cat# E7370L) was utilized for the library preparation and the sequencing was carried out in an Illumina MiSeq (Illumina Inc., United States) platform at MedGenome Labs Pvt. Ltd. (Bengaluru, India) as described by Caporaso et al. (2012).

2.4 Sequence data processing and statistical analysis

The read quality of the raw data obtained from the sequencing provider was analyzed using FastQC version 0.12.1 (Andrews, 2010), which is efficient in spotting potential problems with high-throughput sequencing data. This was followed by primer and adapter trimming

using Trimmomatic 0.39 (Bolger et al., 2014) in the paired-end mode to remove low-quality sequences (Phred quality score – Q-score < 20). The Q-score measures the base calling accuracy in the Illumina sequencing. Selecting a Q-score of greater than 20 would reduce less errors in the obtained reads. The paired-end reads were then overlapped and stitched to form longer reads greater than 400 bp using the NG-merge software 0.3, which corrects errors and ambiguous bases better than other merging programs (Gaspar, 2018). The stitched reads were further processed using the DADA2 pipeline (version 1.16) (Callahan et al., 2016) with default parameters. Further analysis methods were adopted from recent microbiome studies (Hirpara et al., 2024; Nair et al., 2024). Briefly, read quality and error rate correction were performed on single long stitched reads, followed by chimera removal to obtain amplicon sequence variants (ASVs). The ASVs were assigned taxonomy using the naive Bayesian classifier method implemented in DADA2, with a sequence similarity of 97% against the Silva 138.1 prokaryotic small subunit (SSU) taxonomic training dataset. Further analysis was performed using various data analysis packages in R (version 4.1.2) (R Core Team, 2013). The phyloseq object was constructed from the processed ASV table, taxa table, sample metadata, and tree file using phyloseq v.1.38.0 (McMurdie and Holmes, 2013). Later, in the phyloseq object, the reads were filtered to remove mitochondrial and chloroplast-derived sequences. The reads were then rarefied to an even sequencing depth to normalize library size differences. Statistical analyses and plotting were conducted using the *microeco* package (version 0.19.0) in R (Liu et al., 2021). For alpha diversity, beta diversity, and all taxonomy-associated plots, significant differences were tested using the Wilcoxon rank-sum test and permutational multivariate analysis of variance (PERMANOVA), with *p*-value correction by FALSE DISCOVERY RATE (FDR) (Benjamini and Hochberg, 1995). The functional prediction was carried out using the R package *Tax4Fun2*, which is memory efficient in predicting habitat-specific functional profiles and has higher accuracy than PICRUST and Tax4Fun (Wemheuer et al., 2020).

2.5 Isolation of abundant bacterial endophytes from the roots of *Piper colubrinum*

The surface sterilized root tissues of *Piper colubrinum* were macerated aseptically in 1 mL of phosphate-buffered saline solution (pH 7.4) with mortar and pestle. The diluted (10^{-1}) macerate (0.1 mL) was spread on nutrient agar and LB agar plates. The agar plates were incubated at 28°C for 2–3 days. Morphologically different bacterial colonies that were abundant in the plates were subcultured and pure cultures were maintained for further studies (Kollakkodan et al., 2017).

2.6 *In vitro* *Phytophthora* antagonism of the bacterial isolates

The bacterial isolates were screened for their potential to inhibit *Phytophthora capsici* growth on potato dextrose agar using a dual culture plate assay. *P. capsici* mycelial plug was kept at the center of PDA plates and fresh bacterial cultures were streaked on either side of

the mycelial plug equidistantly after 2 days of mycelial growth. The plates were incubated at 28°C for 4–5 days to assess the ability to inhibit the mycelial growth based on the inhibition zone (Aravind et al., 2009; Hyder et al., 2020).

2.7 Molecular identification of the potential bacterial isolates

Genomic DNA from overnight-grown bacterial cultures was isolated using a Macherey-Nagel Bacterial DNA isolation kit () as per the manufacturer's instructions. The 16S rRNA gene was amplified using the primers 1-27F (GAGAGTTTGATCCTGGCTCAG) and 1495R (CTACGGCTACCTGTTACGA) manufactured by Sigma Aldrich, Germany (Hongoh et al., 2003). PCR was in 10 µL total volume 1X initiation cycle at 95°C for 5 min, 30X denaturation cycles at 95°C for 30 s, 30X primer annealing cycles at 55°C for 30 s, 30X extension cycles at 72°C for 2 min, and a 1X elongation cycle at 72°C for 5 min followed by termination at 4°C. Sequencing PCR of the PCR product was conducted using Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, United States) following the manufacturer's protocol. The sequencing of the purified products was conducted using the Sanger method in a 3730xL DNA Analyzer (96 capillary high-throughput DNA Sequencer) at the Genomics facility, Rajiv Gandhi Center for Biotechnology, Kerala, India.

2.8 Phylogenetic analysis

The sequences were used to identify closely related organisms in the National Center for Biotechnology Information (NCBI) BLASTn in the GenBank database¹ and EzTaxon Database (Chun et al., 2007). The phylogenetic tree was constructed using MEGA (version 11.0.13) using multiple sequence alignment by ClustalW and the neighbor-joining method was used with a bootstrap value of 1,000.

2.9 Qualitative plant growth promotion assays of potential bacterial isolates

The following plant growth promotion traits of the bacteria with biocontrol potential were evaluated: indole acetic acid (IAA) production, siderophore production, hydrogen cyanide (HCN) production, and β -1-4-glucanase activity. The methodology followed is briefly explained below:

2.9.1 IAA production

The isolates were incubated in 10 mL of nutrient broth containing 0.2% (v/v) L-tryptophan at 28°C for 10 days. Later, the culture was centrifuged for 20 min at 10000 rpm to check for the presence of IAA in the supernatant (Bric et al., 1991). After adding 2 mL of Salkowski reagent (35% HClO₄, 0.5 M FeCl₃) to 1 mL of culture supernatant, the tubes were incubated at 28 ± 2°C in the dark for 30 min. A color

change from yellow to pink indicates the presence of IAA in the medium.

2.9.2 Siderophore production

Blue Chrome Azur S agar medium was used to test for siderophore production (Louden et al., 2011). Briefly, Blue Agar-CAS medium was made with 100 mL of blue dye, 30 mL of filter-sterilized 10% Casamino acid solution, 10 mL of 20% glucose, and autoclaved MM9 salt medium and agar [added with 32.24 g piperazine-N, N0-bis(2-ethane sulfonic acid (PIPES) at pH 6]. Overnight cultures of the isolates were streaked on the plates. A color change of the medium around the bacterial growth indicates siderophore production.

2.9.3 HCN production

Isolates were inoculated on nutrient agar plates supplemented with glycine at a concentration of 4.4 g L⁻¹. A sterile filter paper (Whatman No.1) saturated with picric acid (0.5%) and sodium carbonate (2%) was placed in the lid of the Petri plate facing the agar inoculated with the fresh cultures of the isolates. The color change of the filter paper from yellow to orange-red indicates HCN production (Lorck, 1948).

2.9.4 β -1-4-glucanase activity

β -1-4-glucanase activity was tested following the protocol of (Hendricks et al., 1995). Spot inoculation of the bacterial isolates was conducted on carboxymethyl cellulose (CMC) agar with Congo red as an indicator. A halo around the bacterial growth indicates positive for β -1-4-glucanase production.

3 Results

3.1 Bacterial abundance and diversity in rhizobiome of *Piper* species

A total of 6,088 good-quality bacterial ASVs were retained after filtering across all soil compartments of both the wild-resistant (*P. colubrinum*) and cultivated susceptible (*P. nigrum*) species from the rarefied datasets, which comprised 6,823 reads per sample. Venn diagram indicated the presence of unique and shared bacterial genera among *P. colubrinum*, *P. nigrum*, and bulk soil (Figure 1C). A total of 31.9% of bacterial genera were shared among wild-resistant and cultivated susceptible species and the bulk soil. *P. colubrinum* (10.2%) shared more bacterial genera with bulk soil than *P. nigrum* (5%), whereas both species shared 11.1% of bacterial genera among them. The distribution of unique bacterial genera varied, and it was found to be highest in bulk soil (18.9%), followed by *P. colubrinum* (15.1%) and *P. nigrum* (7.8%) (Figure 1C).

The Shannon and Chao1 indices showed a higher significant (* $p < 0.05$, Wilcoxon) alpha diversity in bulk soil compared to the rhizobiome compartments of both plant species. (Figures 1A,B). Considering the overall diversity and richness, *P. colubrinum* rhizobiome had higher bacterial diversity compared to that of *P. nigrum*, even though the differences were not statistically significant (Supplementary Table S2). Furthermore, compositional dissimilarities were assessed using principal coordinate analysis (PCoA) based on the Bray–Curtis distance. The findings exhibited significant variation (PERMANOVA; FDR, * $p < 0.05$) among the bacterial communities at

¹ <https://blast.ncbi.nlm.nih.gov/>

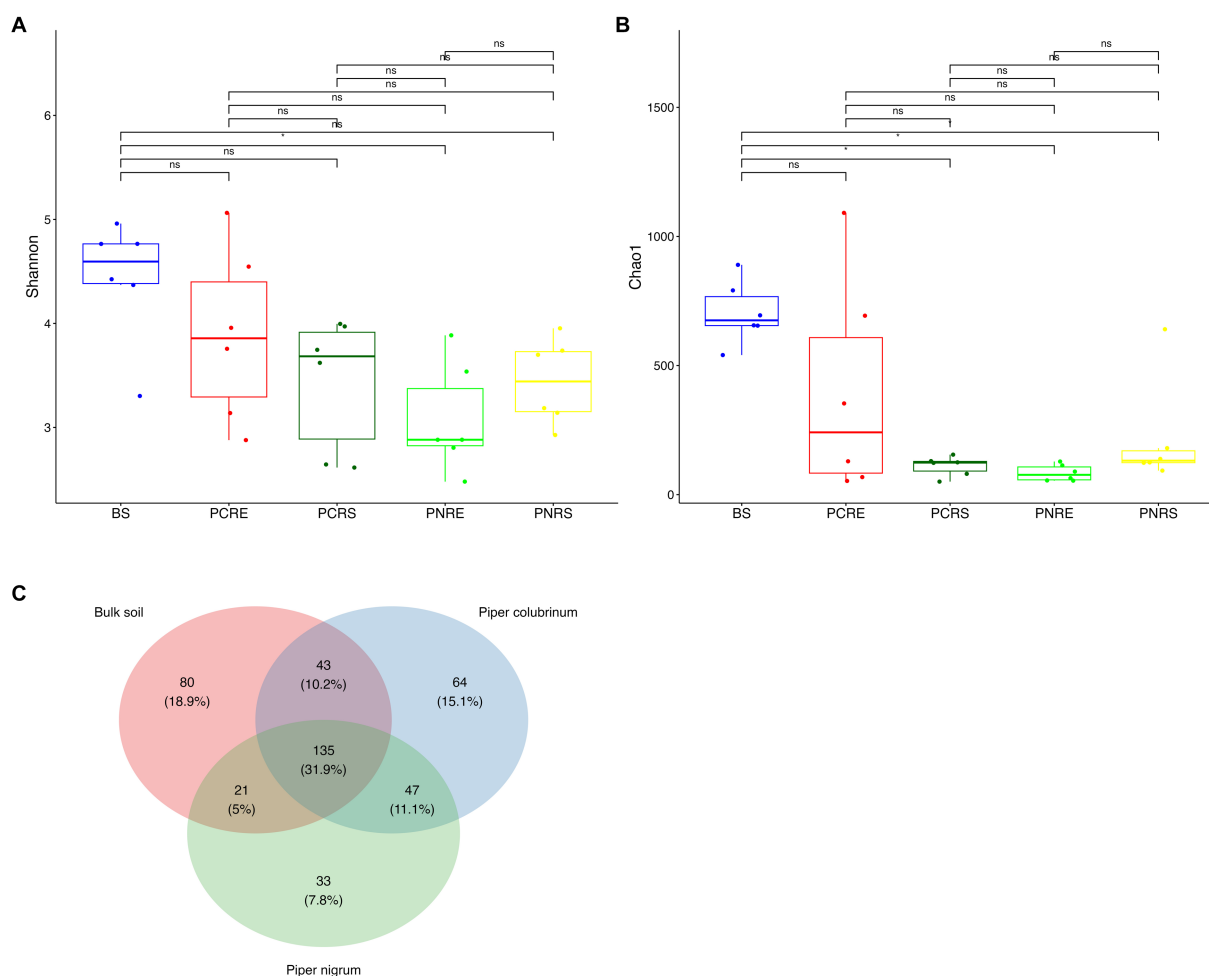


FIGURE 1

Box plots showing the distribution of alpha diversity indices of bacterial amplicon sequence variants (ASVs) (A) Shannon diversity index and (B) Chao 1 index (* $P < 0.05$; Wilcoxon test); (C) Venn diagram illustrating the total number and the shared genera in bulk soil, *Piper colubrinum* and *Piper nigrum* (BS: Bulk soil; PNRE: *Piper nigrum* root endosphere; PNRS: *Piper nigrum* rhizosphere soil; PCRS: *Piper colubrinum* rhizosphere soil; PCRE: *Piper colubrinum* root endosphere).

ASV as well as genus level across bulk soil and rhizobiome of *P. colubrinum* and *P. nigrum* (Table 1). There was an overlap in bacterial ASVs and genera among the rhizosphere and bulk soil compartments, while the endosphere communities remained distinct (Figures 2A,B). These results suggest a marked difference in bacterial community composition among the wild and cultivated *Piper* rhizobiome, with distinct closeness among rhizosphere and bulk soil communities compared to the endosphere.

3.2 Taxonomic composition of bacterial communities in bulk soil and rhizobiome of wild-resistant and cultivated susceptible *Piper*

The dominant phylum in bulk soil, as well as resistant and susceptible *Piper*, was *Proteobacteria*, constituting 76–93% of the bacterial community, followed by *Firmicutes* and *Bacteroidota*, which contributed an abundance up to 4–22% and 1–6%, respectively. The rhizospheric bacterial communities in *P. colubrinum* predominantly consisted of *Proteobacteria* (92.86%), while *P. nigrum* had them in

lower abundance (89.67%), along with *Firmicutes* (4.81%) and *Bacteroidota* (3.98%). In the root endosphere, the abundance proportion of the dominant phyla varied between the two plant species. Abundance was contributed by *Proteobacteria* (76.48%) and *Firmicutes* (22.56%) in *P. nigrum*. In the root endosphere of *P. colubrinum*, the abundance of *Proteobacteria* (93.16%) was higher, whereas *Firmicutes* (4.52%) and *Bacteroidota* (1.07%) were present in lower proportions compared to *P. nigrum* (Supplementary Table S3).

In phylum *Proteobacteria*, the abundance of class *Gammaproteobacteria* was highest among others in bulk soil and rhizobiome of *P. nigrum* and *P. colubrinum*, with a higher abundance proportion in rhizobiome of *P. colubrinum* (91–92%) than *P. nigrum* (75–88%). Furthermore, classification at class level showed *Negativicutes*, *Bacilli*, and *Bacteroidia* as the most abundant members among top 15 in the bulk soil and rhizobiome (Figure 3A). *Negativicutes* showed higher abundance in root endosphere of *P. nigrum* (16.9%) than *P. colubrinum* (2.5%) and *Bacteroidia* was found in higher proportions among the most abundant in rhizosphere of *P. colubrinum* (6.5%) (Supplementary Table S3).

At the genus level, the bacterial community members showed a varied abundance pattern in the bulk soil and rhizobiome. Genus

TABLE 1 Pairwise PERMANOVA statistics show significant differences (***p* < 0.01; **p* < 0.05) in the composition of bacterial communities assessed using PCoA.

ASV level	Groups	Measure	<i>F</i>	<i>R</i> ²	<i>p</i> -value	<i>p</i> . adjusted	Significance
1	BS vs. PCRE	Bray	2.8334	0.2208	0.0040	0.0057	**
2	BS vs. PCRS	Bray	2.8166	0.2198	0.0030	0.0057	**
3	BS vs. PNRE	Bray	4.2478	0.2981	0.0030	0.0057	**
4	BS vs. PNRS	Bray	2.8349	0.2209	0.0090	0.0090	**
5	PCRE vs. PCRS	Bray	1.9713	0.1647	0.0080	0.0089	**
6	PCRE vs. PNRE	Bray	2.2269	0.1821	0.0070	0.0088	**
7	PCRE vs. PNRS	Bray	2.9240	0.2262	0.0030	0.0057	**
8	PCRS vs. PNRE	Bray	4.1304	0.2923	0.0020	0.0057	**
9	PCRS vs. PNRS	Bray	2.8822	0.2237	0.0040	0.0057	**
10	PNRE vs. PNRS	Bray	3.7993	0.2753	0.0030	0.0057	**
Genus level							
1	BS vs. PCRE	Bray	6.8592	0.4069	0.0050	0.0075	**
2	BS vs. PCRS	Bray	6.0880	0.3784	0.0020	0.0075	**
3	BS vs. PNRE	Bray	10.1314	0.5033	0.0050	0.0075	**
4	BS vs. PNRS	Bray	6.7883	0.4043	0.0060	0.0075	**
5	PCRE vs. PCRS	Bray	6.2350	0.3840	0.0040	0.0075	**
6	PCRE vs. PNRE	Bray	3.7869	0.2747	0.0080	0.0089	**
7	PCRE vs. PNRS	Bray	8.5513	0.4610	0.0030	0.0075	**
8	PCRS vs. PNRE	Bray	10.0180	0.5004	0.0060	0.0075	**
9	PCRS vs. PNRS	Bray	2.3528	0.1905	0.0160	0.0160	*
10	PNRE vs. PNRS	Bray	11.1141	0.5264	0.0020	0.0075	**

(BS, Bulk soil; PNRE, *Piper nigrum* root endosphere; PNRS, *Piper nigrum* rhizosphere soil; PCRS, *Piper colubrinum* rhizosphere soil; PCRE, *Piper colubrinum* root endosphere).

Pseudomonas showed higher abundance in the rhizosphere (44–55%), followed by bulk soil (29%) and root endosphere (9–12%). The root endosphere of *P. colubrinum* had a higher percentage (12.75%) of genus *Pseudomonas* than that of *P. nigrum* (9.32%). In root endosphere, genus *Aeromonas* was seen in higher proportion in *P. nigrum* (25.07%) than *P. colubrinum* (3.7%) (Figure 3B). Altogether, these results show clear variation in abundance values of top abundant bacterial members of bulk soil, wild and cultivated *Piper*, with increasing variation in the abundance pattern at lower taxonomic ranks (genus).

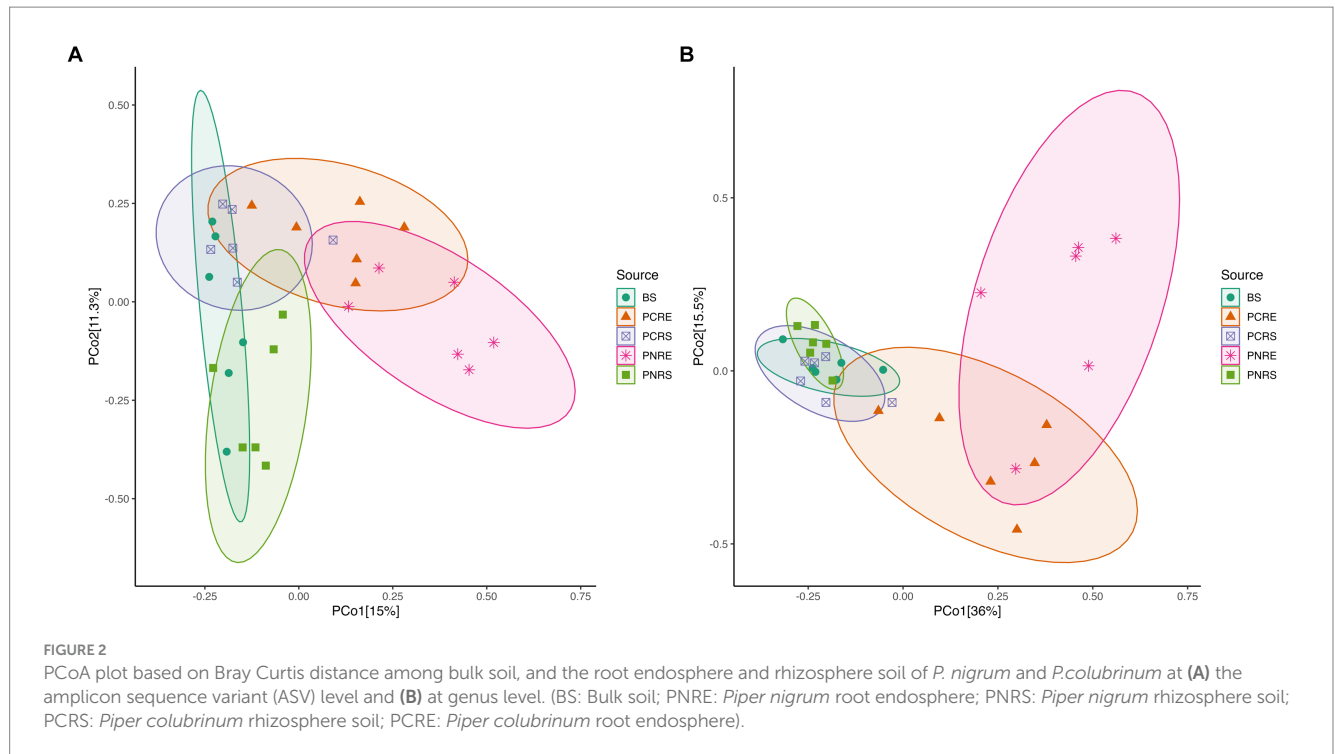
3.3 Differentially abundant bacterial rhizobiome communities in *Piper* species

To further investigate the significance of variation found in the abundance profile of bacterial communities, a differential abundance analysis was performed. The cladogram based on linear discriminant analysis (LDA) effect size (Lefse) analysis of the top 100 most abundant taxa revealed significant differences in the phylogenetic composition of bacterial communities in the rhizobiome of *P. colubrinum* and *P. nigrum*. Twenty-one bacterial clades were found differentially abundant (LDA score > 3.0) among the bulk soil and rhizobiome of *Piper* hosts across all taxonomic ranks, in which resistant *P. colubrinum* rhizobiome had more discriminatory clades than the susceptible *P. nigrum* (Figure 4). These included the genera *Comamonas* and *Variovorax*, which were significantly higher in the

P. colubrinum rhizobiome (Kruskal–Wallis test, **p* < 0.05), along with few plant growth-promoting bacteria such as *Variovorax gossypii* and *Pseudomonas capsici* (Supplementary Figure S3). Furthermore, differential abundance analysis of the bacterial communities using ANCOMBC-II showed that the relative abundances of a few bacterial genera differed significantly at the genus level between the rhizobiome of resistant *P. colubrinum* and susceptible *P. nigrum* (Supplementary Figure S4). In *P. colubrinum* rhizosphere, a significant difference in abundance was found only in the genus *Janthinobacterium* (**p* < 0.05), while other genera such as *Comamonas* and *Variovorax* were differentially abundant but not significant. In *P. nigrum* rhizosphere, *Pseudomonas* showed higher abundance values than *P. colubrinum*; however, the differences were not significant. (Supplementary Figure S4A). In the root endosphere, significant differences were also observed in the abundance values of *Comamonas* (***p* < 0.001) in *P. colubrinum* and *Aeromonas* (***p* < 0.01) in *P. nigrum* (Supplementary Figure S4B). Conclusively, the analysis suggests phylogenetic distinctness among bacterial communities in both *Piper* species, with significant variation in their abundance profile.

3.4 Functional signatures in the rhizobiome of *Piper* species

In metabolic pathways (level 2) predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, there was a higher abundance of pathway categories such as infectious diseases:



bacterial, glycan biosynthesis and metabolism, folding, sorting and degradation, nucleotide metabolism, carbohydrate metabolism, and energy metabolism in the root endosphere of *P. nigrum* compared to *P. colubrinum*, while these differences were not observed in the rhizosphere of these two plants. The metabolic pathway categories such as “Cellular community- prokaryotes” and “Biosynthesis of other secondary metabolites” were found to be highly abundant in *P. colubrinum* compared to *P. nigrum* (Supplementary Figure S5). Furthermore, a cluster analysis was performed for the top 100 KEGG Orthology (KO) functional orthologs among bulk soil, and *Piper* rhizobiome showed a higher abundance of functional orthologs in rhizosphere soil compared to bulk soil and endosphere. KOs such as chemotaxis proteins, osmoprotectants, and other transport systems showed higher abundance in the rhizobiome of resistant *P. colubrinum* compared to susceptible *P. nigrum* (Supplementary Figure S6).

3.5 Isolation, identification, and characterization of the abundant biocontrol genera from the resistant root endosphere

From the morphologically different colonies isolated, those isolates which exhibited maximum percentage inhibition of the oomycete pathogen *P. capsici* in dual plate assay were selected for characterization. They were further characterized phylogenetically and evaluated for their plant growth promotion traits. Among 12 morphologically different isolates, 3 efficiently inhibited *P. capsici* *in vitro* (Figure 5), and they were named B1, B2, and B3. The 16S rRNA gene sequencing and blast analysis in EzTaxon and NCBI showed maximum similarity percentage as B1 - *Pseudomonas aeruginosa* (87–89%), B2 - *Pseudomonas bananamidigenes* (91.58%)/*P. mosselii* (94.17%), and B3 - *Pseudomonas sichuanensis*

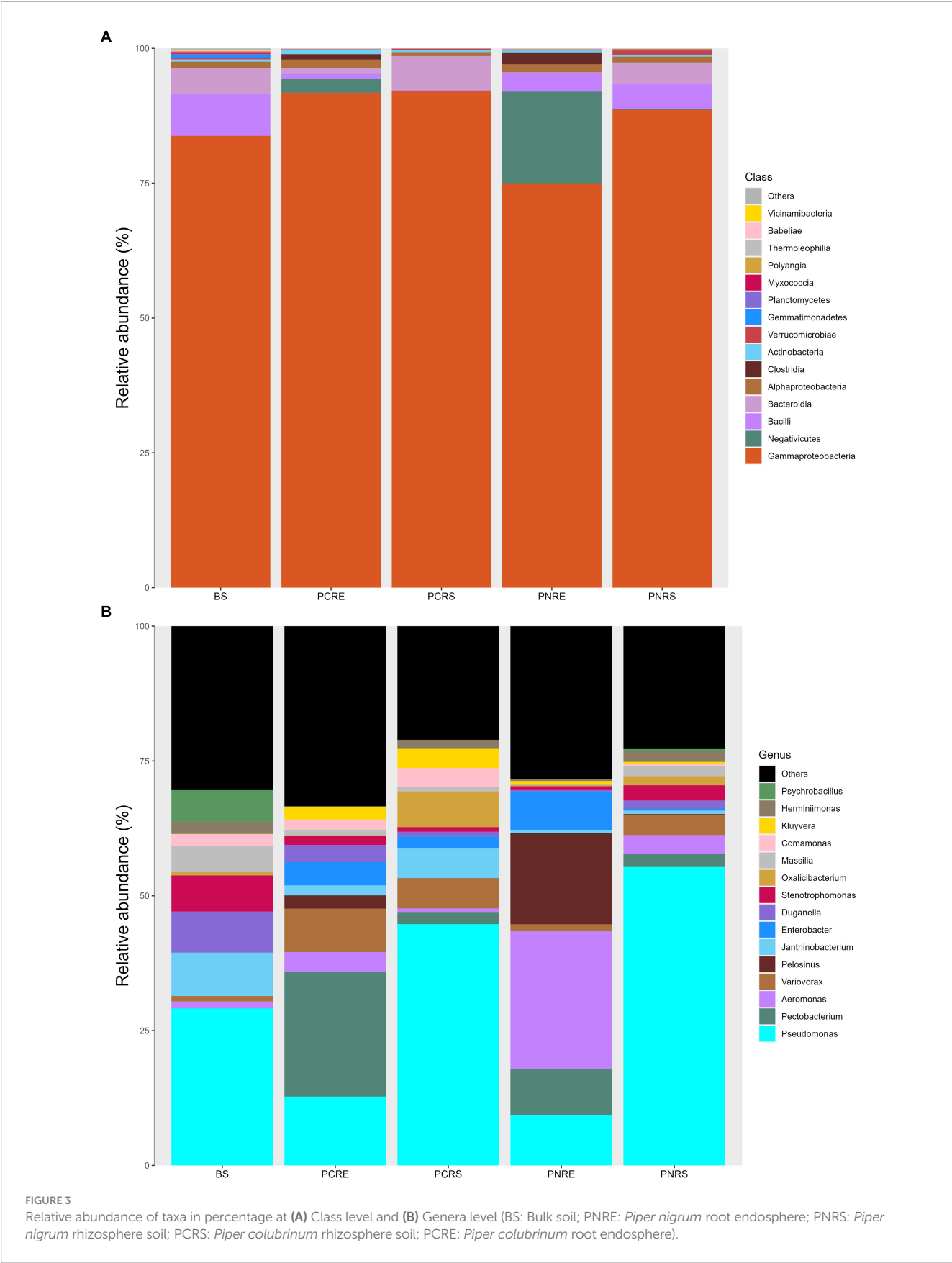
(94.24–94.65%), respectively. The sequences retrieved from EzTaxon and NCBI were used for phylogenetic analysis with MEGA (version 11.0.13). The three isolates (B1, B2, and B3) formed separate clades, indicating that they are different species and they have a paraphyletic relationship with other *Pseudomonas* species (Figure 6). Among the three, B1 exhibited a significantly high percentage inhibition potential against *P. capsici* (Figure 7).

3.6 Determination of IAA, HCN, β -1-4-glucanase, and Siderophore production

The qualitative assay for IAA production revealed that all three isolates were able to produce IAA as they produced a color change from yellow to pink at different intensities. Isolate B1 exhibited maximum IAA production (Figure 8A). HCN production was exhibited by isolates B2 and B3, where the yellow picrate filter paper changed to reddish-brown (Figure 8B). The isolates B1 and B3 produced a halo around the bacterial growth on CMC agar plates, which indicated the production of β -1-4-glucanase (Figure 8C). All the isolates were positive for siderophore production, where they exhibited a color change in the blue CAS agar medium (Figure 8D; Supplementary Table S4).

4 Discussion

The current study encompasses the comparative analysis of the composition and function of bacterial rhizobiome of the resistant and susceptible *Piper* species to reveal its role in plant–pathogen interactions. Venn analysis of the ASVs shared among the wild-resistant and cultivated susceptible rhizobiome revealed compositional



variation in the bacterial diversity (Figure 1C). It is driven by plant species differences even though a portion of the community retains the niche homeostasis (Shi et al., 2022). The higher bacterial diversity

indices in the rhizobiome of the wild-resistant *P. colubrinum* compared to the *Piper nigrum* are noteworthy. The enriched microbial community diversity in resistant plants compared to susceptible plants

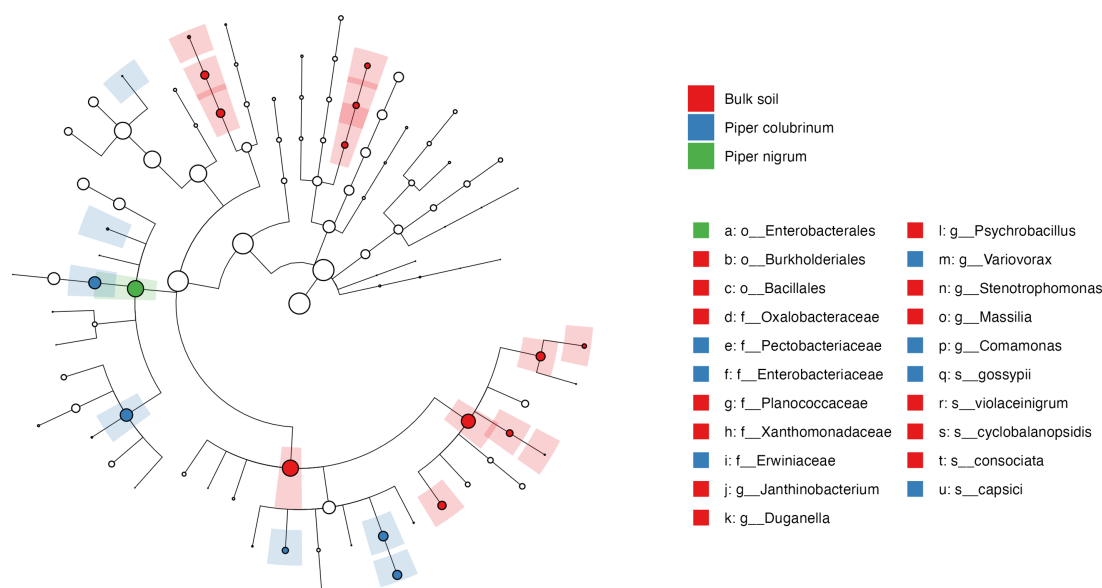


FIGURE 4
Cladogram of Linear discriminant analysis (LDA) effect size (LEfSe) analysis of microbial abundance in the bulk soil and the hosts.

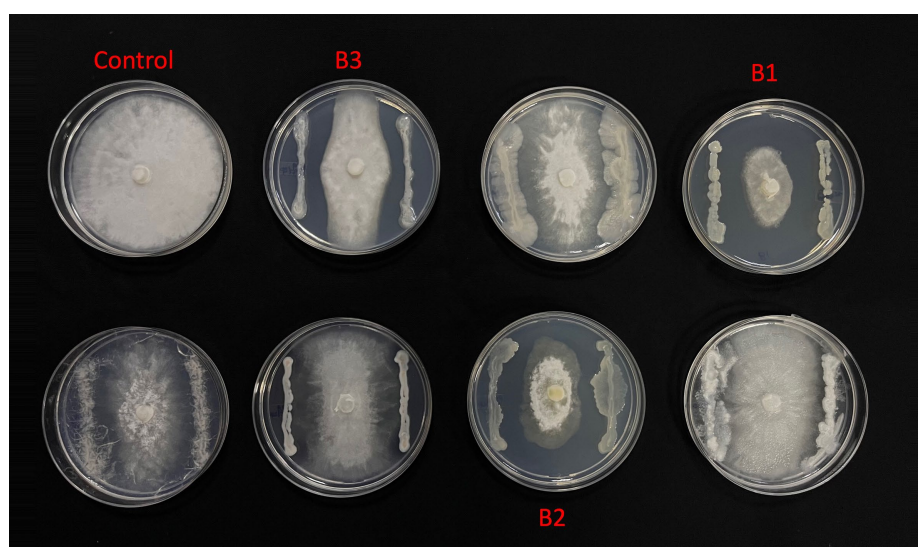


FIGURE 5
In vitro Phytophthora antagonism of bacterial isolates (B1: *Pseudomonas aeruginosa*; B2: *Pseudomonas mosselii*; B3: *Pseudomonas sichuanensis*).

has been discussed in earlier studies in tobacco (Shi et al., 2022) and tomato (Zhang et al., 2020). A study reported that the resistant cultivars had more bacterial operational taxonomic units (OTUs) as they exhibited higher Chao1 richness indices even though there were no significant differences in the alpha diversity between the resistant and susceptible cultivars (Wei et al., 2019). The alpha diversity was higher in the resistant rhizobiome of *P. colubrinum* compared to *P. nigrum* even though the differences were not significant (Figures 1A,B). Li et al. found that the rhizosphere microbial community significantly differs by distinctly clustering based on the resistance and susceptibility of the different wheat varieties to *Fusarium* head blight (Li et al., 2023a). The diversity, structure, and

composition of bacterial communities in the rhizosphere varied among potato scab-resistant and susceptible plant species (Kopecky et al., 2019). The PCoA analysis conducted on the bacterial community among the bulk soil and the rhizobiome of the *Piper* species revealed that the bacterial communities primarily clustered by the soil compartments as well as the species (Figure 2). Few studies revealed that the rhizosphere and bulk soil bacterial microbiome cluster together, while the endophytic communities cluster distinctly as it is highly dependent on the plant species (Afzal et al., 2019; Tkacz et al., 2020; Dutta et al., 2021). The root endosphere communities clustered distinctly between the susceptible and resistant *Piper* species (Figure 2). This finding remains consistent with the findings of Li et al.

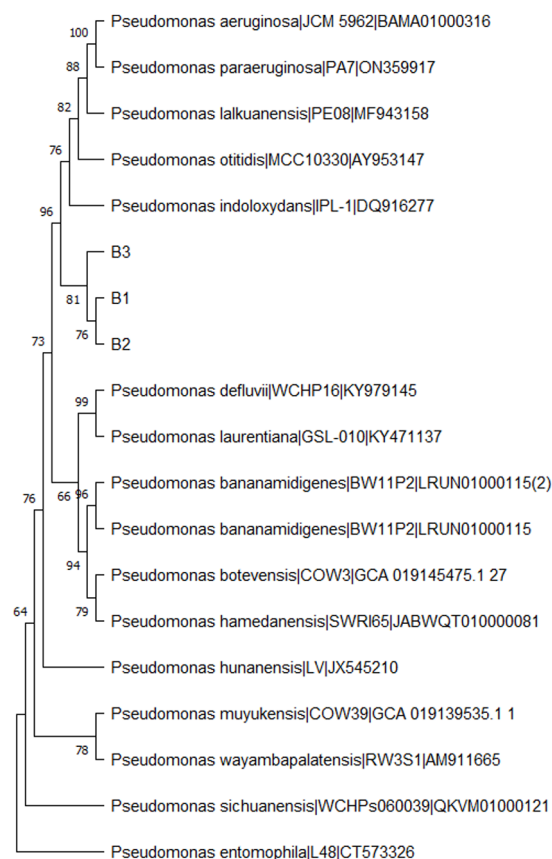


FIGURE 6
Phylogenetic tree of bacterial isolates (B1: *Pseudomonas aeruginosa*; B2: *Pseudomonas mosselii*; B3: *Pseudomonas sichuanensis*) constructed using MEGA (Version 11.0.13) by neighbour-joining method with a bootstrap value of 1000.

who found that the endophytic bacterial communities of the resistant peach cultivars clustered differently (Li et al., 2019). A recent study that explored the temporal dynamics of the rhizo-microbiome in eggplant discussed that the host plant recruits a diverse disease-suppressive rhizo-microbiome diversity which in turn influences its disease resistance (Zhang et al., 2024). Our diversity analysis falls in line with these studies as we could find a distinct diversity pattern in the bacterial rhizobiome of the wild-resistant and cultivated susceptible *Piper* species.

Phylum *Proteobacteria* are associated with a multitude of plant-associated bacteria endowed with the ability to fix nitrogen, cycle nutrients, stimulate plant development, and antagonize several soil-borne plant pathogens (Inderbitzin et al., 2018; Aqueel et al., 2023). The prominence of *Proteobacteria* in *P. nigrum* rhizosphere was evident in a study conducted at Yok Don national park in the Central Highlands of Vietnam (Tran et al., 2022). Obieze et al. characterized the abundance of the phylum *Proteobacteria* in the bacterial rhizosphere communities of cultivated black pepper (Obieze et al., 2023). The rhizobiome of *P. nigrum* and *P. colubrinum* showed the presence of *Proteobacteria* as the top abundant phylum (Supplementary Table S3). Acuña et al. reported the presence of *Proteobacteria* as the most abundant bacterial phylum in the rhizosphere and root endophytic compartments of wheat (Acuña et al., 2023). We found that the

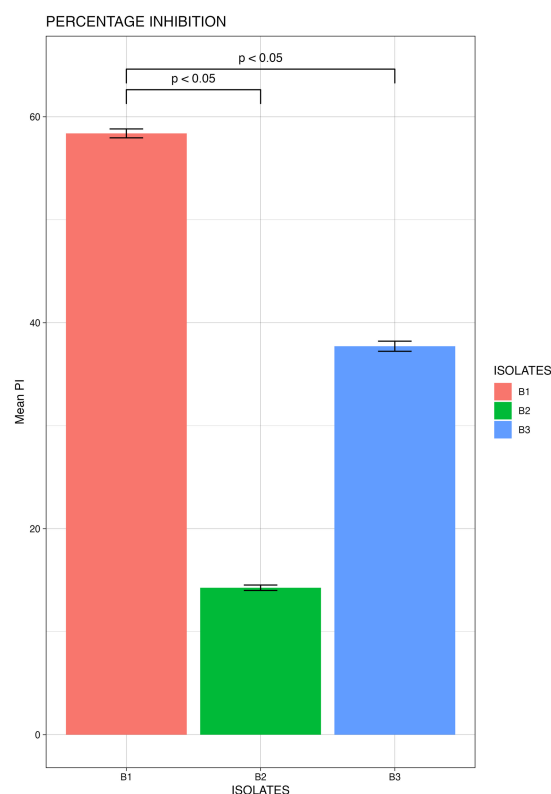


FIGURE 7
Mean Percentage inhibition *P. capsici* by the bacterial isolates (B1: *Pseudomonas aeruginosa*; B2: *Pseudomonas mosselii*; B3: *Pseudomonas sichuanensis*) (significance $p < 0.05$; mean of three replicates each $n = 3$).

higher abundance of *Proteobacteria* was realized in the rhizobiome of wild-resistant *P. colubrinum* which may indicate its role in disease resistance. *Firmicutes* and *Bacteroidota* (*Bacteroidetes*) were the other two dominant phyla of which *Bacteroidota* dominated the resistant rhizobiome. This is in line with the findings of Tang et al., in which they showed that the phylum *Bacteroidota* was associated with rhizosphere where there was reduced occurrence of fusarium wilt and was negatively correlated to disease incidence. This reveals its role in inducing soil-borne disease resistance to associated crop plants (Cheng et al., 2020; Tang et al., 2020). At the genus level, the bacterial diversity differences were evident and the prominent genera was *Pseudomonas*, especially in the root endosphere of *P. colubrinum* compared to *P. nigrum* (Figure 3). *Pseudomonas* has great significance as a plant root endophyte and it plays an important role in promoting plant health by stimulating plant growth and suppressing soil-borne pathogens (Lu et al., 2022; Qian et al., 2022). The relevance and abundance of *Pseudomonas* as a root endophyte in suppressing a soil-borne disease such as *Verticillium* wilt in cotton was discussed by Zeng et al. (2022). Endophytic *Pseudomonas* species from *Piper tuberculatum* was found antagonistic to the *Fusarium* root rot in black pepper (Da Costa Pereira et al., 2019). These studies support our findings on *Pseudomonas* as a dominant endophyte in the resistant *P. colubrinum* root endosphere which can be prospected as a potential biocontrol agent against the oomycete pathogen.

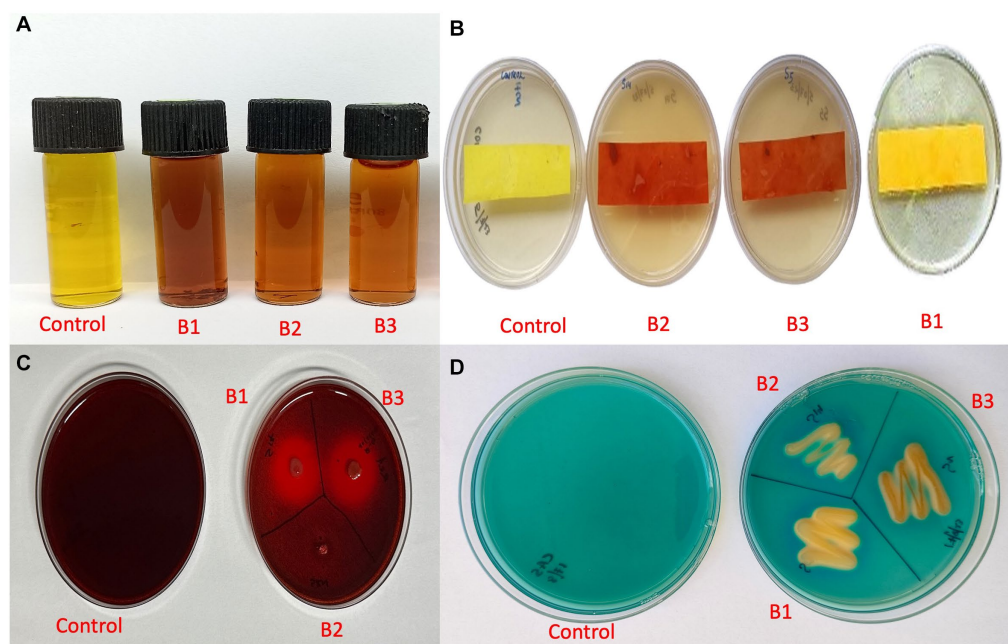


FIGURE 8

Qualitative assay for plant growth promotion traits (A) IAA production (B) HCN production (C) β -1-4-glucanase activity (D) Siderophore production (B1: *Pseudomonas aeruginosa*; B2: *Pseudomonas mosselii*; B3: *Pseudomonas sichuanensis*).

The phylogenetic distinctness and differential abundance analysis revealed other significantly different genera in the resistant *P. colubrinum* rhizobiome such as *Comamonas*, *Variovorax* (root endosphere), and *Janthinobacterium* (rhizosphere) (Supplementary Figures S3, S4). A prior study reported the increased abundance of *Comamonas* associated with reduced prevalence of *Phytophthora* blight in sweet pepper discussing its role in the biocontrol of soil-borne plant pathogens (Zhang et al., 2019). Booth et al. showed the potential use of *Comamonas* as a biopesticide against oomycetes pathogens in crops such as tomato, cabbage, and chickpea (Booth et al., 2022). The higher abundance of *Comamonas* was also associated with the healthy root endosphere of *Nicotiana tabacum* plants compared to *Ralstonia solanacearum*-infected plants (Li et al., 2023b). The genera *Variovorax* was associated with plant endophytic communities with potential biocontrol against *Phytophthora infestans* in grape vines (Bruissson et al., 2019; Vaghari Souran et al., 2023). *Janthinobacterium* present in rhizosphere soil displayed broad antagonism against the soil-borne oomycete pathogen *Pythium ultimum* and other fungal pathogens such as *Rhizoctonia solani* and *Fusarium graminearum* (Yin et al., 2021) and is known to produce antifungal compounds such as janthinopolyenemycins (Anjum et al., 2018). In our findings, we observed the significant differential abundance of these anti-oomycete genera in the resistant rhizobiome which indicates their potential role in the pathogen resistance in *P. colubrinum*.

In general, KEGG: level 2 metabolic pathway abundance showed a high degree of variability among the bulk soil, rhizosphere, and endophytic compartments of *P. colubrinum* and *P. nigrum* (Supplementary Figure S5). The pathway category “Cellular community- prokaryotes” is majorly related to quorum sensing and biofilm formation which significantly contributes to the colonization

and establishment of beneficial bacteria in the rhizosphere (Herrera et al., 2016) “Biosynthesis of other secondary metabolites” includes gene functions such as phenyl propanoid biosynthesis, and biosynthesis of many antimicrobial compounds. Many of these compounds have proven antifungal activity and are synthesized by beneficial bacteria such as *Pseudomonas* and *Janthinobacterium* (Anjum et al., 2018; Ackermann et al., 2021). Osmoprotectants are small organic compounds that aid in maintaining the cell turgor and safeguarding cellular structures during plant stress. The ATP-binding protein is important for the active transport of osmoprotectants into bacterial cells via the osmoprotectant transport mechanism (Mishra et al., 2022). Methyl-accepting chemotaxis proteins (MCPs) are essential sensory receptors in bacteria that enable them to recognize and react to changes in their surroundings. As a result of this, beneficial bacteria such as *Pseudomonas* can detect infections and react by either moving away from potentially hazardous circumstances or producing antimicrobial compounds that hinder the dissemination of the pathogen (Feng et al., 2021; Ogura et al., 2021). KOs such as chemotaxis proteins, osmoprotectant, and other transport systems showed higher abundance in the rhizobiome of resistant *P. colubrinum* compared to susceptible *P. nigrum* (Supplementary Figure S6). Yang et al. studied the potential of the indirect manipulation of the plant disease resistance by metabolites that trigger the interaction of disease-suppressive microbiota to suppress the soil-borne *Ralstonia solanacearum* (Yang et al., 2023). Hence, these plant-specific metabolites may have the potential to induce disease resistance by recruiting these beneficial microbiota. Our analysis of the functional potential of the rhizobiome of *P. colubrinum* and *P. nigrum* suggests plant species can modulate specific metabolic pathways and may lead to better survival and resistance against pathogens in *P. colubrinum* compared to *P. nigrum*.

This study is the first of its kind even though the culturable diversity is lower possibly due to the limitations in cultural conditions. Studies have recognized that the identification of bacteria through metagenomic screening does not essentially correlate with their cultivability (Tan et al., 2015; Tlou et al., 2024). However, in our study, the resistant *P. colubrinum* root endosphere harbored the dominant genus *Pseudomonas* as the most effective biocontrol agent against *P. capsici*, in line with the 16S rRNA amplicon data. A previous report suggested that the *P. colubrinum* housed more antagonistic endophytic bacterial genera than that of *P. nigrum* (Kollakkodan et al., 2017). The diversity of *Pseudomonas* in the root endosphere of *Piper* species was previously discussed in a few studies (Da Costa Pereira et al., 2019; Ashajyothi et al., 2020). The phylogenetic distinctness suggests the possible role of this genera in plant disease resistance. The biocontrol potential and plant growth promotion trait of *Pseudomonas* has been proven in many studies as discussed earlier (Da Costa Pereira et al., 2019; Lu et al., 2022; Qian et al., 2022; Zeng et al., 2022). This study is a first step toward exploring the root microbiome diversity of resistant *Piper* species in order to explore the role of these beneficial bacteria in conferring resistance against *Phytophthora capsici*.

5 Conclusion

In this study, we characterized the bacterial rhizobiome of resistant *Piper colubrinum* and susceptible *Piper nigrum* using metagenomic sequencing of the 16S rRNA gene. We compared the bacterial rhizobiome in these two plant species to ascertain the differences in their community composition, diversity, and function. Our hypothesis was based on the assertion that disease resistance in *P. colubrinum* may be contributed by the host genome along with its microbiome. We found significant differences in the bacterial community composition between the two species. In *P. colubrinum* rhizobiome, genera such as *Pseudomonas*, *Janthinobacterium*, *Variovorax*, and *Comamonas* were found to be differentially abundant compared to *P. nigrum*. Functional prediction revealed the abundance of KEGG pathways and KOs, which specifically support the colonization and deployment of beneficial bacteria with biocontrol activity in *P. colubrinum* rhizobiome. Our study confirms that the susceptible (*P. nigrum*) and resistant (*P. colubrinum*) species could harbor distinct bacterial genera in their rhizobiome although growing in the same environmental conditions. These findings could probably indicate the significance of plant species in shaping its rhizobiome. The culturable diversity of the resistant root endosphere, which harbors efficient biocontrol agents such as *Pseudomonas*, strengthens our hypothesis about the possible role of root microbiome in conferring resistance against soil-borne pathogens. Furthermore, this study lays the groundwork for gaining mechanistic insights into the plant species specificity to the bacterial rhizobiome in the *Piper* genus. These findings can be further substantiated by assessing the potential of the culturable isolates for plant growth promotion and biocontrol traits. However, to obtain a broader pattern of the plant–rhizobiome interaction, studies should also investigate the other underexplored species. To expand upon this study, future research could identify keystone taxa for microbiome engineering

that can supplement resistance breeding approaches. Ultimately, these efforts could facilitate sustainable agriculture through microbiome-based plant breeding to mitigate crop diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/>, PRJNA991100.

Author contributions

AP: Conceptualization, Writing – review & editing, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. RS: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – review & editing. ES: Conceptualization, Writing – review & editing, Funding acquisition, Project administration, Supervision.

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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/fmicb.2024.1458454/full#supplementary-material>

FIGURE S1

Sampling location (Site A and B) at Western Ghats, Kerala, India.

FIGURE S2

Field Sampling and collection.

FIGURE S3

LDA score assessments of the size of differentiation among bulk soil and the hosts (score threshold 3).

FIGURE S4

Differential abundance analysis of bacterial genera in (a)Rhizosphere and (b) Root Endosphere in Piper spp. (ANCOM-BC; *P<0.05) (PNRE: Piper nigrum root endosphere; PNRS: Piper nigrum rhizosphere soil; PCRS: Piper colubrinum rhizosphere soil; PCRE: Piper colubrinum root endosphere).

FIGURE S5

Heat map analysis of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways at LEVEL 2(BS: Bulk soil; PNRE: Piper nigrum root endosphere; PNRS: Piper nigrum rhizosphere soil; PCRS: Piper colubrinum rhizosphere soil; PCRE: Piper colubrinum root endosphere).

FIGURE S6

Heat map analysis of top 100 predicted Kyoto Encyclopaedia of Genes and Genomes (KEGG) functional orthologs (BS: Bulk soil; PNRE: Piper nigrum root endosphere; PNRS: Piper nigrum rhizosphere soil; PCRE: Piper colubrinum root endosphere; PCRS: Piper colubrinum rhizosphere soil).

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Possible biocontrol of bacterial blight in pomegranate using native endophytic *Bacillus* spp. under field conditions

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Bacterial blight in pomegranate, caused by *Xanthomonas citri* pv. *punicae* (Xcp), is one of the most devastating diseases, leading to substantial economic losses in pomegranate production. Methods for blight management in pomegranate production are scarce and not well established. To date, the major control strategy is targeting the pathogen with antibiotics and copper-based compounds. However, excessive use of antibiotics has resulted in the development of antibiotic resistance in the field population of Xcp. Hence, as a means of eco-friendly and sustainable management of bacterial blight, the use of native endophytes was investigated under field conditions in the current study. Endophytic bacteria were isolated from micro-propagated nodal explants of pomegranate and were identified as *Bacillus haynesii*, *B. tequilensis*, and *B. subtilis*. They were found to produce volatiles that inhibited Xcp growth during *in vitro* antibiosis assay. GC-MS-based volatile profiling revealed the presence of several bioactive compounds with reported antimicrobial activities. These endophytes (CFU of 10⁸/mL) were then spray-inoculated on leaves of 6-month-old pomegranate plants in the polyhouse. They were found to induce ROS-scavenging enzymes such as catalase and peroxidase. This alteration was a manifestation of host tissue colonization by the endophytes as ROS scavenging is one of the mechanisms by which endophytes colonize the host plants. Furthermore, two-season field trials with endophytes for blight control resulted in a reduction of disease index by 47–68%, which was considerably higher than the reduction due to the chemical immune modulator (2-bromo-2-nitro-1, 3-propanediol) currently being recommended for blight control. In addition, these endophytes also exhibited reduced sensitivity to this immune modulator; thus, the current study advocates the use of *B. haynesii*, *B. subtilis*, and *B. tequilensis* as biocontrol agents for bacterial blight of pomegranate either alone or as a part of integrated disease management.

KEYWORDS

Bacillus spp., biological control agents, integrated disease management, *Punica granatum* L., volatile organic compounds, *Xanthomonas citri* pv. *punicae*

1 Introduction

Pomegranate (*Punica granatum* L.), also known as the “Fruit of Paradise,” has edible red arils that are highly rich in anti-oxidants and have proven health benefits (Seeram et al., 2006; Viuda-Martos et al., 2013). These benefits include reduction of inflammation, muscle damage, and an increase of platelets blood levels to name a few. In addition to edible parts, non-edible

parts of the tree can also be used commercially owing to their several beneficial properties, for example, peel powder can be used as a food preservative (Lacivita et al., 2021; Giri et al., 2024). Owing to its innumerable benefits and uses, the global pomegranate market is expected to expand to USD 33.86 (by 2028) from USD 24.8 billion (2021). Furthermore, due to its adaptability to a wide range of weather and soil conditions, fresh pomegranate fruit is available in India all throughout the year. Therefore, India has become one of the global leaders in pomegranate production. Currently, India ranks seventh in the production of pomegranate in the world with a total area under cultivation of approximately 275,500 hectares, producing 3,215,000 metric tons of pomegranate fruits (DA& FW, GoI, 2023). Notably, in 2022–2023, 62,280 metric tons of pomegranates worth USD 58.36 million were exported from India (APEDA, 2024). Consequently, pomegranate plantation has played a very significant role in improving the socio-economic scenario of small landholders (2.5 million), particularly in the Indian states of Maharashtra, Karnataka, Gujarat, Rajasthan, and Andhra Pradesh. However, as with any other crop, pomegranate production is threatened by several pests and diseases due to the unavailability of any resistant germplasm.

One of the most devastating diseases of pomegranate is bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Syn = *Xanthomonas citri* pv. *punicae*: Xcp), which was first reported in India in the 1950s (Hingorani and Mehta, 1952). The bacterial blight symptoms mostly appear on the fruits and leaves, sometimes the stem too, leading to damage to the whole plant and economic losses to the grower (Sharma et al., 2017). In India, bacterial blight, also called oily spot, can result in 60–80% and in some cases 100% losses in fruit yield (Sharma et al., 2022a). Until now, the main strategy for bacterial blight control in pomegranate production relied upon the application of antibiotics and copper-based chemical compounds (Benagi et al., 2012; Sharma et al., 2022b). Extensive use of antibiotics in agriculture may, however, cause a reduction in the efficacy of antibiotics by facilitating the emergence of antibiotic resistance among the pathogens. For instance, *Erwinia amylovora* has been reported to have developed antimicrobial resistance in a number of geographic locations where antibiotics have been used indiscriminately in apple and pear orchards (Tancos et al., 2016). The impact of streptomycin-resistant *E. amylovora* in orchards remains a subject of ongoing scientific debate (Förster et al., 2015). Recently, reduced sensitivity to antibiotics and copper compounds in the field populations of pomegranate bacterial blight pathogen has also been reported from India (Krishna et al., 2020; Sharma et al., 2022a). Moreover, the majority of antibiotics are non-specific, meaning they not only act against the target pathogen but may also affect other beneficial bacteria naturally present in or on the plants (Daisley et al., 2022). Consequently, the Government of India issued a draft gazette notification on “Prohibition of Streptocycline (Streptomycin sulfate 90% w/w + Tetracycline hydrochloride 10% w/w) in agriculture and horticulture” dated 17 December 2021, released by the Ministry of Agriculture and Farmer’s Welfare. The notification imposes a ban on the manufacture and import of this product, w.e.f. with effect from 1 February 2022, with the ban on its use set to take effect from 1 January 2024. This gazette was brought out by the government because of the growing concerns over the risk of the development of resistance to these antibiotics in human beings and animals. Thus, the development of efficient and eco-friendly technologies focused on the elimination or reduction of the application of antibiotics in agriculture is highly

desirable. In addition, globally the export of residue-free fruits is highly promoted, therefore providing an impetus to find new alternatives for organic production of pomegranate.

One such promising approach is the utilization of microbes for the biological control of pathogens (Berg and Hallmann, 2006; Morales-Cedeño et al., 2021). Biocontrol agents (BCA) can mediate plant protection through direct mechanisms such as the production of secondary metabolites (Pretali et al., 2016) or hydrolytic enzymes that have antagonistic effects on the growth and survival of plant pathogens (Oukala et al., 2021); or indirect mechanism of defense involving the phenomenon of induced systemic resistance (ISR) (Wei et al., 1991), through jasmonic acid/ethylene pathway (Kannoja et al., 2019). ISR induction occurs when microbe-associated molecular patterns (MAMPs) of the BCAs are recognized by the plant/host and a defense response is mounted (Pieterse et al., 2014). This type of resistance is durable because the chances of the development of resistance in a pathogen against this type of resistance are very low as the pathogen does not directly interact with the BCAs or the resistance-stimulating agent (Romanazzi et al., 2016). Consequently, more recently attention has been focused on the beneficial aspects of endophytes and the application of these endophytes as BCAs (Backman and Sikora, 2008; Strobel, 2018; Collinge et al., 2022). Endophytes, derived from the Greek words “endo” meaning “inside” and “phyte” meaning “plant,” are symbiotic groups of microorganisms that colonize the internal tissues of plants asymptotically (Nair and Padmavathy, 2014; Santoyo et al., 2016). These endophytes, apart from being effective BCAs, also have plant growth-promoting (PGP) activities through (i) production of enzyme 1-Aminocyclopropane-1-carboxylase (ACC) deaminase, which is the precursor to plant growth regulator ethylene, hence reducing the levels of ethylene; (ii) nutrient acquisition such as nitrogen, phosphate, potassium, and iron; and (iii) synthesis of phytohormones such as auxin, cytokinin, and GA (Santoyo et al., 2016). A significant range of endophytic bacteria and fungi have been isolated from a variety of plants and shown to have a number of positive effects on the host plant (Bacon and Hinton, 2007; Manjunatha et al., 2022; Salvi et al., 2022). Such benefits embrace the lessening of chemical fertilizer usage, the enhancement of soil quality, the conservation of the environment, and sustainable agriculture. The well-studied and most abundant organisms isolated from plant tissues belong to Actinobacteria, Proteobacteria, and Firmicutes and include members of the genera *Streptomyces*, *Pseudomonas*, *Azoarcus*, *Enterobacter*, *Burkholderia*, *Stenotrophomonas*, and *Bacillus* (Malfanova et al., 2013; López et al., 2018).

Horticultural crops, including vegetables and fruits, harbor endophytic bacteria (*Pseudomonas* and *Bacillus*), and many of them have been reported to possess antimicrobial properties against a wide range of pathogens (Ray et al., 2016; Pavithra et al., 2021). Despite adequate knowledge of the diversity of bacterial endophytes in fruit crops, studies on their biocontrol potential against pomegranate disease are scarce. In one of the studies, the antagonistic activity of endophytic bacteria or fungi isolated from different wild genotypes or cultivars of pomegranate was tested against Xcp (Karn et al., 2022), and in another study, the endophytes were isolated from pomegranate roots and tested against wilt causing pathogen (Maruti and Sriram, 2021). However, both these studies were preliminary as they focused only on the *in vitro* antagonism. More recently, *in vitro* and *in planta* antagonistic effects of bacterial endophytes against Xcp, the most dreaded bacterial pathogen of pomegranate, were reported (Singh

et al., 2023). It was the first report of endophytes isolated from micro-propagated pomegranate plants, and the isolates could inhibit the pathogen *in vitro* and under polyhouse conditions. However, there are still no reports of utilization of endophytes for biocontrol of *Xanthomonas citri* pv. *punicae* under field conditions. Moreover, none of the abovementioned studies deciphered the probable mechanism by which the endophytes exert their antagonistic effect on the pathogen.

Therefore, the current study was undertaken to decipher the probable mechanisms behind the biocontrol action of endophytes and to evaluate their effectiveness against *X. citri* pv. *punicae* under field conditions. Specifically, the study aimed to: (i) isolate and identify endophytes with potential biocontrol activity against the bacterial blight pathogen; (ii) assess the antagonistic activity of volatile compounds produced by these endophytes against the pathogen using an *in vitro* approach, and profile the volatile organic compounds (VOCs) through GC–MS to identify the antimicrobial compounds; (iii) evaluate the alteration in the host's biochemical response upon endophyte inoculation and confirm *in vivo* host colonization by the endophytes; (iv) conduct on-field trials to assess the effectiveness of endophytic bacteria against bacterial blight, as well as test the sensitivity of endophytic bacteria to bactericides and copper compounds (used in current practices for blight management) *in vitro*. Although the results of the present study advocate the use of potential bacterial endophytes for biocontrol of pomegranate bacterial blight under field conditions, it will be important to standardize the mode of application of these endophytes in the future for better results. In addition, there is a need to evaluate the scalability of this endophyte technology for commercial use and its long-term impact on pomegranate production.

2 Materials and methods

2.1 Isolation and selection of endophytic bacteria as biocontrol agent

Bacterial endophytes were isolated from tissue-cultured (TC series) pomegranate plants of Bhagwa variety in our previous study (Singh et al., 2023). Isolated pure colonies were then picked up and maintained as pure culture until further use. The isolates were tested for their antagonistic activity against the bacterial blight pathogen (*Xanthomonas citri* pv. *punicae*: Xcp) using *in vitro* dual culture assays (Supplementary Figure S1A) and under polyhouse conditions. On the basis of the screening results, three effective isolates were selected for further studies in the present investigation. These isolates exhibited equal to or more than 50% inhibition of pathogen growth *in vitro*, and under polyhouse conditions, they either completely checked the incidence of bacterial blight or reduced the severity of the symptoms. Pure cultures of these isolates have been deposited at a culture repository under the National Biodiversity Act, 2002 with accession numbers NAIMCC-B-03178 to NAIMCC-B-03180.

2.2 Molecular identification of potent endophytic bacteria

Effective bacterial endophytes were identified at the molecular level based on a partial 16S rRNA gene sequence. Cultures grown in

nutrient glucose broth (NGB) for 24 h with shaking at 120 rpm at $28 \pm 1^\circ\text{C}$ were used for genomic DNA (gDNA) isolation using HiPurATM Bacterial Genomic DNA Purification kit (HiMedia) as per the manufacturer's instructions. For each isolate, PCR was performed using good quality gDNA and amplified using universal primers for 16S rRNA region (F-AGAGTTTGATCCTGGCTCAG and R-GGTTACCTTGTACGACTT) (Patel, 2001) with initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s, and extension at 72°C for 1 min, followed by final extension at 72°C for 8 min (Manjunatha et al., 2023).

2.3 Phylogenetic analysis of potent endophytic bacteria

Phylogenetic analysis was carried out to confirm the identity and understand the genetic relatedness of the effective isolates with other bacteria belonging to the same genus and species. The 16S rRNA region amplified from bacterial endophytes and deposited at the GenBank NCBI with accession numbers ON629736, KY575578, and KY575582, along with reference sequences downloaded from the database was aligned using the MUSCLE algorithm. BLASTn search, limiting the search to type material, was performed, and the reference sequence with the maximum similarity was included in the phylogenetic analysis. Neighbor-joining trees were drawn, evolutionary distances were computed using the Tamura-Nei method, 1,000 bootstrap replications were performed, and the consensus tree was rooted to *Paenibacillus polymyxa* and reported. Since *Paenibacillus polymyxa* has been found to be phylogenetically distinct from *Bacillus* spp. (Ash et al., 1993), it was used as an out-group in the current study. This analysis involved 20 nucleotide sequences including three from the isolates examined in the current study. All ambiguous positions were removed for each sequence pair (pairwise deletion option) after which there were a total of 1,496 positions in the final dataset. Phylogenetic analyses were conducted in MEGA11 (Tamura et al., 2021).

2.4 Study of antimicrobial properties of the potent endophytic bacteria

2.4.1 Antibiosis test for production of inhibitory volatile compounds

Inhibitory effects of volatiles secreted by endophytic *Bacillus* spp. on the growth of Xcp were assayed using a method that prevented direct contact between the two microbes, thereby excluding any contact-dependent inhibitory effects of the endophytic bacteria on the pathogen. In this method, adopted from the study by Dennis and Webster (1971), two nutrient glucose agar (NGA) plates were used in which one of the plates was inoculated with the pathogen and kept on top of the other plate that was inoculated with the endophyte (Supplementary Figure S1B). Volatiles would be emitted by the endophytes in the upward direction; hence, the endophyte plate was kept below, and the pathogen plate was kept on top. For the control setup, one of the plates was inoculated with the pathogen, and the other plate contained un-inoculated NGA only such that if any effect is produced by the media components, it will be visible in the control

plates. The plates were sealed with parafilm and incubated at $28 \pm 1^\circ\text{C}$, making sure that the pathogen-containing plate was on top of the endophyte-containing plate and the growth of Xcp was monitored and recorded. The growth of Xcp was monitored in terms of (i) zone of inhibition in the area just above the endophyte-inoculated area, (ii) reduction in cells/colonies, and (iii) reduction in yellow pigment production.

2.4.2 Identification of antimicrobial compounds through gas chromatography–mass spectrometry

Solvent (methanol and ethyl acetate) extracts of *Bacillus* culture were subjected to GC–MS to identify their volatile organic compound profile. In brief, bacterial isolates were inoculated in nutrient glucose broth (NGB) and incubated at $28 \pm 1^\circ\text{C}$ for 24–48 h on a shaker incubator (120 rpm). When the OD_{600} was 1.5, then $1:10^5$ dilution was performed to obtain an initial inoculum of 200–300 CFU per 10 μL , and 10 μL of the culture was added in 50 mL NGB and incubated again after which volatile collection was performed. In brief, the culture flask was placed with a volatile collection assembly customized to have two lateral openings in an aseptic chamber (Nagrale et al., 2022). Volatile collection was carried out for 4 h, and volatiles were consecutively eluted in the desired solvent (methanol or ethyl acetate) in a final volume of 250 μL (Nagrale et al., 2022). GC–MS-QP2020 (Shimadzu) equipped with a DB5-MS column was used for volatile separation and spectrum analysis. The GC–MS columns and settings were as detailed in Nagrale et al. (2022). Identification of metabolites was performed based on mass spectra similarity with library spectra available at the National Institute of Standards and Technology (NIST-2014 version).

2.5 *In planta* experiments for the evaluation of host biochemical response upon endophyte inoculation

The effective endophytes were exogenously sprayed onto pomegranate plants, under polyhouse conditions. In brief, endophytes were inoculated in NGB and kept on a shaker incubator at 28°C for 24–48 h. The bacterial suspension was then diluted to 10^8 cells per ml ($\text{OD}_{600} \text{ nm} = 0.4$), of which approximately 25 mL solution was sprayed on each plant. Three biological replicates were used for each treatment, i.e., TC-4, TC-6, and TC-310 inoculated plants as well as the control (not inoculated with any endophyte). Host biochemical response was observed by measuring (i) the activity of enzymes involved in the anti-oxidative defense system, namely catalase, peroxidase (POD), and superoxide dismutase (SOD), and (ii) total phenolic content (TPC) in the host.

2.5.1 Total phenolic content estimation

Total phenols were extracted by crushing 0.5 g of leaf tissue in 5 mL of 30% ethanol. The sample was centrifuged at 10,000 rpm for 20 min, and the supernatant was collected. Five milliliters of 30% ethanol was added to 1 mL supernatant. The solvent was evaporated by incubating in a hot air oven at 60°C for 3 h. The sample was resuspended in water and used for TPC estimation (Waterhouse, 2002). TPC was measured against a standard curve of gallic acid with absorption at 765 nm, and gallic acid equivalents (GAEs)/g of dry plant material were calculated for control (un-inoculated) and treatment (endophyte-inoculated).

2.5.2 Enzyme extraction and activity estimation

Enzymes were extracted by crushing 0.5 g leaf tissue in 10 mL of 0.1% trichloroacetic acid (TCA). The sample was centrifuged at 10,000 rpm for 20 min, and the supernatant was collected as a source of enzymes.

Catalase activity was measured using a hydrogen peroxide (H_2O_2) standard curve with absorption at 240 nm (Aebi, 1984). One unit of enzyme activity was defined as the amount of enzyme required to decompose 1 μmole of H_2O_2 per min. Total peroxidase activity (POD) was measured using guaiacol as a standard with absorption at 470 nm (Chance and Maehly, 1955). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of guaiacol per min.

Superoxide dismutase (SOD) activity was measured following the nitroblue tetrazolium (NBT) reduction method (Dhindsa et al., 1981). The reaction mixture (3 mL) contained 50 mM phosphate buffer, pH 7.8, 26 mM methionine, 20 μM riboflavin, 750 μM NBT, and 1 μM EDTA. After adding enzyme solution (0.1%) and distilled water, the reaction was allowed to run for 15 min under 4,000 lx light. The absorbance was recorded at 560 nm, and one unit of enzyme activity was defined as the amount of enzyme that reduced the absorbance by 50% of the absorbance of no enzyme control.

Fold change, in enzyme activity or accumulation of TPC, between endophyte-inoculated (treatment) and un-inoculated (control) plants was calculated and finally reported.

2.6 Host colonization by the endophytes

The host colonization pattern of the endophytes was checked *in vivo*. In brief, pomegranate plants were sprayed with endophyte culture [CFU of $10^8/\text{mL}$ (absorbance at 600 nm ≈ 0.4)] in a polyhouse. Then, 25 mL of endophyte culture was sprayed manually on each plant, and plants that were not inoculated with this endophyte served as control. Leaves from inoculated plants were excised at 24-h intervals, and periodic isolations were taken to check the colonization pattern of the endophytes. The leaf tissues were cut into small pieces and surface-sterilized with sodium hypochlorite (1%) for 30 s and ethanol (70%) for 30 s followed by three rinses with sterile distilled water (Anjum and Chandra, 2015). The sterile leaf tissues were then placed onto nutrient glucose agar media containing Petri dishes. Water after the last rinse was also plated to confirm the endophytic nature of the organism. Isolations in the same way were also taken from control plants. The bacterial colonies obtained in treated plants that were different from those obtained in control plants were only considered; then, these were checked for their morphological similarities with TC-310 that was sprayed onto plants. On these two bases, the frequency of endophyte colonies was measured, and percent recovery was calculated. Furthermore, 16S sequencing was performed to confirm the isolation of endophytes.

2.7 Bio-efficacy of potent endophytic bacteria under field conditions

The effective endophytes were utilized for field trials at research farms of the ICAR-National Research Centre on Pomegranate (Hiraj, Solapur, Maharashtra, India) and compared to the performance of the immune modulator (2-bromo-2-nitro-1, 3-propanediol) and water

control. The research plot (H-22) was a 5-year-old (in 2022) pomegranate orchard cv. Bhagwa with a spacing of 4.5 m × 3.0 m. This plot was selected for the study and is dedicated to all ongoing bacterial blight-related studies at the center. No bacterial control sprays were applied throughout the trial period, up to harvesting. The season selected for the study was *Mrig* bahar (Rainy season), during which there is maximum incidence of bacterial blight naturally due to conducive weather conditions. The age of the plants in the research orchard was 5 years at the time of fruit regulation during the 2022 season trials.

A randomized block design experiment was carried out with five treatments, three replicates, and two plants per replicate. The treatments included T1: TC-4 (*B. haynesii*), T2: TC-6 (*B. subtilis*), T3: TC-310 (*B. tequilensis*), T4: immunomodulator (bactronol), and T5: control (water). Bacterial endophytes were grown in NGB overnight. The next day, the culture was used as a primary culture to inoculate larger volumes of NGB to obtain a final volume of the culture (6 L) with a total CFU of 10⁸/mL or higher (absorbance at 600 nm ≈ 0.4). This culture was sprayed onto plants, with 1 L of the culture or chemical solution sprayed per plant, and 0.3 mL of sticker was added to all solutions. In the case of the control, 1 L of water with 0.3 mL of sticker was sprayed. A total of five sprays, at an interval of 5 days each, were performed after fruit setting and when blight Percent Disease Index (PDI) reached 5% in at least one of the treatments. For observation of disease incidence and severity, five branches with maximum fruits on each branch were labeled, and data were recorded for only these branches. Hence, for one plant, the data were mean of five branches. Observations for disease incidence and severity on leaves and fruits were recorded weekly, and the percent disease index (PDI) was calculated using the formula given below (Sharma et al., 2017). To confirm the pathogen, isolation of Xcp was performed in the laboratory from infected leaves. Such field trials were conducted for 2 consecutive years (2022 and 2023) during *Mrig* bahar (May–June crop; second or monsoon flowering)

$$PDI = \left[\frac{\sum \text{Number of observation} \times \text{Severity grade}}{\text{Total observations} \times \text{Maximum grade}} \right] \times 100$$

2.8 Chemical sensitivity test

The selected endophytes were tested for their sensitivity to commercially available bactericides and other chemicals using a good diffusion assay (Holder and Boyce, 1994). These chemicals included the immune modulator: 2-bromo-2-nitro-1, 3-propanediol (0.5 g/L); and other copper-based compounds: copper hydroxide (2 g/L) and copper oxychloride (2 g/L). Their doses were chosen as per the recommendation of the center to pomegranate farmers for field application during bacterial blight management. In brief, culture suspension of bacterial colony with a CFU of 10⁸/mL was made in sterile distilled water and spread on NGA plates. A well of 6 mm was dug in the middle of the plate using a sterile cork borer, and the required volume (100 µL) of the test chemical was added to the well. The clear zone around the well, which was devoid of any bacterial growth, i.e., the zone of inhibition (ZOI), was measured using zone scales (HiMedia) after 24 h of growth at 28 ± 1°C in a BOD incubator.

Percent inhibition (PI) in the growth of endophytes on the chemical was calculated by the following formula and plotted.

$$PI = 100 - \left[\frac{\text{Diameter of colony on control plate} - \text{Zone of inhibition on treatment plate}}{\text{Diameter of colony on control plate}} \right] \times 100$$

2.9 Statistical analysis

All experiments were carried out with a minimum of three replicates with appropriate controls. For biochemical response, a *t*-test was carried out to determine whether the difference (fold change) between treatment and control was statistically significant at a *p*-value of < 0.05. For polyhouse experiments, five plants were taken per treatment considering each plant as one replicate, and the experiment design was completely randomized design (CRD). For field studies, three replicates per treatment were taken with two plants per replicate, and the experiment design was randomized block design (RBD). Statistical analysis was performed using WASP 2.0¹ (Jangam and Wadekar, 2004), and the critical difference was expressed at a *p*-value of ≤ 0.05.

3 Results

3.1 Isolation and selection of endophytic bacteria

Microbes isolated from plant endosphere, inhabiting the internal tissues in a symptomless manner, are known as endophytes. Such microbes can also be isolated from tissue-cultured plants and may have a diverse impact on the growth of hosts. In our previous study (Singh et al., 2023), we reported, for the first time, the isolation of endophytes from nodal segments of micro-propagated pomegranate plants. These endophytes (TC series) along with endophytes isolated from field-grown pomegranate plants (EB series) were tested for their antagonistic effects on the growth of bacterial pathogen *Xanthomonas citri* pv. *punicae* (Xcp) *in vitro* and *in planta*. We observed higher inhibitory effects were shown by TC series endophytes as compared to EB series endophytes both *in vitro* and *in planta*, for example, three endophytes, namely TC-4, TC-6, and TC-310 showed equal to or more than 50% inhibition of Xcp *in vitro*, while none of the EB series endophytes showed equal to or more than 50% inhibition. During *in planta* antagonistic assays, the endophytes were applied in prophylactic mode, i.e., sprayed prior to pathogen challenge inoculation at an interval of 8 days. Plants inoculated with TC-4 or TC-310 did not show any symptoms, indicating that these endophytes had controlled blight incidence completely, while plants inoculated with TC-6 exhibited lower disease incidence and severity. Based on these results, it was deduced that these three isolates showed varying mechanisms by which

¹ <https://ccari.icar.gov.in/wasp2.0/index.php>

pathogen's growth was inhibited, and therefore in the current study, these isolates and their inhibition mechanisms were investigated further.

3.2 Molecular characterization and identification of potent endophytic bacteria

To understand the nature of the antagonistic mechanism, it is important to first identify the bioagent. In the current study, bacterial endophytes were identified based on BLASTn homology search of 16S rDNA sequence followed by phylogenetic analysis. When we did BLASTn for TC-310 (KY575582), limiting the search to type material, the maximum similarity was obtained with *B. tequilensis* (NR104919) followed by another *B. tequilensis* (MN543830). Similarly, for the other isolate TC-4 (ON629736), the maximum similarity was obtained with *B. haynesii*, and for TC-6 (KY575578), it was obtained with *B. subtilis*. Although these results are more reliable as they are based on type material confirming the identity, we did not just rely on the BLASTn results. We further performed phylogenetic analysis and as shown in [Figure 1](#), TC-4 grouped separately with *B. haynesii* isolates, away from the clade containing *B. subtilis* and *B. tequilensis*, with a high bootstrap support of 98–99%. Hence, the identity of the isolates was further confirmed based on the phylogenetic relationship between effective endophytes and type strains' sequences retrieved from the NCBI database ([Figure 1](#)). All these sequences have been deposited at GenBank NCBI, and the pure cultures have been deposited at a designated microbial repository for agriculturally important microorganisms (AIMs) under the National Biodiversity Act, 2002 and a member of World Federation of Culture Collections (WFCC; [Table 1](#)).

3.3 Antimicrobial properties of the potent endophytic bacteria

An antibiosis test was performed using a method that prevented direct contact between the pathogen and endophyte. This means that whatever inhibition was observed in the growth of the pathogen could be due to the volatiles released by the endophytic bacteria. The antibiosis results revealed that the endophytes could inhibit the growth of the pathogen and reduce the production of yellow pigment ([Supplementary Figure S1C](#)). Thus, volatile organic compound (VOC) profiling of the isolates was performed using GC–MS. Indeed, GC–MS-based VOC profiles of the isolates (*B. subtilis* and *B. tequilensis*) were found to be composed of some really useful bioactive compounds, such as fatty acids and benzimidazole derivatives, which have reported antimicrobial activities. Some of these compounds also exhibit their anti-bacterial activity by inhibiting biofilm production by pathogens and interfering with quorum sensing of the bacteria ([Table 2](#); [Supplementary Figure S2](#)). Hexadecanoic acid methyl ester and Pyrrolo[1,2-a] pyrazine-1,4-dione hexahydro-3-(phenylmethyl) were identified in *B. subtilis* and *B. tequilensis*. N-acetyl-3-methyl-1,4-diazabicyclo [4.3.0] nonan-2,5-dione was identified in *B. tequilensis* only, while 2-(p-(Dimethyl amino) phenyl) benzimidazole and 9,9-dimethyl-Xanthene were identified in *B. subtilis* only.

3.4 Evaluation of host biochemical response upon endophyte inoculation

In our previous study, the effect of endophytes on host physiological parameters was studied. Therefore, in the current study, we further evaluated the effect of the exogenous application of endophytes on the anti-oxidant machinery of the host plants ([Figure 2](#)). It was found that the effect of endophytes on different enzyme activities varied. *B. haynesii* and *B. subtilis* upregulated catalase and peroxidase activity and downregulated SOD activity, while the trend was reversed in the case of *B. tequilensis*. The accumulation of total phenolics also differed under the influence of these endophytes. *B. haynesii*-treated plants accumulated more phenolics as compared to the control, while *B. subtilis* and *B. tequilensis* treatment caused a reduction in the levels of total phenolics as compared to the control. This reaffirmed that the endophytes exhibited their antagonistic effects via different mechanisms. Hence, the endophytes can be used to form a consortium to be used as a biocontrol agent against Xcp.

3.5 Host colonization by the endophytes

To validate any endophyte-mediated response, it is important to understand the colonization pattern. Since all the endophytes altered the host's biochemical response and host–pathogen interaction, we further validated the colonization of host leaf tissue by the endophytes following artificial inoculation. One of the most effective endophyte *B. tequilensis* (TC-310) was sprayed onto plants and recovered from leaves after periodic isolations. The endophyte could be recovered up to 7 days post-artificial inoculation of endophytes on host plants indicating their successful colonization. Hence, the observed changes in host biochemical response or reduction in blight incidence mediated by the endophytes can be attributed to the successful colonization by the endophytes ([Supplementary Figure S3](#)). However, more elaborate studies are required to illustrate colonization traits of the endophytic bacteria.

3.6 Bio-efficacy of potent endophytic bacteria under field conditions

The current study reports, for the first time, the successful application of endophytes for biocontrol of bacterial blight in pomegranate under field conditions ([Supplementary Figure S4](#)). Endophytic bacterial isolates (*B. haynesii*, *B. subtilis*, and *B. tequilensis*) were used for field trials against blight, which were conducted during *Mrig* bahar (May–June crop; second or monsoon flowering) for 2 consecutive years (2022 and 2023). The percent disease index (PDI) on pomegranate fruits ranged from 2.2 to 36.4% during season I (2022) and 5.6 to 17.7% during season II (2023) in the control ([Figure 3](#)). All the endophytes could reduce the disease in the range of 47 to 68% over the control. Interestingly, this reduction of disease over control was higher than the reduction due to chemical immune modulators in both seasons ([Supplementary Table S1](#)). PDI on leaves and stems remained below 1% throughout the trials during both seasons.

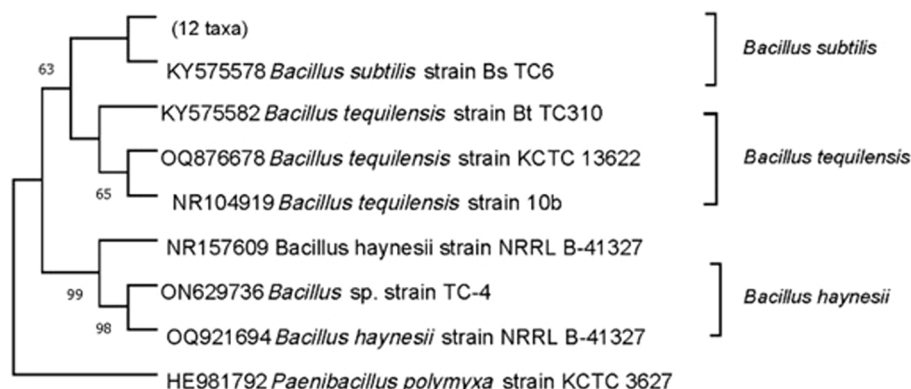


FIGURE 1

Molecular characterization and identification of endophytic bacteria used as biological agents in the study. Neighbor-joining tree depicting the phylogenetic relationship between effective endophytes (TC-4, TC-6, and TC-310) and type strains' sequences retrieved from the NCBI database. The Tamura-Nei method was used as the best model for constructing the phylogenetic tree with 1,000 replicates. The tree shown represents the bootstrap consensus tree inferred from 1,000 replicates, and numeric values on the branches indicate bootstrap support values in percentage. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. *Paenibacillus polymyxa* was used as an out-group.

TABLE 1 Details of the most promising endophytic bacterial bioagents against *Xanthomonas citri* pv. *punicae* causing bacterial blight of pomegranate.

Isolate code	Molecular identity	GenBank accession number	NAIMCC accession number*
TC-4	<i>Bacillus haynesii</i>	ON629736	NAIMCC-B-03178
TC-6	<i>Bacillus subtilis</i>	KY575578	NAIMCC-B-03179
TC-310	<i>Bacillus tequilensis</i>	KY575582	NAIMCC-B-03180

*National Agriculturally Important Microbial Culture Collection, NAIMCC, Mau, India.

3.7 Chemical sensitivity of the bacterial endophytes

All three endophytes showed reduced sensitivity with the chemicals such as immune modulator (2-bromo-2-nitro-1, 3-propanediol) and copper-based compounds (copper hydroxide and copper oxychloride) at their recommended doses (Supplementary Figure S5). *Bacillus subtilis* was found to be the least sensitive to all three chemicals with a maximum inhibition of 23% in 2-bromo-2-nitro-1, 3-propanediol. Similarly, 2-bromo-2-nitro-1, 3-propanediol inhibited the growth of *B. tequilensis* by 24%, and maximum inhibition was found in *B. haynesii* (33%). However, none of the chemicals exhibited growth inhibition of 50%, and hence, the endophytes can be called less sensitive to these chemicals (Figure 4).

4 Discussion

Xanthomonas genus containing 27 species can infect more than 400 plant hosts and hence is notorious for causing huge economic losses to agriculture and horticulture, worldwide (Ryan et al., 2011). The use of microbial biological control agents (BCAs) for biocontrol of such phytopathogens is one of the eco-friendly and sustainable approaches (Marin et al., 2019). However, in several examples of biocontrol of *Xanthomonas* spp., field studies have not been carried

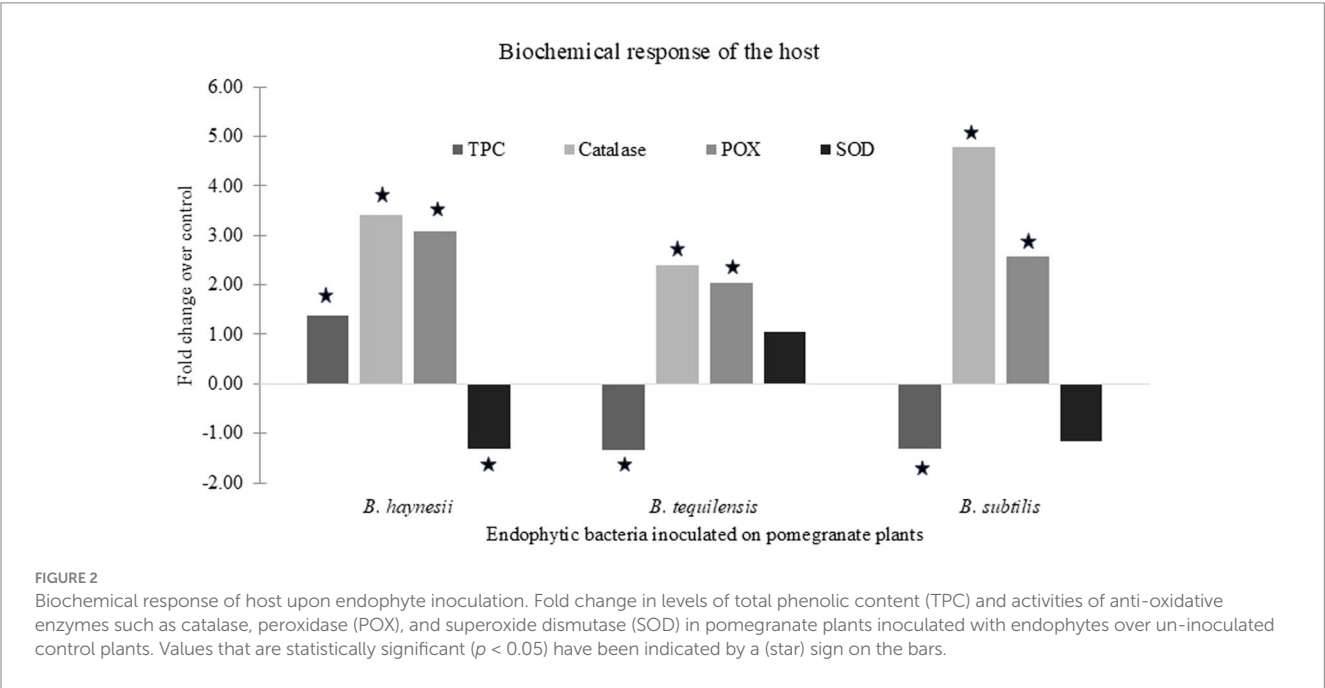
out. Therefore, the current study provides the first-ever successful on-field application of endophytic *Bacillus* spp. to control bacterial blight causing *X. citri* pv. *punicae* (Xcp), a deadly pathogen of pomegranate. The study also provides novel insight into the antimicrobial activity of these endophytic *Bacillus* spp.

Endophytes isolated from micro-propagated pomegranate plants were able to successfully reduce the bacterial blight incidence and severity in pomegranate under polyhouse conditions (Singh et al., 2023). In the current study, the mode of action of these isolates (TC-4, TC-6, and TC-310) that showed maximum inhibition of Xcp was further validated. Based on their genetic relatedness to the type material sequences, these endophytes were identified as *Bacillus haynesii* (TC-4), *B. tequilensis* (TC-310), and *B. subtilis* (TC-6) (Figure 1). While the majority of the studies have reported *B. subtilis* as a successful biocontrol agent, only a few studies have reported *B. tequilensis* as a biocontrol agent and that too mostly against fungal pathogens (Li et al., 2018; Xu et al., 2019; Baard et al., 2023). Very recently, Eltokhy et al. (2024) reported the capability of *B. haynesii* to produce antimicrobial metabolites, which were antagonistic to several multi-drug-resistant pathogens. Nevertheless, to the best of our knowledge, this is the first study where endophytic *B. haynesii* has been reported as a BCA against any bacterial pathogen in plants.

In the current study, the results of the dual plate antibiosis assays suggested that the endophytic *Bacillus* spp. produced volatiles that not only inhibited the growth of the pathogen but also reduced the production of the yellow pigment by the pathogen. The yellow xanthomonadin pigments produced by phytopathogenic *Xanthomonas* spp. are crucial for the epiphytic survival of the pathogen during pathogen–host interaction (Basu and Wallen, 1967; Poplawsky and Chun, 1998; He et al., 2020). Several studies report epiphytic colonization as the first step in *Xanthomonas* infection cycle. While the pathogen remains on the leaf surface epiphytically, it has to protect itself from UV rays (sunlight) and other oxidative stressors. These yellow pigments also provide protection to the bacteria against such oxidative stress (He et al., 2020). Hence, a reduction in pigment production by Xcp, under the influence of *Bacillus* spp. as observed in

TABLE 2 List of bioactive compounds identified in VOC profiles of bacterial endophytes.

Volatile organic compound	Retention time (min)	% Area	Chemical formula	Molecular weight (g/mol)	Bioactivity/role/function reported in literature
Hexadecanoic acid methyl ester (palmitic acid)	22.06	15 and 74%	C ₁₇ H ₃₄ O ₂	270.4507	Antimicrobial (Rajaofera et al., 2019)
N-acetyl-3-methyl-1,4-diazabicyclo [4.3.0] nonan-2,5-dione	23.14	3%	C ₁₀ H ₁₄ N ₂ O ₃	210.23	Anti-bacterial (Sharma and Thakur, 2020; Awan et al., 2023)
2-(p-(Dimethylamino)phenyl) benzimidazole	23.46	0.08%	C ₁₅ H ₁₅ N ₃	237.31	Anti-fungal (Wright, 1949; Beč et al., 2023)
Pyrrolo[1,2-a] pyrazine-1,4-dione hexahydro-3-(phenylmethyl)	23.91	1.7 and 9%	C ₁₄ H ₁₆ N ₂ O ₂	244.29	Antimicrobial: Inhibit bacterial biofilm formation, quorum sensing (Gopi et al., 2014; Kannabiran, 2016; Kiran et al., 2018; Maia et al., 2022)
9,9-dimethyl-Xanthene	21.32	0.02%	C ₁₅ H ₁₄ O	210.27	



the current study, indicates reduced survival fitness of the pathogen and compromised anti-oxidative activity.

The endophytic *Bacillus* spp. can have several ways of inhibiting the growth of the target pathogen, including the emission of volatiles (Bruissson et al., 2019) or induction of host defense response (Chandrasekaran and Chun, 2016; Oukala et al., 2021). In the current study, GC–MS–based VOC profiling of the endophytes confirmed the production of several bioactive compounds, by the isolates (Supplementary Figure S2). Additionally, an induction in anti-oxidant enzyme activities along with an increase in phenolic levels (Figure 2) in host cells suggested the possible defense priming of the host by the endophytes. These volatile compounds with known antimicrobial functions included hexadecanoic acid, which has been reported to be synthesized by different species of *Bacillus* and implicated to be an important component of their anti-fungal activity (Rajaofera et al., 2019); however, in the current study, it could be an important component of their anti-bacterial activity against *Xanthomonas*. Another compound found in the profiles of the effective endophytes was N-acetyl-3-methyl-1,4-diazabicyclo [4.3.0] nonan-2,5-dione, which belongs to the piperazine group of compounds and has recently

been reported in profiles of a strain of *B. subtilis* that showed anti-fungal activity (Awan et al., 2023). This compound has also been reported as a major component of bioactive profiles of actinobacteria isolated from forest soil (Sharma and Thakur, 2020); however, its direct antimicrobial effects have never been reported earlier. While the antimicrobial activities of other piperazine compounds are known (Jadhav et al., 2017), there is a need to discern the specific antimicrobial effects of N-acetyl-3-methyl-1,4-diazabicyclo [4.3.0] nonan-2,5-dione. A pyrrole derivative, Pyrrolo [1,2-a] pyrazine-1,4-dione hexahydro-3-(phenylmethyl), was observed in the current VOC profiles. This compound has been reported in *Bacillus* spp. (Gopi et al., 2014) and has proven antimicrobial activities mediated by inhibition of quorum sensing of the pathogen (Kannabiran, 2016; Kiran et al., 2018). Similarly, xanthene derivatives also inhibit the growth of the pathogen by disrupting quorum sensing and biofilm synthesis by the pathogen (Maia et al., 2022); however, specific antimicrobial activity for 9,9-dimethyl xanthene (observed in VOC profiles in the current study) has not been reported yet and calls for further research. *Bacillus* VOCs can have other biocidal actions by disturbing the cell physiology and cell membrane integrity as they try to enter the aqueous phase of

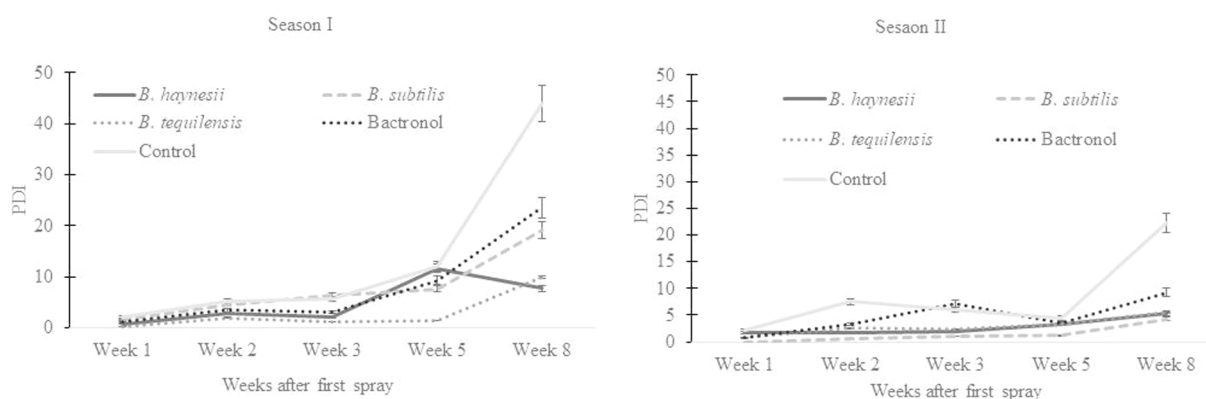


FIGURE 3

Biocontrol of bacterial blight of pomegranate under field conditions using endophytic bacteria indicating a reduction in disease (PDI: percent disease index) as compared to chemical check [2-bromo-2-nitro-1, 3-propanediol (bactronol)] and un-treated control. (A) Trials during the 2022 rainy season crop. (B) Trials during the 2023 rainy season crop. Values presented are mean of three replicates, and error bars are based on standard deviation.

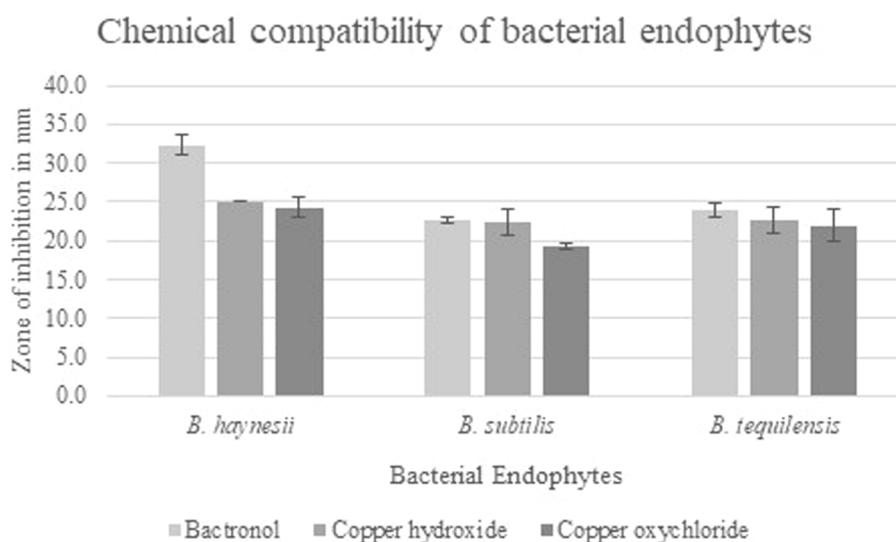


FIGURE 4

Chemical sensitivity of bacterial endophytes. Growth inhibition in terms of zone of inhibition of three bacterial endophytes *B. haynesii*, *B. subtilis*, and *B. tequilensis* on chemicals such as 2-bromo-2-nitro-1, 3-propanediol (bactronol), copper hydroxide, and copper oxychloride. Values presented are mean of three replicates, and error bars are based on standard deviation.

the cell membrane (Massawe et al., 2018). However, more elaborate studies are required to ascertain the mode of action of these inhibitory volatiles observed in the current study.

Non-phytopathogenic bacteria, such as endophytes, trigger plant immunity by recognizing microbe-associated molecular patterns (MAMPs) through the host cells. This results in a burst of reactive oxygen species (ROS) as a part of the host's first line of defense (Newman et al., 2013). To overcome this burst and gain entry into plant cells, endophytes induce ROS-scavenging mechanisms (Liu et al., 2017). ROS scavenging could be the mechanism by which the endophyte *B. tequilensis* colonized pomegranate leaves. This is because the activities of all three enzymes, which are crucial in ROS scavenging, were induced upon inoculation with *B. tequilensis*.

Since there are only a few reports on successful on-field biological control of bacterial blight in pomegranate, the final objective of the

study was to ascertain the applicability of endophytes and test their efficacy under field conditions. Many studies report antagonistic activities of biocontrol agents against pathogens *in vitro* or under polyhouse conditions but fail to extend their results to the field. Earlier researchers have used lactic acid bacteria (LAB) for successful integrated management of pomegranate bacterial blight where they reported the effect of LAB to be at par with the antibiotic streptomycin (Gajbhiye et al., 2023). In the current study, the performance of endophytes was better than or at par with the performance of the immune modulator (2-bromo-2-nitro-1, 3-propanediol) under field conditions where a reduction (in disease) of 47–61% during season I and 53 to 68% in season II was observed. Hence, for the first time, the biocontrol of bacterial blight, with the application of endophytes, is reported under field conditions. Under field conditions, it is difficult to say conclusively whether the sprays (5 sprays at 5-day intervals) acted

in the prophylactic mode or curative mode, but the observed reduction in disease index could be attributed to the various underlying antagonistic mechanisms employed by *Bacillus* spp. The regular sprays could have primed the defense response of the host plants against Xcp, or the emission of the volatiles by the endophytes could have inhibited the growth and epiphytic survival of the pathogen itself by disrupting biofilm formation. Biofilm formation is an important component in *Xanthomonas* virulence, and strains lacking the ability to form biofilm lose their pathogenicity over the host (Mina et al., 2019).

Moreover, the tested endophytes were less sensitive to chemicals such as 2-bromo-2-nitro-1, 3-propanediol, copper hydroxide, and copper oxychloride at the tested doses. This is noteworthy because once the ban on the antibiotic streptomycin is imposed, these chemicals are the only effective measures to manage bacterial blight in pomegranate. Copper nanoparticles have also been found to be effective in controlling bacterial blight disease in pomegranate (Chikte et al., 2019). Hence, given the lower sensitivity of the bacterial endophytes with these chemicals, the results of the current study support and advocate the use of endophytes, namely, *B. haynesii*, *B. subtilis*, and *B. tequilensis* for bacterial blight management either alone or as a part of integrated disease management practices. Such an integrated management approach, utilizing biocontrol agents and copper, has been recently reported to manage center rot in onion (Koirala et al., 2024). However, the strain of *B. subtilis* used in that study was sensitive to copper at 250 ppm, while the isolates reported in the current study were compatible with 2,000 ppm of copper compounds. Similarly, the application of fungicide-compatible endophytic *Trichoderma* spp. has also been recommended for the management of rubber leaf fall disease caused by *Corynespora* (Seekham et al., 2024). Hence, it will be worth investigating in the future whether the integration of these biocontrol agents with other chemicals results in better bacterial blight management in pomegranate.

Based on the results of the current study, we conclude that endophytic *B. haynesii*, *B. subtilis*, and *B. tequilensis* control the bacterial blight incidence and severity in the following two ways (i) by priming the plant defense response and (ii) by hampering the infection mechanism of the pathogen. The host plant (pomegranate) behaves in a similar way against any alien microorganisms trying to enter its tissues, whether they are pathogens or useful endophytes. When endophytes are inoculated on the plants, the plants recognize their MAMPs and mount the first line of defense, which is manifested as a burst of ROS. To overcome this ROS burst and gain entry into host cells, endophytes induce the activity of ROS-scavenging enzymes such as catalase, peroxidase, and superoxide dismutase. This induction of the anti-oxidative machinery of the host against beneficial endophytes also facilitates defense priming against a subsequent pathogen attack. On the other hand, the beneficial endophytes also secrete volatiles that reduce the yellow pigments produced by *Xanthomonas*. These pigments are crucial for the epiphytic survival of the pathogen during its infection cycle on the host and also provide protection against oxidative stress; hence, reduction of pigment indicates reduced pathogen survival ability. Some of the volatiles also possess antimicrobial activities, which can directly kill the cells of the pathogen or hamper the infection process via the disruption of biofilm production. However, more elaborate studies are required to evaluate the molecular mechanism behind the induction of host defense response or the endophyte-mediated induced systemic resistance and understand the role of volatiles in inhibiting biofilm production during Xcp–pomegranate interaction.

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

NM: Conceptualization, Data curation, Formal analysis, Writing – review & editing. SP: Conceptualization, Formal analysis, Writing – review & editing. RA: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. NS: Data curation, Formal analysis, Resources, Writing – review & editing. JS: Conceptualization, Formal analysis, Project administration, Writing – review & editing. MH: Resources, Writing – review & editing. RM: Project administration, Resources, Writing – review & editing.

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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/fmicb.2024.1491124/full#supplementary-material>

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Exploring *Trichoderma* diversity in the Western Ghats of India: phylogenetic analysis, metabolomics insights and biocontrol efficacy against Maydis Leaf Blight disease

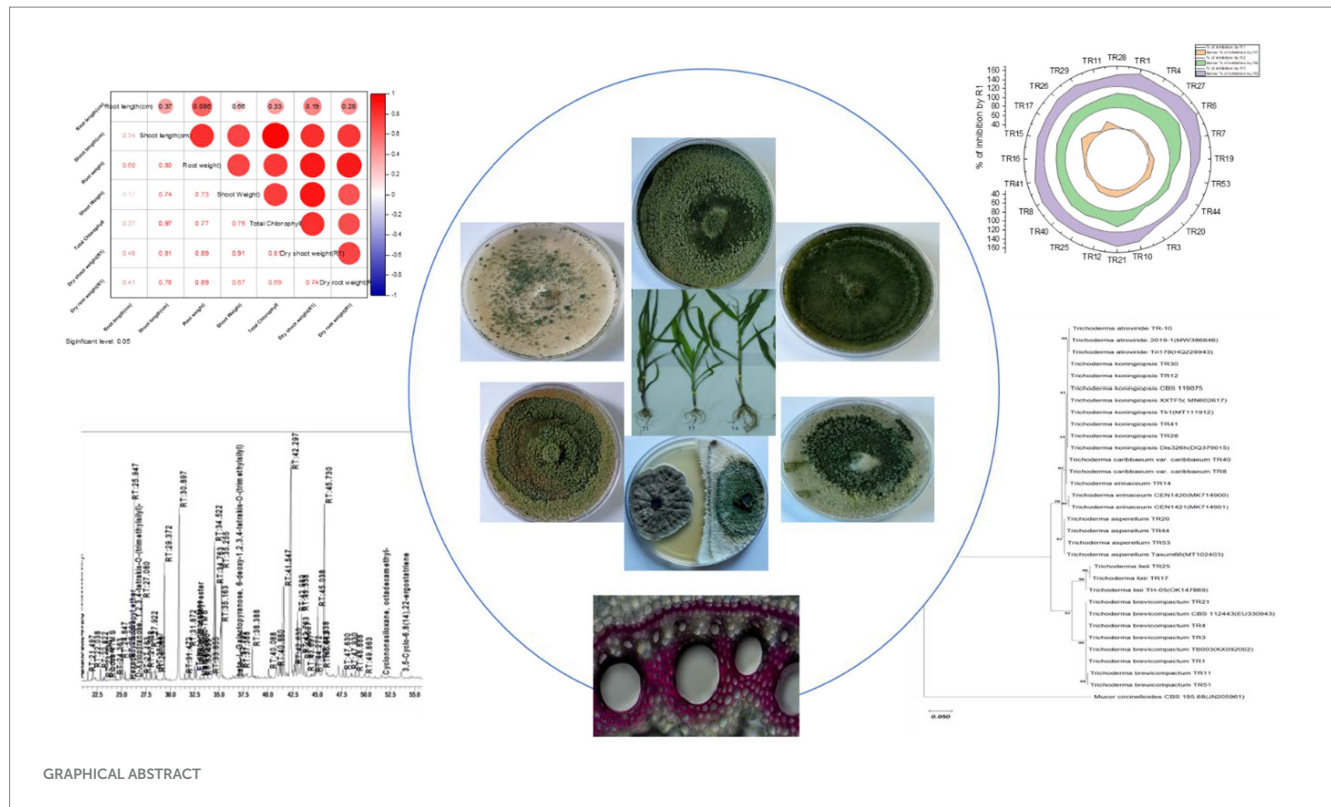
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The Western Ghats of India is recognized as one of the world's eight "hottest hotspots" of biological diversity. *Trichoderma*—a well-known biocontrol agent, was explored from this hotspot. A total of 260 *Trichoderma* spp. isolates were studied, with 9% exhibiting strong biocontrol potential and crop growth-promoting activity. Furthermore, this study identified three novel isolates—*Trichoderma caribbaeum* var. *caribbaeum*, *Trichoderma lixii*, and *Trichoderma brevicompactum*—which are reported for the first time from the Western Ghats making a significant contribution to the field. Based on internal transcribed spacer ribosomal RNA (ITS-rRNA) and translation elongation factor 1- α (tef-1 α) gene sequence analysis, molecular characterization, identified major isolates as *Trichoderma koningiopsis*, *Trichoderma asperellum*, *T. caribbaeum* var. *caribbaeum*, *T. lixii*, *T. brevicompactum*, *Trichoderma atroviride*, and *Trichoderma erinaceum*. Seed biopriming with the effective *Trichoderma* strain TR11 reduced the maydis leaf blight (MLB) disease index to 32.92% and improved plant growth-promoting attributes in maize. Defensive enzyme activities were increased 2.5–4.2-fold in various treatments with the TR11 isolate, along with enhanced lignification postpathogen inoculation, indicating strengthened plant defense mechanisms. The promising strain *T. brevicompactum*-TR11 produces secondary metabolites; among them, 5% were found to have a role in biocontrol activity such as octadecanoic acid, palmitic acid-TMS, 5-(4-nitrophenoxy)methyl, furane-2-carboxaldehyde, and stearic acid-TMS, phosphoric acid-3TMS, galactopyranose, 5TMS. This study explored *Trichoderma* diversity in the Western Ghats of India with phylogenetic relationship, metabolomics insights, and biocontrol efficacy against MLB disease.

KEYWORDS

galactopyranose, *Trichoderma* spp., secondary metabolites, host-pathogen interaction, diversity



Introduction

The Western Ghats of India—a UNESCO World Heritage site, is one of the world's eight major biodiversity hotspots. It stretches from the southern tip of Gujarat through the Satpura range in the north, passing through Maharashtra, Goa, Karnataka, and Kerala, and ends at the southernmost part of India (Mehta and Shah, 2021). This region hosts a diverse range of organisms, including many novel and undiscovered microorganisms with significant potential for different applications (Nampoothiri et al., 2013). *Trichoderma*, common ascomycetes fungi found in soils globally, are widely studied for managing soil- and foliar-borne diseases. They are prevalent in various environments such as agricultural areas, grasslands, forests, and deserts (Jaklitsch, 2011). Multiple species of *Trichoderma* act as biocontrol agents against fungal pathogens (Li et al., 2013). A major threat is maydis leaf blight (MLB) or southern corn leaf blight (SCLB), caused by the fungus *Bipolaris maydis*, poses a significant threat to maize production globally (Manzar et al., 2022b). MLB can cause grain yield reductions of over 40%, with historical epidemics in the 1970s leading to losses of 28–91% (Zhu et al., 2021; Gogoi et al., 2014). Chemical control methods, though widely practiced, have

several drawbacks, including environmental contamination, pathogen resistance, and adverse effects on non-target organisms. Hence, the use of biocontrol agents such as *Trichoderma* offers a sustainable and environmentally friendly alternative (Aggarwal et al., 2024). However, the efficacy of *Trichoderma* as a biocontrol agent can vary significantly among species and strains. Therefore, exploring the diversity of *Trichoderma* in different ecosystems and understanding their phylogenetic relationships is critical for identifying strains with superior biocontrol potential. Due to the lack of distinct morphological features and the rise of cryptic species, identifying *Trichoderma* based solely on morphology is challenging (Hoyos-Carvajal et al., 2009; Sun et al., 2012; Rodriguez et al., 2021; Manzar et al., 2022a). Molecular techniques now enable accurate identification of *Trichoderma* isolates and potential new species through gene sequence analysis (Druzhinina et al., 2005; Kubicek et al., 2008). The diverse genus *Hypocrea* *Trichoderma* includes around 160 species—the majority of them were identified via molecular phylogeny from pure cultures and herbaria specimens (Druzhinina et al., 2005). Surveys of *Trichoderma* spp. have been conducted in regions such as Siberia, the Himalayas (Kullnig et al., 2000), China (Zhang et al., 2005), Central and South America, and Egypt (Gherbawy et al., 2004). These studies identified unique species in each area and discovered new ones, possibly indicating species' preference for specific regions or climates (Bissett et al., 2003; Kraus et al., 2004). The ability of *Trichoderma* spp. to induce resistance in maize plants against foliar pathogens has been proven (Harman, 2011; Jie et al., 2014). In response to pathogen attacks, plants exhibit various biochemical reactions, among which the antioxidative response holds significance due to the generation of numerous reactive oxygen species (ROS). This antioxidative defense mechanism involves the activation of enzymes such as peroxidase (POX), superoxide

Abbreviations: ITS, Internal transcribed spacer; tef-1 α , Translation elongation factor 1- α ; DNA, Deoxyribonucleic acid; BLAST, Basic local alignment search tool; NCBI, National Center for Biotechnology Information; rRNA, Ribosomal ribonucleic acid; SOD, Superoxide dismutase; PPO, Polyphenol oxidase; PO, Peroxidase; PDI, Percent disease index; ROS, Reactive oxygen species; H₂O₂, Hydrogen peroxide; DAB, 3,3'-Diaminobenzidine; GC-MS, Gas chromatography-mass spectrometry; MLB, Maydis leaf blight; SCLB, Southern corn leaf blight; rpb2, RNA polymerase II second largest subunit; DDBJ, DNA data bank of Japan.

dismutase (SOD), and polyphenol oxidase (PPO). Plant pathogens trigger the induction of these enzymes through oxidative damage (Chugh et al., 2011). POX, a pivotal enzyme, participates in the biosynthesis of lignin and phytoalexins (Fan et al., 2006), and SOD operates to neutralize oxygen free radicals and active oxygen, thereby shielding plant membranes and essential enzymes within the salicylic acid pathway (Guo et al., 2006). *Trichoderma* spp. create a broad range of secondary metabolites that belong to many chemical groups, including alcohols, aldehydes, monoterpenes, sesquiterpenes, aromatic compounds, esters, furans, hydrocarbons, ketones, and compounds comprising S and N elements (Nieto-Jacobo et al., 2017; Esparza-Reynoso et al., 2021). These metabolites might involve in several biological activities, including microorganism-to-microorganism communication and biocontrol (Crutcher et al., 2013). The *Trichoderma* species are considered as plant growth-promoting fungi (PGPF), capable of colonizing and multiplying in the rhizosphere and strengthening plant defense systems. They can promote plant development in several indirect ways; however, little is known about metabolites-based interactions now. *Trichoderma* metabolites have been demonstrated to promote plant growth by acting as antibiotics against fungal pathogens (Vinale et al., 2014). This study aimed to ascertain the species diversity of *Trichoderma* gathered from the hot spot regions of Western Ghats of India and to find out the most potent *Trichoderma* isolates for managing MLB disease. This study focuses on characterizing *Trichoderma* isolates collected from the rhizosphere soil of various crops or plants in the Western Ghats region of India. The isolates were molecularly characterized and evaluated for their efficacy in reducing MLB severity caused by the pathogen *B. maydis*. Histochemical studies were conducted to assess disease reduction and to visualize lignin and callose deposition as the first line of defense, and also investigated the impact of these isolates on biochemical responses in maize plants, specifically the activity of defense enzymes (POX, SOD, and CAT), and analyzed secondary metabolites produced by the most effective *Trichoderma* isolates.

Materials and methods

The study sampled various regions within the Western Ghats, including part of Shimoga, Coorg, Kodagu's Somvarpet Taluk, Dakshina Kannada, Channarayapatna, Sakleshpur, Madikeri, Piriapatna, Karwar, Begar, Sulya, and Udupi during an exploration survey conducted in December 2021. A total of 36 soil samples were collected, and the *Trichoderma* isolates were obtained and characterized in this study were obtained.

Isolation and storage of pure cultures

Using the serial dilution plating approach, the *Trichoderma* selective medium described by Manzar and Singh (2020) was employed to isolate *Trichoderma* species. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), K_2HPO_4 (0.9 g), KCl (0.15 g), NH_4NO_3 (1.0 g), glucose (3.0 g), Rose Bengal (0.15 g), and agar (20 g) make up 1 L of *Trichoderma* selective medium. Putative *Trichoderma* colonies were purified using potato dextrose agar (PDA) for two rounds of subculturing. Mineral oil was used to keep pure cultures at 4°C.

Morphological analysis

Strains were cultivated on PDA for 7–8 days at 27°C to perform morphological examination using microscopic observations. After 3 days of incubation, pustules with white conidia were used to make microscopic slides for morphological features. To prepare the slides, a drop of 3% KOH was applied to each slide. KOH wets the conidia in the slide to provide better observation and aids in the conidiophores' dissemination. According to Gams and Bissett, *Trichoderma* species were identified by Samuels (2006). Supplementary Table S1 lists microscopic observation and cultural morphology.

DNA extraction and amplification

To extract DNA, *Trichoderma* isolates were grown on a PDA medium at 28°C for 4 days. After incubation, 3–4 mycelial plugs were transferred into a 250-ml conical flask containing 100 mL of potato dextrose (PD) broth, followed by shaking at 150 rpm at 28°C. The mycelial matrix was filtered using Whatman filter paper 42 following 6 days of development. It was cleaned using sterile distilled water and centrifuged at 10,000 rpm for 20 min at 4°C. Liquid nitrogen was then used in a pestle and mortar to pulverize it. The Nucleopore GDNA Fungus Kit-NP-7006D (Genetix Biotech Asia Pvt. Ltd., New Delhi, India) was utilized to extract genomic DNA (Manzar et al., 2021). Using a spectrophotometer (Shimadzu, Tokyo, Japan), the concentration and purity of the DNA were ascertained by measuring the absorbance at 260 and 280 nm. Using the primers ITS1 (TCCG TAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGA TATGC), the nuclear small-subunit rRNA gene's ITS region was amplified in an automated thermocycler (Bio-rad-C1000 Thermal Cycler) by White et al.'s 1990 report. A 25- μL volume was used for the PCR, which contained 1X Taq Buffer with KCl (2.5 μL), 200 μM deoxynucleotide triphosphate (dNTP, 0.5 μL), 1.5-mM MgCl_2 (1.5 μL), 1 U/reaction Taq polymerase (0.2 μL), 1X nuclease-free water (17.3 μL , Genetix brand), ITS 1 (10 pm) primer (1 μL), ITS4 (10 pm) primer (1 μL), and 100 ng genomic DNA (1 μL). The *Trichoderma* isolates ITS region was amplified at 95°C (initial denaturation) for 2 min, 40 denaturation cycles at 94°C for 1 min, 55°C (annealing) for 1 min, 72°C (extension) for 1 min, and at 72°C (final extension) for 10 min. Gel electrophoresis was performed for the polymerase chain reaction (PCR) products (10 μL), bands were recorded, and photographed in the gel documentation unit (Bio-Rad, Philadelphia, PA, USA). Bidirectionally Sanger sequencing method (Eurofins Pvt. Ltd) followed by sequence assembly with (Hall et al., 1999) was performed. For all ITS1, ITS4, and translation elongation factor 1- α (tef-1 α) region sequences, multiple sequence alignment was performed using (Tamura et al., 2021) and CLUSTAL W (Thompson et al., 1994; Kashyap et al., 2023).

The ITS1 and ITS4 gene sequences of each *Trichoderma* isolate underwent alignment and basic local alignment search tool (BLAST) interface analysis using TrichoOKey, the National Center for Biotechnology Information (NCBI) database, and the TrichoBLAST program for tef-1 α gene sequences. Sequence similarity percentages confirmed matches. Aligned sequences were then submitted to the DNA Data Bank of Japan (Mashima et al., 2017) and (Sayers et al., 2022) to obtain accession numbers for ITS and tef-1 α regions. The molecular phylogenetic analysis employed the Maximum Likelihood

technique using (Tamura et al., 2021) and (Tamura et al., 1993). A consensus tree was inferred, and nodal robustness was assessed using the bootstrap method with 1,000 replications to determine phylogenetic relationships among isolates and *Trichoderma* species.

In vitro* assessment of *Trichoderma* isolates for antagonistic activity against *Bipolaris maydis

The test pathogen *B. maydis* and the 19 *Trichoderma* isolates were inoculated at the opposing corners of Petri plates to see if the isolates were antagonistic to each other (Morton and Stroube, 1955). *B. maydis* inoculation was performed 3 days ahead of the *Trichoderma* isolates since it developed slowly in a culture. The observations were reported on the sixth day after the dual culture experiment was run in three replications. The mycelial growth area of *B. maydis* was measured. Utilizing the formula described by Mokhtar and Aid (2013), the percent inhibition of the pathogen vis-à-vis the control was ascertained: mycelial growth inhibition (MGI) (%) = $(C - T) \times 100/C$. Here, MGI represents the percent inhibition of mycelial growth, where C denotes pathogenic control, and T signifies the area of *B. maydis* mycelial growth in dual culture with *Trichoderma*.

Inoculum preparation for maize plant treatment using selected bioagents

Water was used to soak the cleaned sorghum seeds for 12 h. The seeds were soaked, and then cooked for 20–25 min in a boiling water bath to soften the grains and then inoculated with a 3-day-old, actively growing *Trichoderma* isolate culture with the help of the sterilized aseptic cork borer, subsequently grown for 2 weeks at 28°C in a biochemical oxygen demand (BOD) incubator. The flasks were shaken rapidly to prevent clumping and disrupt the mycelial mat after 7 days of inoculation. The sorghum seeds that the *Trichoderma* isolates had colonized were allowed to air dry in the shade for 14 days before being pulverized in a mixer grinder.

Pure bioagent powder was obtained after being concurrently run through 50–80 mesh sieves. Talc powder and the finely ground *Trichoderma* isolates grown in sorghum seeds were combined in a 1:2 ratio, and the mixture was allowed to air dry for 3 days at room temperature in the shade. Approximately 5 g/kg of tapioca starch was added to the mixture. Pot tests were conducted using the final *Trichoderma* inoculum, which was adjusted in the formulation to 5×10^8 cfu/g and then packed in polythene bags at room temperature ($25 \pm 2^\circ\text{C}$). In the fully grown culture of *B. maydis*, the conidia in the culture were scraped with the help of a spatula, and were suspended in deionized water containing 0.02% tween 20. This process produced the conidial suspension of *B. maydis*. As recommended by previous research, the final pathogen concentration was adjusted to 1×10^6 conidia L^{-1} (Manzar et al., 2022b; Muimba-Kankolongo and Bergstrom, 2011). Using a hand-operated sprayer, the conidial suspension of *B. maydis* (10^6 conidia L^{-1}) was sprayed on plants that were 25-days old in the evening to induce leaf blight symptoms. Plants treated with water only were considered as control plants, and plants inoculated with *B. maydis* as pathogen-induced controls.

Pot preparation and plant material

The glasshouse experimental setup comprised 5-kg plastic pots filled with sterilized autoclaved soil. Within each 30-cm plastic pot, healthy and surface-sterilized seeds of the susceptible Kanchan variety of maize were meticulously planted. The experiment included nine distinct treatments: T1 (CM212 + E7 + TR11), where CM212 is a moderately susceptible cultivar of maize, E7 is the virulent isolate of *B. maydis*, and TR11 is the isolate of *Trichoderma*; T7 (Dhiari local + TR11 + E7), where Dhiari local is the susceptible cultivar of maize, E7 is the virulent isolate of *B. maydis*, and TR11 is the isolate of *Trichoderma*; T4 (VL78 + E7 + TR11), where VL78 is the resistant cultivar of maize, E7 is the virulent isolate of *B. Maydis*, and TR11 is the isolate of *Trichoderma*; the pathogen inoculated control T8 (Dhiari local+E7), where Dhiari local is the susceptible cultivar of maize and E7 is the virulent isolate of *B. Maydis*; T2 (CM212 + E7), where CM212 is a moderately susceptible cultivar of maize and E7 is the virulent isolate of *B. Maydis*; T5 (VL78 + E7), where VL78 is the resistant cultivar of maize and E7 is the virulent isolate of *B. maydis*; the uninoculated untreated control T6 (VL78), where VL78 is the resistant cultivar of maize; T3 (CM212), where CM212 is a moderately susceptible cultivar of maize; and T9 (Dhiari local) where Dhiari local is the susceptible cultivar of maize. Ten seeds were sown per pot, with one healthy plant retained after germination. Each treatment was replicated five times in a completely randomized design. Pathogen inoculation occurred 25 days after sowing. Maize seeds underwent biopriming, involving surface sterilization with a 1% sodium hypochlorite solution followed by immersion in water for 24 h. *Trichoderma*-treated seeds received a talc-based formulation containing 5×10^8 cfu/g @10 g/kg of seeds in a 2% gum arabic solution, following established protocols (Lewis et al., 1991). Samples were collected for each biochemical assay from individual treatments and from plants either inoculated with the pathogen or left uninoculated, at specific intervals postinoculation—namely, 0, 24, 48, and 72 h—to evaluate the induction of defense-related enzymes in maize plants under glasshouse conditions, with an average of three replications. The samples were stored at -80°C . Enzyme activity data, including assays for superoxide dismutase, POX, and polyphenol oxidase, were obtained using a spectrophotometer. SOD was estimated by the method described (Beauchamp and Fridovich, 1971). Polyphenol oxidase (PPO) activity was estimated by the method described (Mayer et al., 1966). POX activity was determined by the standard method (Hammerschmidt et al., 1982). Plant growth traits and disease severity were assessed 45 days after sowing. Disease severity was quantified using the percent disease index (PDI), calculated based on leaf area infection scores ranging from 1 to 9. The PDI was calculated using the following formula: $\text{PDI (\%)} = \Sigma (\text{Scale} \times \text{Amount of plants}) / (\text{Maximum grade} \times \text{Total number of plants})$ described by Wheeler (1969).

Qualitative assay for ROS, callose deposition, and lignification

A maize leaves assay was conducted to measure reactive oxygen species (ROS) using the 3',3'-diaminobenzidine (DAB) staining

method. The DAB used in the assay was obtained from Sigma–Aldrich (USA), with catalog number D8001. The technique used in this assay was previously described by Thordal-Christensen et al. (1997), Bindschedler et al. (2006), and Daudi and O'Brien (2012). Leaf segments from various treatments were excised and submerged in a 1 mg/mL DAB solution (pH 3.8) for 8 h. This allowed the leaf segments to undergo a reaction with H₂O₂ and peroxidase. Leaf segments were treated with a solution containing 1.5 g/L trichloroacetic acid in ethanol:chloroform (4:1 v/v) for 48 h to achieve fixation. Subsequently, the segments were fixed in a solution consisting of 50% glycerol, which is commonly used in microscopy. Subsequently, digital photographs of leaf segments dyed with DAB were captured using a Canon digital camera from Japan. Each treatment consisted of four biological replicates. For callose deposition, leaves were cut into 5-mm strips; leaf samples were immersed in the fixative (ethanol/acetic acid, 3:1) and incubated for 12–24 h at room temperature. The fixed leaves were transferred into 1 N NaOH solution and incubated at 60°C for 1–2 h. Wash the cleared leaves several times with distilled water to remove NaOH. Stain the leaves by immersing them in aniline blue solution (in 1% [wt/vol] aniline blue in 0.01 M K₃PO₄, pH 12, in darkness for 30–60 min in the dark at room temperature. After staining, wash the leaves gently with potassium phosphate buffer. The stained sections were mounted on slides and fixed using 95% ethanol before being observed under a compound light microscope manufactured by Nikon, Japan. The presence of blue color indicated callose deposition. The plants with different treatments were collected 15 days after being inoculated with *B. maydis* for lignin deposition. The roots were cut by hand and stained with a solution containing 1% phloroglucinol in 20% HCl. The stained sections were mounted on slides and fixed using 95% ethanol before being observed under a compound light microscope manufactured by Nikon, Japan. The presence of a pink color indicated lignin deposition, as described by Singh et al. (2011).

Gas chromatography–mass spectrometry

Fungal culture filtrates were analyzed using gas chromatography–mass spectrometry (GC–MS) to detect active biomolecules. Secondary metabolites were identified using a single quadrupole mass spectrometer detector, following methods described by Stoppacher et al. (2010) and Siddiquee et al. (2012). The secondary metabolites in the culture filtrate of *Trichoderma* species were analyzed by GC–MS. Hydrocarbons and other secondary metabolites were separated from *Trichoderma* methanol extracts using a Gas Chromatograph GC-2020 (Shimadzu).

The mycelial mat from the petri plates was harvested. Liquid nitrogen grinding was carried out, and the obtained powder was collected into the tubes. A 6-ml metabolite extraction buffer (methanol:chloroform:water = 62.5:25:12.5) was added. Homogenization was carried out for 15–20 min. The sample was vortexed at retention time (RT) for 30 min. Centrifugation was performed at 13,000 rpm for 30 min at 4°C. After centrifugation, two layers were observed. The lower layer containing metabolites was taken and vacuum dried. To the pellet obtained, 100 µL of derivatizing reagent was added and incubated at 80°C for 30 min. *N,O*-Bis(trimethylsilyl) trifluoro acetamide + trimethylchlorosilane (BSTFA + TMCS) is used as a derivatization reagent for GC. This derivatized sample was further taken for GC–MS analysis; column:

Spincotech DB-5 Column, flow rate: 1 mL/min, injection volume: 1 µL, run time: 60 min, and *m/z* range: 50–800 were used. The identified compound spectra were compared with those in the GC–MS database of the National Institute of Standards and Technology (NIST), with a threshold for detection set at over 90% resemblance.

Statistical analysis

The data obtained from laboratory and glasshouse studies were analyzed using a one-way analysis of variance (ANOVA) method, as described by Trillas et al. (2006). Duncan's multiple range test (DMRT) was employed to compare the means. The Statistical Product and Service Solution (SPSS) software version 16.0 was used for this analysis—created by SPSS, Inc., which is now known as IBM SPSS.

Results

The exploration survey was conducted to study the *Trichoderma* spp. from the rhizospheric soil of the Western Ghats. A total of 260 *Trichoderma* isolates were obtained and subjected to morphological, biochemical, and molecular analyses.

Morphological description

Upon obtaining a pure isolate of *Trichoderma* spp., morphological characterization was conducted according to Barnett and Hunter's (1972) method. This entailed assessing concentric rings, pigmentation, and mycelium texture, as detailed in Table 1 and illustrated in Figures 1A,B. Microscopic identification involved examining cultural structures such as phialides, hyphae, conidial shape, and conidiophore number. Conidia exhibited a mean length ranging from 3.4–3.5 µm and a mean width ranging from 2.7–3.0 µm.

Molecular characterization of *Trichoderma* isolates

Molecular identification of 19 *Trichoderma* isolates was conducted using DNA sequencing of the internal transcribed regions (ITS1 and ITS4) and a segment of the *tef-1α* gene. This approach confirmed species identity, complementing earlier morphological assessments. Amplicon sequencing revealed consistent fragment sizes of 650 bp for ITS and 600 bp for *tef-1α* across all isolates. Sequence analysis and BLAST search confirmed species classification with five isolates identified as *Trichoderma koningiopsis* (TR41, TR28, TR12, TR28, and TR30), six as *Trichoderma brevicompactum* (TR11, TR51, TR4, TR1, TR3, and TR21), two as *Trichoderma caribbaeum* var. *caribbaeum* (TR8 and TR40), one as *Trichoderma atroviride* (TR10), three as *Trichoderma asperellum* (TR44, TR53, and TR20), two as *Trichoderma lixii* (TR17 and TR25), and one as *Trichoderma erinaceum* (TR14). Accession numbers were obtained for submitted gene sequences in the NCBI GenBank database. Geographical coordinates and other relevant data are provided (Table 1). The outgroup species were selected to illustrate phylogenetic relationships, *Mucor circinelloides* (JN205961) for ITS and *Mucor rongii* (MT815277) for *tef-1α* were chosen as an outgroup.

TABLE 1 Detailing *Trichoderma* isolate data, including isolate codes, geographical information, host sources, genetic sequence accessions, and morphological characteristics of conidia.

Isolate code	GPS location	Country	Host	Source of isolation	GenBank accession no.		Identity of isolates	Conidia size Length x width	Conidia shape
					ITS	TEF			
TR41	13.87428 N 75.23412 E	India	Coconut	Rhizospheric soil	PP175190	LC799601	<i>Trichoderma koningiopsis</i>	3.7 μm × 2.3 μm	oblong to ellipsoidal or ovoidal and smooth
TR8	12.44 N 75.93 E	India	Bamboo	Rhizospheric soil	PP153914	LC799821	<i>Trichoderma caribbaeum</i> var. <i>caribbaeum</i>	4.8 μm × 2.5 μm	Conidia ellipsoidal to nearlyoblong, smooth.
TR40	12.43 N 75.89 E	India	<i>Artocarpus hirsutus</i>	Rhizospheric soil	PP153421	LC799822	<i>T. caribbaeum</i> var. <i>caribbaeum</i>	3.1–3.2 μm × 3 μm	Conidia ellipsoidal to nearly oblong, smooth
TR28	14.00 N 75.55 E	India	Arecanut	Rhizospheric soil	PP152293	LC799823	<i>T. koningiopsis</i>	3.9 μm × 2.7 μm	oblong to ellipsoidal or ovoidal and smooth
TR25	12.51 N 75.49 E	India	Rubber	Rhizospheric soil	PP141160	LC799824	<i>Trichoderma lixii</i>	3.5 μm × 1.9 μm	globose or subglobose conidia
TR12	12.92 N 76.40 E	India	<i>Curcuma longa</i>	Rhizospheric soil	PP141036	LC799825	<i>T. koningiopsis</i>	2.7 μm × 2.1 μm	oblong to ellipsoidal or ovoidal and smooth
TR21	12.93 N 75.76 E	India	Sugarcane	Rhizospheric soil	PP140921	LC800024	<i>Trichoderma brevicompactum</i>	2.7–3.5 μm × 2.5–3.0 μm	Conidia were sub-spherical or oval and smooth
TR-10	12.47 N 75.59 E	India	<i>Piper nigrum</i>	Rhizospheric soil	PP140887	LC800025	<i>Trichoderma atroviride</i>	3.0–4.0 μm × 2.0–3.0 μm	Conidia were sub-globose to globose, smooth, and thick-walled
TR30	12.41 N 75.71 E	India	Coffee	Rhizospheric soil	PP140873	LC800026	<i>T. koningiopsis</i>	3.9 μm × 2.5 μm	oblong to ellipsoidal or ovoidal and smooth
TR3	12.39 N 75.87 E	India	Senegalia catechu	Rhizospheric soil	PP140845	LC800027	<i>T. brevicompactum</i>	3.2 μm × 2.8 μm	Conidia were sub-spherical or oval and smooth, conidiophores was 1.8–2.9 × 0.9–2.1 μm
TR1	12.51 N 75.50 E	India	Rubber	Rhizospheric soil	PP140831	LC800028	<i>T. brevicompactum</i>	3.1 μm × 2.9 μm	Conidia were sub-spherical or oval and smooth
TR4	12.33443 N 76.1507 E	India	Tobacco	Rhizospheric soil	PP112347	LC800245	<i>T. brevicompactum</i>	3.5 μm × 3.0 μm	Conidia were sub-spherical or oval and smooth
TR17	12.33 N 76.15 E	India	Ragi	Rhizospheric soil	PP112276	LC800246	<i>T. lixii</i>	3.5 μm × 2.1 μm	globose or subglobose conidia
Tr14	12.42 N 77.20 E	India	CocoaCoconut	Rhizospheric soil	PP112272	LC800247	<i>Trichoderma erinaceum</i>	1.3 μm × 1.5 μm	ellipsoidal to broadly ellipsoidal
TR20	14.91 N 74.25 E	India	Maize	Rhizospheric soil	PP217768	LC800357	<i>Trichoderma asperellum</i>	3.32 μm × 3.18 μm	<i>globose toovoidal</i>
TR44	13.50 N 75.21 E	India	Rubber	Rhizospheric soil	PP229207	LC800358	<i>T. asperellum</i>	2.6 μm × 2.32 μm	<i>globose toovoidal</i>
TR53	15.02 N 74.77 E	India	Ragi	Rhizospheric soil	PP229206	LC800359	<i>T. asperellum</i>	3.09 μm × 2.95 μm	<i>globose toovoidal</i>
TR11	12.512045 N 75.509370 E	India	Rubber	Rhizospheric soil	PP346030	LC800360	<i>T. brevicompactum</i>	3.5 μm × 3.0 μm	Conidia were sub-spherical or oval and smooth
TR51	13.34 N 75.00E	India	Rubber	Rhizospheric soil	PP340179	LC800361	<i>T. brevicompactum</i>	2.8 μm × 2.1 μm	Conidia were sub-spherical or oval and smooth

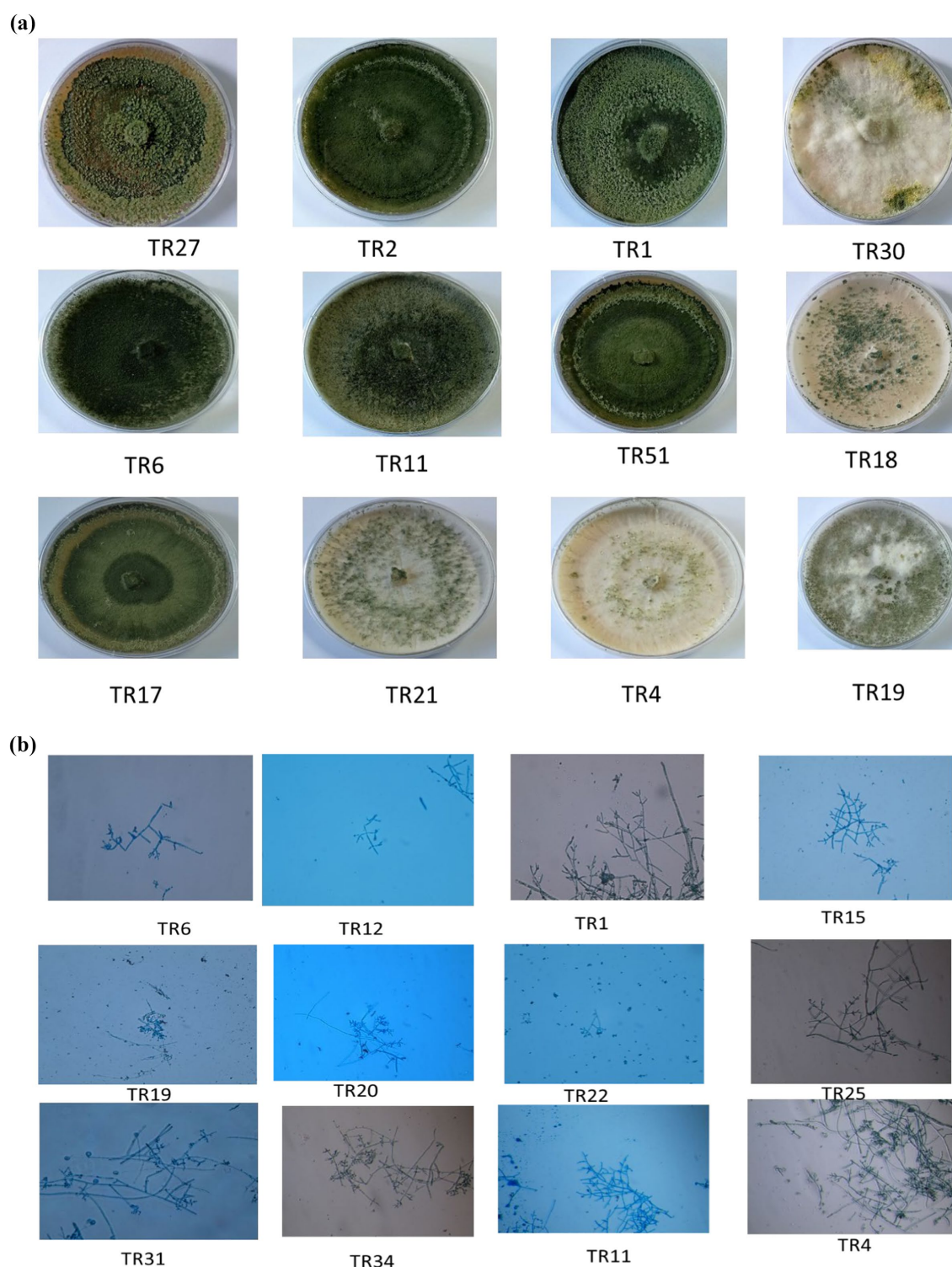


FIGURE 1

(A) Representative images showing the diverse colonies morphology of *Trichoderma* isolates on potato dextrose agar (PDA) media after 8 days of incubation at 27°C. (B) Microscopic characteristics of representative *Trichoderma* strains, showcasing phialides in groups emerging from small terminal clusters, conidiophores, and conidia (10x magnification).

Phylogenetic relationship based on ITS rRNA and tef-1 α gene sequences

Phylogenetic analysis was performed on 19 *Trichoderma* isolates (TR41, TR8, TR40, TR28, TR25, TR12, TR10, TR30, TR3,

TR1, TR17, TR14, TR20, TR44, TR53, TR11, and TR51) using ITS1, ITS4, and tef-1 α sequences. The analysis confirmed their classification within the *Trichoderma* genus. The bootstrap analysis assessed nodal robustness, with numerical values indicating bootstrap support. Based on these values, the 19 *Trichoderma* spp.

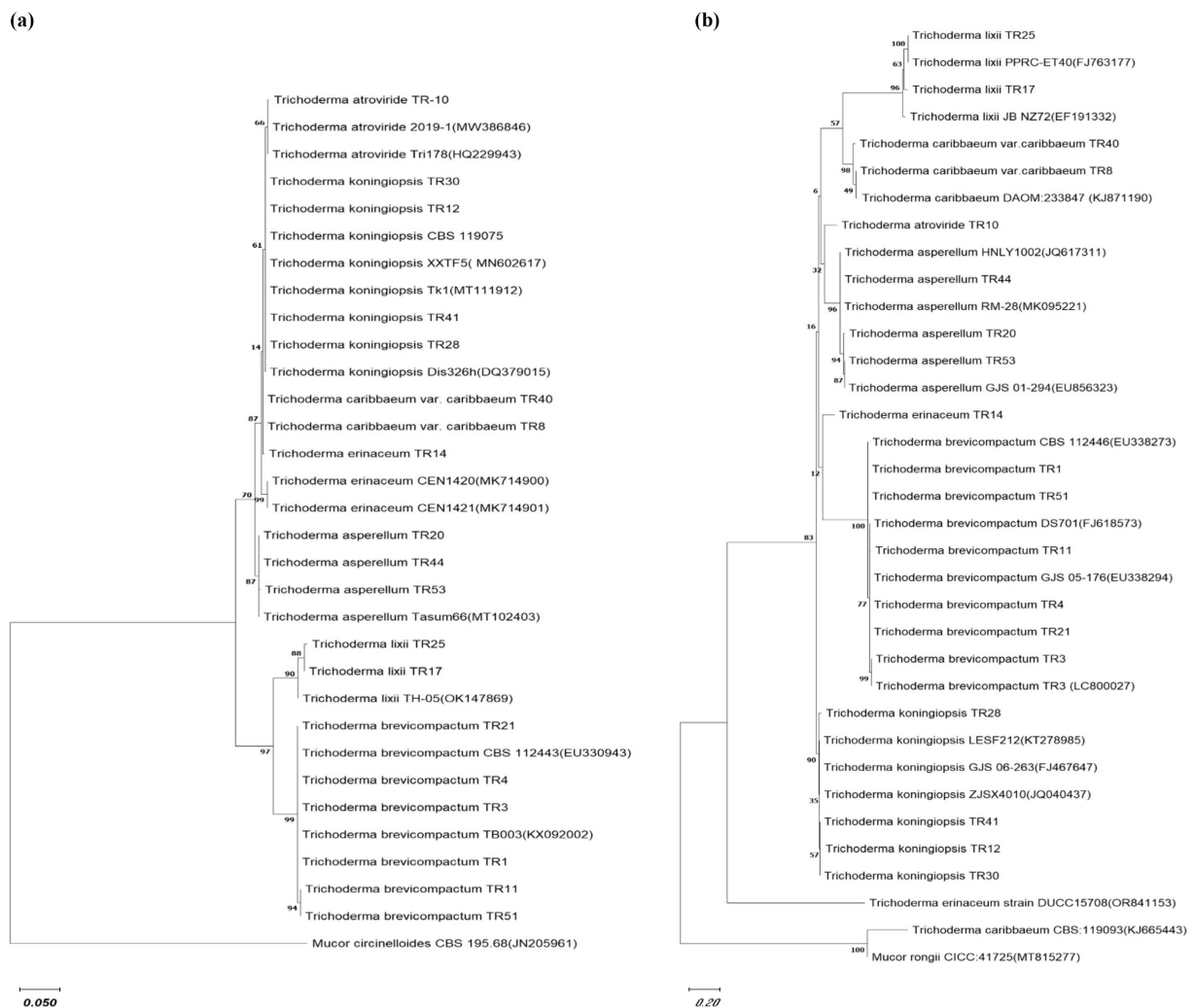


FIGURE 2

Phylogenetic relationship among *Trichoderma* isolates. Cladogram illustrating the phylogenetic relationships among *Trichoderma* isolates, constructed using the maximum likelihood method based on (A) ITS sequences and (B) Tef gene sequences. Bootstrap replication was set to 1,000. The comparison utilized sequences obtained from NCBI GenBank with the accession numbers listed in the manuscript. *Mucor circinelloides* CBS 195.68 (JN205961) and *Mucor rongii* CICC:41725 (MT815277) were selected as an outgroup for ITS and Tef gene phylogenetic tree, respectively. The scale bar indicates 0.1 and 0.2 substitutions per site for ITS and Tef gene phylogenetic tree, respectively.

isolates were categorized into two distinct clades, designated as clade A and clade B, using ITS1 and ITS4 gene sequences, respectively. The *T. atroviride* isolate TR10, clustered with *T. atroviride* 2019-1 (MW386846) and Tri178 (HQ229943) with a bootstrap value of 66%. The isolates of *T. koningiopsis* TR12, TR41, and TR28 clustered with *T. koningiopsis* XXTF5 (MN602617), Tk1 (MT111912), and Dis326h (DQ379015), respectively, with a bootstrap value 63%, whereas *T. caribbaeum* var. *caribbaeum* TR8 clustered with *T. caribbaeum* var. *caribbaeum* TR10 with a bootstrap value 87%. *T. erinaceum* TR14 clustered with *T. erinaceum* (MK714900 and MK714901) with a bootstrap value of 99%. *T. asperellum* (TR44, TR20, and TR53) formed as a distinct clade and clustered with *T. asperellum* (MT102403) at a bootstrap value of 87%. The separated clade B formed two subclades where subclade B1 consists of *T. lixii* (TR25 and TR17) clustered with *T. lixii* (OK147869) and subclade B2 consists of *T. brevicompactum* (TR21,

TR4, TR3, and TR1) clustered with *T. brevicompactum* (CBS112443 and TB003) and the outgroup were *M. circinelloides*, respectively (Figure 2A). The bootstrap values indicated the division of the 19 *Trichoderma* spp. isolates into two distinct clades based on tef-1 α gene sequences. Clade A consists of four subclades, subclade A1 consists of two subclades, one subclade consists of *T. lixii* (TR25 and TR17) clustered with *T. lixii* (J763177 and EF191332) with a bootstrap value of 100%, and the other subclade consists of *T. caribbaeum* var. *caribbaeum* isolates (TR40 and TR8) clustered with *T. caribbaeum* (KJ871190) with a bootstrap value of 98%. The subclade A2 consists of two clades where *T. atroviride* formed a separate clade and *T. asperellum* (TR44, TR20, and TR53) clustered as a distinct clade with *T. asperellum* (MK095221, JQ617311, and EU856323) with a bootstrap value of 96%. The subclades A3 consists of two subclades *T. erinaceum* isolates TR14 formed a distinct clade, whereas the other subclades consist of

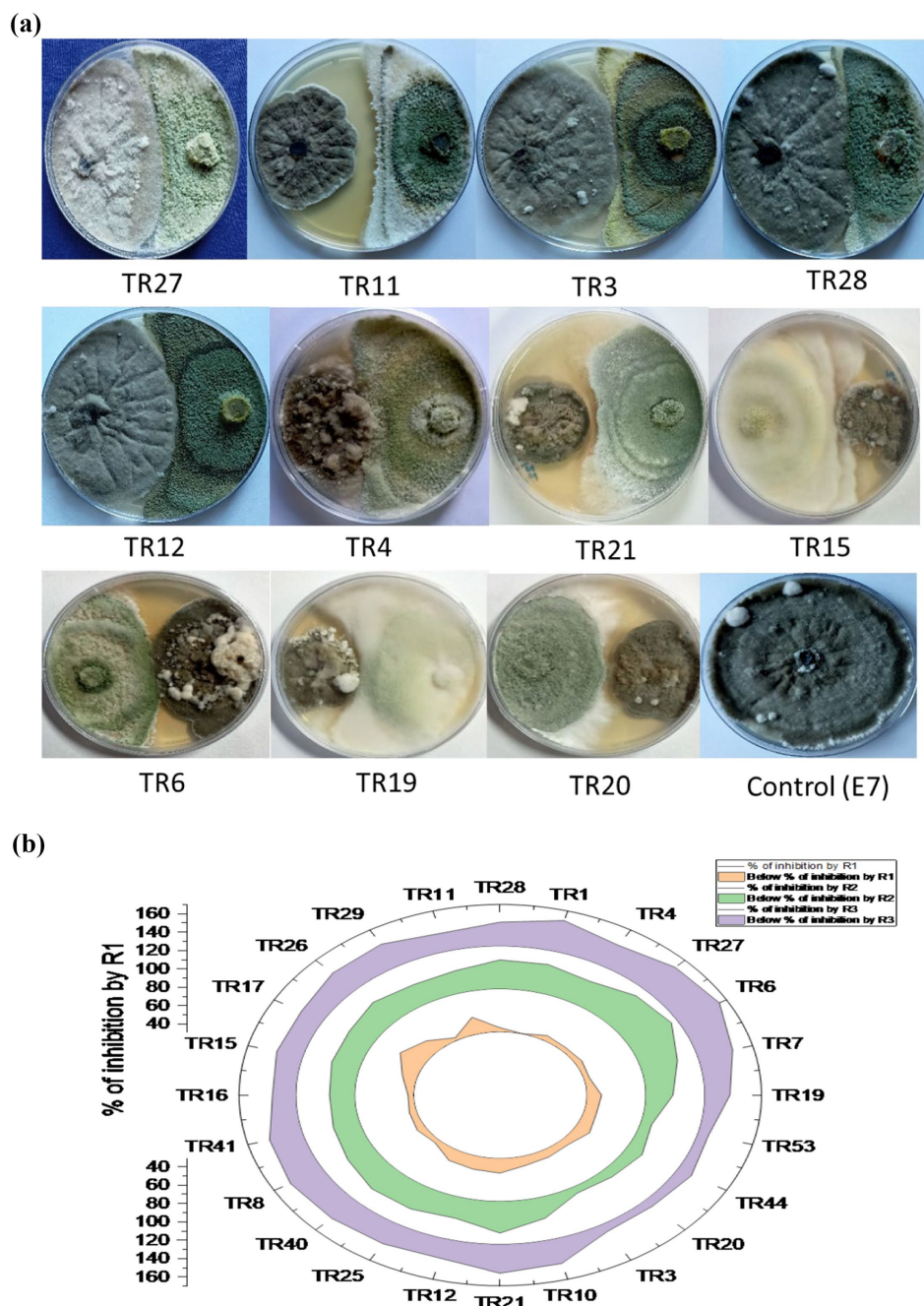


FIGURE 3

(A) Plates illustrate the representative pictures of the antagonistic activity of *Trichoderma* isolates against *Bipolaris maydis* (control) in a dual culture assay under *in vitro* laboratory conditions at $28 \pm 2^\circ\text{C}$. Observations were recorded on the sixth day. (B) Stacked radial plot depicts the percentage inhibition among multiple variables in a circular format. Results are expressed as the average of three replications.

T. brevicompactum TR1, TR51, TR11, TR21, TR4, and TR3 clustered with *T. brevicompactum* (EU338273, FJ618573, and EU338294). The subclades A4 consists of *Trichoderma koningiopsis* (TR28, TR41, TR12, and TR30), clustered with *Trichoderma koningiopsis* (KT278985, FJ467647, and JQ040437) with a bootstrap value of 90%. Clade B consists of *T. erinaceum* (OR841153), which formed a distinct clade, and clade C consists *T. caribbaeum* (KJ665443) with a distinct clade and an outgroup *M. ronii* formed a separate clade (Figure 2B).

In vitro assessment of *Trichoderma* isolates for antagonistic potential

The *in vitro* screening of *Trichoderma* isolates revealed a noteworthy reduction in the mycelial growth of *B. maydis*, ranging from 39.52 to 60.00%. Among the isolates, TR11 exhibited the highest inhibition rate at 60.00%, followed by TR21 and TR06, which showed inhibition rates of 58.57 and 58.38%, respectively (Supplementary Table S2 and Figures 3A,B).

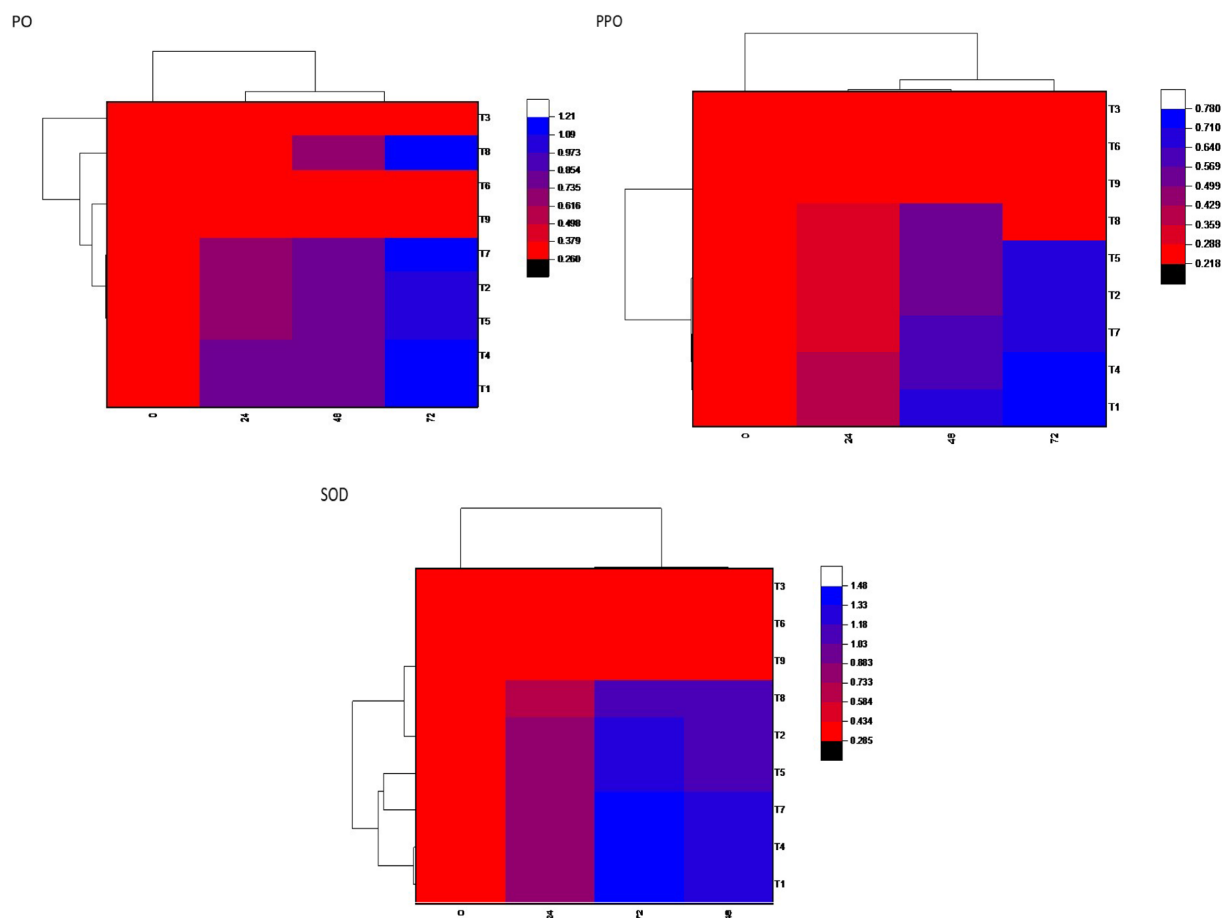


FIGURE 4

Heatmap showing defensive enzymatic activities in maize plants, postinoculated with *Bipolaris maydis*. Observations were recorded at different time intervals, whereas the x-axis shows different time intervals: 0, 24, 48, and 72 h for superoxide dismutase (SOD), polyphenol oxidase (PPO), and peroxidase (PO) activities in maize leaves; and y-axis shows different treatments T1: CM212 + E7 + TR11; T2: CM212 + E7; T3: CM212; T4: VL78 + E7 + TR11; T5: VL78 + E7; T6: VL78; T7: Dhiari local + TR11 + E7; T8: Dhiari local + E7; and T9: Dhiari local, where CM212, Dhiari local and VL78 are moderately susceptible cultivars of maize; E7 is the virulent isolate of *Bipolaris maydis*; and TR11 is *Trichoderma* isolate.

Exploring biochemical defensive enzyme cascade in maize against *Bipolaris maydis* under glasshouse conditions

The enzymatic activities of superoxide dismutase (SOD), polyphenol oxidase (PPO), and POX were assessed at 0, 24, 48, and 72-h postinoculation with *B. maydis* in 30-day-old maize plants. In all treatments, including seed biopriming with *Trichoderma* isolates, activities of SOD, PPO, and PO were significantly elevated compared to both the pathogen-treated control and the untreated healthy control. Treatment T7 (Dhiari Local+TR11 + E7) exhibited the highest SOD activity, 27.11% higher than the pathogen control at 72 h, followed by treatment T1 (CM212 + TR11 + E7) with a 20.38% increase, where CM212 is a moderately susceptible cultivar of maize, E7 is the virulent isolate of *B. maydis*, and TR11 is the isolate of *Trichoderma*. Treatment T4 showed the highest POX activity, while T1 recorded the highest PPO activity, both exceeding the pathogen control by 13.02 and 12.50%, respectively, at 72 h postinoculation. These enzyme activities remained stable at 0 h, increased at 24 h, and peaked at 72 h postinoculation across all treatments. Conversely, the untreated pathogen control showed no significant increase in enzyme activity (Figure 4).

Enhancing growth and reducing disease in maize through seed biopriming with *Trichoderma* isolates

Significant enhancements in crop physiological growth parameters, such as root length, shoot length, fresh shoot, and root weight, as well as dry shoot and root weight, were observed following seed biopriming treatments compared to the control under controlled glasshouse conditions after a 45-day period post sowing. Among these treatments, treatment T1 (CM212 + E7 + TR11) demonstrated the highest measurements for root length (57.03 cm), shoot length (113.33 cm), fresh shoot weight (25.57 g), root weight (9.15 g), dry shoot weight (3.70 g), dry root weight (1.38 g), and total chlorophyll content (31.12) (Supplementary Table S3). The findings indicate that all *Trichoderma* isolates led to a reduction in the percent disease index (PDI) relative to the pathogen-inoculated control. Treatment T4 (VL78 + E7 + TR11) exhibited the lowest PDI (31.88%), followed by T1 (CM212 + E7 + TR11) (35.22%), which statistically resembled T7 (Dhiari local + TR11 + E7) (37.81%) (Figure 5). Conversely, the highest PDI was recorded in the pathogen-inoculated control across different varieties, with T8 (Dhiari local+E7) (46.60%) showing the highest, followed by T2

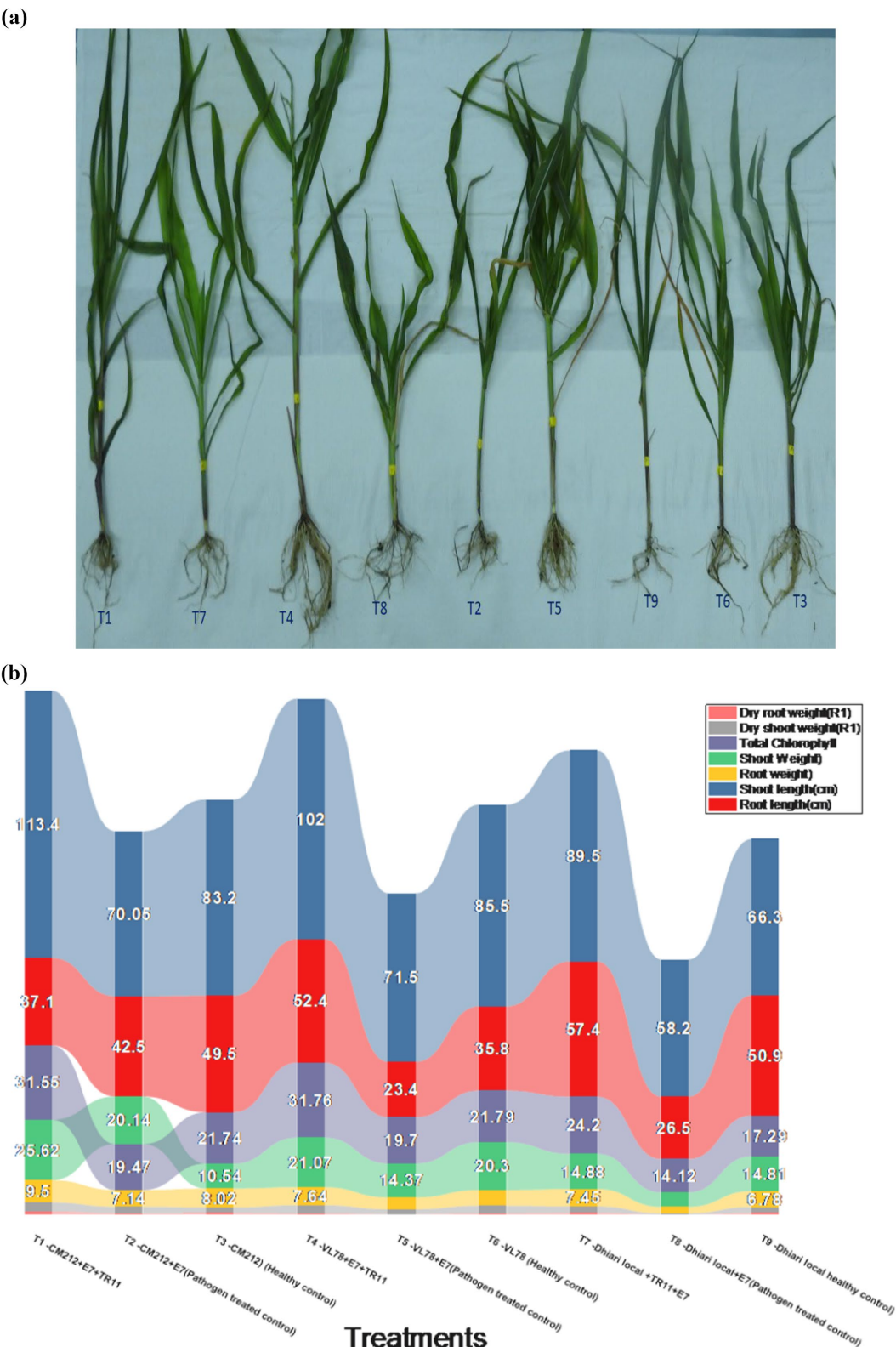


FIGURE 5 (Continued)

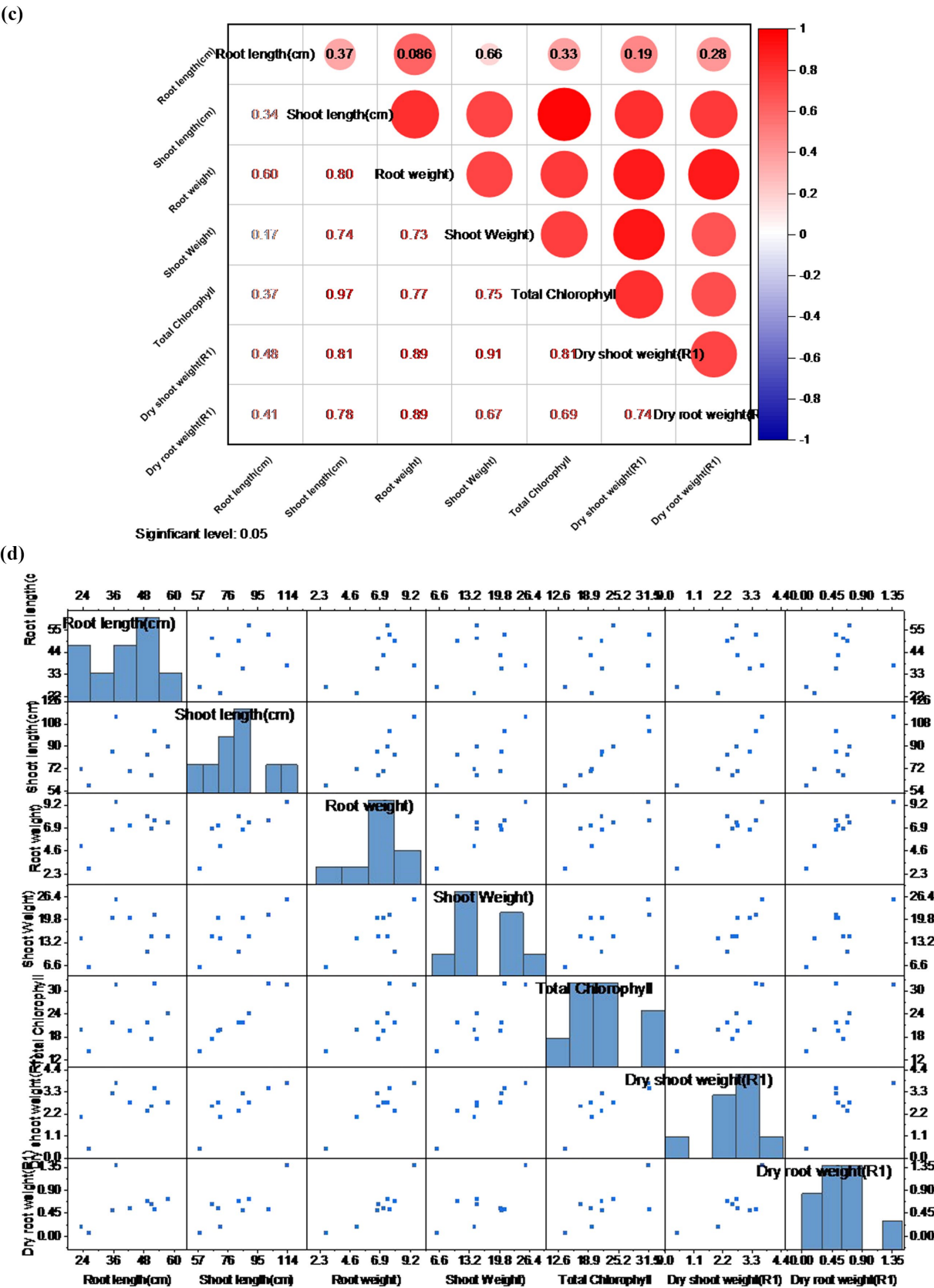


FIGURE 5 (Continued)

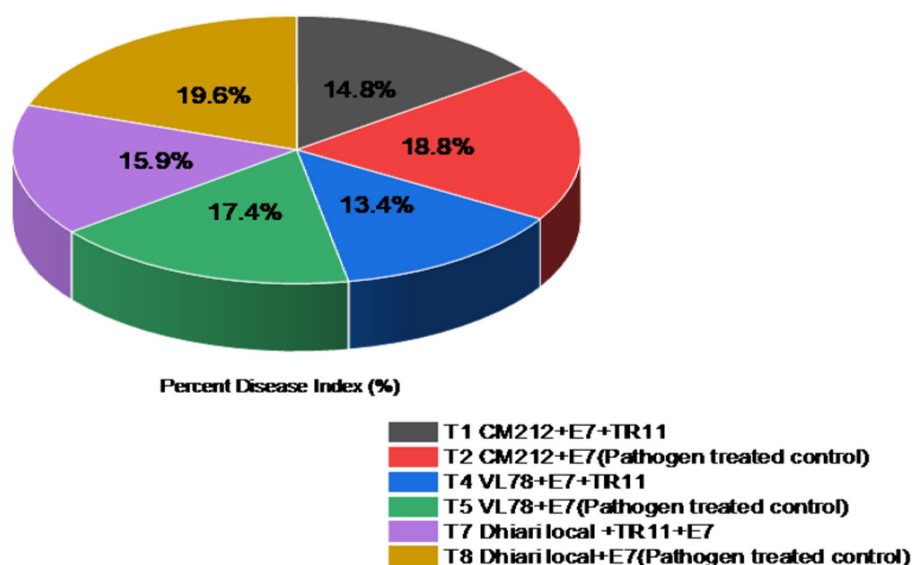


FIGURE 5

Growth-promotion efficiency of *Trichoderma* strains seed biopriming on various growth parameters of maize crop with different treatments: T1: CM212 + E7 + TR11; T2: CM212 + E7; T3: CM21; T4: VL78 + E7 + TR11; T5: VL78 + E7; T6: VL78; T7: Dhiari local + TR11 + E7; T8: Dhiari local+E7; and T9: Dhiari local, where CM212, Dhiari local, and VL78 are moderately susceptible cultivar of maize; E7 is the virulent isolate of *Bipolaris maydis*; and TR11 is *Trichoderma* isolate. (A) Experimental crop after harvest to compare physiological growth, (B) growth parameters among various treatments, (C) correlation analysis shows all parameters are positively correlated, (D) correlation coefficient matrix and associated *p*-values, and (E) percent disease index. Results are expressed as the average of three replications.

(CM212 + E7) (44.74%), at the 45th day after sowing (Supplementary Table S4).

Induction of lignin deposition in maize roots, induction of callose deposition in maize leaves against pathogen invasion and ROS

Lignin deposition within the cell wall serves as a defensive mechanism against plant pathogen invasion into vascular tissues. Histochemical staining of transverse sections of maize root tissues treated with various methods revealed differences in lignin deposition. The treatments, T1 (CM212 + E7 + TR11), T7 (Dhiari local + TR11 + E7), and T4 (VL78 + E7 + TR11), with *Trichoderma* isolate (TR11), showed the highest lignification in all cultivars compared to the pathogen inoculated and healthy controls. Lignin deposition increased after pathogen inoculation. The strongest increase was observed after treatment with the pathogen only and after combined treatment with TR11 isolate. (Supplementary Figure S1A).

The assessment of reactive oxygen species (ROS) accumulation and callose deposition in maize tissues was conducted to elucidate the innate immune response of plants following inoculation with *Trichoderma*. Notably, ROS, particularly hydrogen peroxide (H₂O₂), exhibited marked accumulation in maize leaves 48 h postpathogen inoculation, as evidenced by *in situ* DAB staining. The *Trichoderma*-treated plants also demonstrated induction of H₂O₂ accumulation in leaves (Supplementary Figure S1B). Moreover, the analysis of callose deposition, an additional marker of plant innate immune response, in maize leaf tissue after 48 h of treatment, demonstrated a notable rise in both the quantity and

area percentage of callose deposition compared to mock controls (Supplementary Figure S1C).

Analysis of secondary metabolites produced by *Trichoderma* spp. under *in vitro* conditions

To identify metabolites that might be involved in growth promotion and biocontrol effects we further analyzed *T. brevicompactum* TR11 isolate by GC–MS as it was the most promising strain. A total of 88 compounds were identified from the GC–MS analysis. Results of the GC–MS analysis (Supplementary Figure S2 and Table 2), indicated that the main components of *Trichoderma brevicompactum* TR11 filtrate were octadecanoic acid (peak area: 16.27%), palmitic acid-TMS (peak area: 9.44%), phosphoric acid-3TMS (peak area: 7.35%) galactopyranose, 5TMS (peak area: 6.52%), 5-(4-nitrophenoxy)methyl (peak area: 5.43%), furane-2-carboxaldehyde (peak area: 5.43%), stearic acid-TMS (peak area: 4.05%), laevulinic acid – TMS (peak area: 3.48%), sucrose, 8TMS (peak area: 3.18%) D-fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)- α -D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl) (peak area: 2.83%), linoleic acid trimethylsilyl ester (peak area: 2.83%), 2-palmitoylglycerol, 2TMS (peak area: 2.53%), 3-methyl-2-furoic acid, TMS (peak area: 2.12%), whereas, other components were present in low percentages; hexadecanoic acid, methyl ester (peak area: 1.39%), D-(–)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 2) (peak area: 1.38%), butylated hydroxytoluene (peak area: 1.13) elaidic acid-TMS (peak area: 1.56%), *cis*-5,8,11,14,17-eicosapentaenoic acid, *tert*-butyldimethylsilyl ester (peak area: 1.51%), lactose, 8TMS (peak area: 1.21%), butylated hydroxytoluene (peak area: 1.13%). On the contrary, there are many components present in traces (peak area less than 1%).

TABLE 2 Detailing compound analysis data.

S. No.	Compound name	RT	Area	% of compound
1	Benzenamine, 2,5-dimethyl-	3.193	362,48	0.06
2	N-Trifluoroacetylmorpholine	3.248	54,455	0.09
3	3,6,9-Trioxaundecanedioic acid, bis(trimethylsilyl) ester	3.967	290,536	0.46
4	Dodecane, 2,6,11-trimethyl-	4.062	130,118	0.21
5	Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester	4.276	132,476	0.21
6	Alanine, 2TMS	4.827	63,366	0.10
7	Dodecane, 2,6,11-trimethyl-	4.91	35,302	0.06
8	Ethyl(2-hydroxyethyl)carbamic acid, O,O'-bis(trimethylsilyl)	5.505	466,303	0.74
9	Levulinic acid – TMS	5.575	2,208,558	3.48
10	α -Hydroxyisovaleric acid, (2TMS)-	6.229	61,147	0.10
11	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	6.323	24,358	0.04
12	Furane-2-carboxaldehyde, 5-(4-nitrophenoxy)methyl-	6.751	3,439,276	5.43
13	Pentanoic acid, 4-methyl-2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	8.143	16,889	0.03
14	2-Acetylamino-2-methyl-1-propanol, trimethylsilyl ether	8.565	100,668	0.16
15	RT:9.088	9.475	102,771	0.16
16	Phosphoric acid-3TMS	9.326	4,660,031	7.35
17	Hexadecane-1,2-diol, bis(trimethylsilyl) ether	9.59	16,314	0.03
18	Niacin, TMS	9.947	33,460	0.05
19	Phenylacetic acid, monoTMS	10.051	303,526	0.48
20	3-Methyl-2-furoic acid, TMS	10.561	1,346,054	2.12
21	Uracil, 2TMS	11.236	54,149	0.09
22	3,5-Dimethoxymandelic acid, di-TM	11.608	39,513	0.06
23	Fumaric acid, bis-TMS	11.725	63,769	0.10
24	2-Amino-2-(hydroxymethyl)propane-1,3-diol, N-acetyl-, tris(trimethylsilyl) ether	11.942	70,679	0.11
25	Tetradecane	13.286	38,264	0.06
26	Glutaric acid-2TMS	13.417	84,494	0.13
27	Eicosane	15.951	188,097	0.30
28	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	15.977	178,561	0.28
29	Butylated hydroxytoluene	16.4	718,658	1.13
30	2-Aminobenzoxazole, 2TMS	17.339	209,419	0.33
31	3-Phenyllactic acid, 2TMS	18.622	16,709	0.03
32	2-Amino-2-(hydroxymethyl)propane-1,3-diol, N-acetyl-, tris(trimethylsilyl) ether	19.106	140,325	0.22
33	Hexadecane	19.264	43,029	0.07
34	Cyclooctasiloxane, hexadecamethyl-	20.198	68,688	0.11
35	Tartaric acid-4TMS	20.355	55,492	0.09
36	3,5-Di- <i>t</i> -butyl-4-methoxy-1,4-dihydrobenzaldehyde	21.499	302,202	0.48
37	Eicosane	22.039	318,666	0.50
38	Phosphorin, 2,4,6-tris(1,1-dimethylethyl)-	22.819	249,523	0.39
39	Ribose-4TMS	23.068	98,130	0.15
40	Eicosane	23.231	160,961	0.25
41	Cyclononasiloxane, octadecamethyl-	24.371	138,897	0.22
42	Fructose-5TMS	24.849	630,750	1.00
43	D-(–)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 2)	25.067	876,181	1.38
44	Hexadecyl isopropyl ether	25.665	44,500	0.07
45	β -D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	25.942	1,021,065	1.61

(Continued)

TABLE 2 (Continued)

S. No.	Compound name	RT	Area	% of compound
46	D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	26.26	99,809	0.16
47	Galactopyranose, 5TMS	27.076	2,688,256	4.24
48	Eicosane	27.536	341,517	0.54
49	Hexadecanoic acid, methyl ester	27.921	881,521	1.39
50	Cyclononasiloxane, octadecamethyl-	28.081	174,837	0.28
51	n-Pentadecanoic acid	28.446	157,927	0.25
52	Eicosane	28.583	103,152	0.16
53	Galactopyranose, 5TMS	29.371	4,133,277	6.52
54	Palmitic acid-TMS	30.896	5,983,399	9.44
55	Cyclooctasiloxane, hexadecamethyl-	31.475	128,950	0.20
56	Linoleic acid, methyl ester	31.868	463,054	0.73
57	Elaidic acid, methyl ester	32.029	89,988	0.14
58	Methyl stearate	32.657	255,866	0.40
59	Tetrapentacontane	32.508	339,509	0.54
60	Margaric acid-TMS	33.041	93,640	0.15
61	Tetrapentacontane	33.454	105,928	0.17
62	Nonadecanamide	33.866	176,443	0.28
63	Linoleic acid trimethylsilyl ester	34.523	1,791,127	2.83
64	Elaidic acid-TMS	34.646	990,576	1.56
65	cis-5,8,11,14,17-Eicosapentaenoic acid, tert-butyldimethylsilyl ester	35.157	957,151	1.51
66	Stearic acid-TMS	35.255	2,565,588	4.05
67	β -L-Galactopyranose, 6-deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)	36.603	67,454	0.11
68	Tetrapentacontane	37.034	271,038	0.43
69	Cyclononasiloxane, octadecamethyl-	37.403	147,322	0.23
70	Myristic acid, 2,3-bis(trimethylsiloxy)propyl ester	38.39	696,415	1.10
71	Cyclononasiloxane, octadecamethyl-	40.09	173,936	0.27
72	2-Palmitoylglycerol, 2TMS	41.546	1,606,078	2.53
73	Cyclononasiloxane, octadecamethyl-	42.627	1,96,162	0.31
74	Lactose, 8TMS	42.766	765,944	1.21
75	Sucrose, 8TMS	42.98	2,016,418	3.18
76	D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)- α -D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	43.338	1,790,957	2.83
77	D-(–)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 2)	43.592	751,036	1.19
78	Heptadecanoic acid, glycerine-(1)-monoester, bis-O-trimethylsilyl-	43.895	100,435	0.16
79	Trehalose-8TMS	44.833	473,701	0.75
80	2-Monostearin, 2TMS	45.038	918,991	1.45
81	3- α -Mannobiose, octakis(trimethylsilyl) ether (isomer 2)	45.513	128,986	0.20
82	Per-O-trimethylsilyl-(3-O- α -D-mannopyranosyl-4-O- β -D-glucopyranosyl-D-glucitol)	45.513	128,986	0.20
83	Glycerol monostearate, 2TMS	45.724	10,311,392	16.27
84	Maltose, 8TMS	47.626	360,735	0.57
85	Maltose, octakis(trimethylsilyl) ether (isomer 1)	47.931	255,695	0.40
86	2,3-Dihydroxypropyl icosanoate, 2TMS	48.882	131,999	0.21
87	Cyclononasiloxane, octadecamethyl-	51.602	81,090	0.13
88	3,5-Cyclo-6,8(14),22-ergostatriene	53.616	148,948	0.24

This includes secondary metabolite compound names, retention times (RT), peak areas (area), and the percentage of each compound in the total composition (% of compound).

Discussion

In this study, *Trichoderma* strains were isolated and characterized from the Western Ghats of India, reflecting *Trichoderma* as a key mycobiome component in varied ecosystems, such as forests, supported by the studies by Kamala et al. (2015) and Ghorbanpour et al. (2018). In our study, a multigene approach involving the *tef-1 α* , ITS1, and ITS4 gene sequences was used to accurately classify 19 *Trichoderma* isolates into seven distinct species: *T. koningiopsis*, *T. brevicompactum*, *T. caribbaeum* var. *caribbaeum*, *T. lixii*, *T. asperellum*, *T. atroviride*, and *T. erinaceum*. Similarly, Zheng et al. (2021) used *tef-1 α* and RNA polymerase II second largest subunit (*rpb2*) sequences to identify *Trichoderma* isolates, including species such as *T. achlamyosporum* and *T. aquatica*.

Trichoderma spp. exhibit strong antagonistic potential through mycoparasitism, competition, antibiosis and induce systemic resistance in plants, which helps reduce the incidence and severity of diseases caused by phytopathogens (Atanasova et al., 2013; Vargas et al., 2014; Cherkupally et al., 2017). According to the *in vitro* results, the *T. brevicompactum* TR11 isolates exhibited significant inhibitory potential against the *B. maydis* E7 isolates, restricting its mycelial growth by up to 60% compared to other isolates in the dual culture assay. Numerous studies have supported the antagonistic potential of certain *Trichoderma* species, against *Exserohilum turcicum*, *Bipolaris oryzae*, and *Colletotrichum graminicola* (Ma et al., 2023; Khalili et al., 2012; Manzar et al., 2021). The findings from the glasshouse experiments highlight the antagonistic capabilities of *Trichoderma* isolates against *B. maydis*, in maize plants. Treatment T1 (CM212 + E7 + TR11), which comprised the moderately susceptible maize variety CM212, the virulent strain E7, and the *Trichoderma* isolate TR11, exhibited significant improvements in several plant growth parameters, such as root and shoot lengths, fresh and dry weights of shoots, as well as a reduction in disease severity compared to the pathogen-inoculated control under glasshouse conditions. In a previous study, seeds of *Celosia cristata* treated with *Trichoderma harzianum* Th62 also showed significantly increased plant height, diameter of the stem, number of branches, dry weight of root, stem, leaf, and flower and enhanced resistance against *Alternaria alternata*, *Rhizoctonia solani*, *Cystospora chrysosperma*, *Sclerotinia sclerotiorum*, and *Fusarium oxysporum* (Hou et al., 2021).

In our study treatment T1, which combined the moderately susceptible maize variety CM212, the *B. maydis* virulent strain E7, and the *Trichoderma* isolate TR11, showed a marked increase in the activities of defense enzymes such as POX, polyphenol oxidase, and superoxide dismutase compared to pathogen-treated control and the untreated, unchallenged healthy control. This increased defense enzymatic activity is vital for reinforcing the plant defense mechanisms and, as a result, slows the progression of the disease. Our findings align with existing literature that highlights the importance of enzymes such as polyphenol oxidase and POX in biological control and plant disease resistance (Zhang et al., 2008; Petrov and Van Breusegem, 2012; Singh and Singh, 2012; Kashyap et al., 2021). In our experiments, maize plants treated with *Trichoderma* isolates TR11 displayed significant callose deposition and lignification levels, which contributed to their resistance against MLB. This is consistent with the findings that *Trichoderma* spp. treated plants exhibited increased activities of various defense enzymes, including glucanase, POX, and polyphenol oxidase, alongside enhanced cell wall lignification and callose deposition compared to untreated controls (Singh et al., 2019; Keswani et al., 2019; Keswani et al., 2014; Siddaiah et al., 2017; Ben

Amira et al., 2018; Kashyap et al., 2022). The TR11 isolate exhibited strong biocontrol potential, demonstrating both growth promotion and antagonism through experiments on antimicrobial activity, *in vitro* antibiosis, pot trials for plant growth promotion, and induction of systemic resistance. A total of 88 compounds were detected in the ethyl acetate extracts. The primary constituents include fatty acids, esters, aldehydes, hydrazides, pyrazines, imidazoles, triazines, and fatty amides. The primary active ingredient in our investigations *T. brevicompactum* TR11 isolate acetonetic extract was glycerol monostearate 2TMS, followed by palmitic acid-TMS and phosphoric acid-3TMS in the *Trichoderma* TR11 filtrate. Similar findings were found in *Trichoderma* species, such as *T. harzianum* and *Trichoderma viride* produce secondary metabolites such as butanoic acid, propanoic acid, and hexadecanoic acid, which inhibit the growth of phytopathogenic fungi, such as *Colletotrichum lagenarium*, *S. sclerotiorum*, and *Cercospora beticola* (Agoramoorthy et al., 2007; Liu et al., 2008; Altieri et al., 2007; Avis et al., 2000; Pohl et al., 2011). The fatty acids such as pentadecanoic acid, butanedioic acid, dodecanoic acid, heptadecanoic acid, palmitic acid, and octadecenoic acid exhibit antifungal and antibacterial properties against various plant pathogens.

Conclusion

Exploring the biodiversity hotspot of the Western Ghats of India, *Trichoderma* isolates were meticulously screened, revealing 7% with robust biocontrol potential and growth-promoting activities. Notably, three novel *Trichoderma* isolates—*T. caribbaeum* var. *caribbaeum*, *T. lixii*, and *T. brevicompactum*—were reported for the first time from this region. Molecular characterization, based on ITS-rRNA and *tef-1 α* gene sequencing, identified major isolates, enhancing our understanding of genetic diversity. *In vitro* screening demonstrated substantial mycelial growth inhibition by 24 *Trichoderma* spp. isolates against *B. maydis*, underscoring their biocontrol efficacy. The biochemical defense mechanisms in maize, including elevated enzymatic activities and seed biopriming with *Trichoderma* isolates TR11, showcased promising disease reduction and growth promotion attributes. Induction of lignin and callose depositions in maize roots and leaves postpathogen invasion highlighted intricate defense responses. This study provides a comprehensive insight into the multifaceted roles of *Trichoderma* spp. in boosting plant health and combating phytopathogens, emphasizing their potential in sustainable agriculture and paving the way for the future research endeavors in crop protection and productivity enhancement.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI database.

Author contributions

NM: Conceptualization, Data curation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review &

editing. AK: Formal analysis, Software, Supervision, Visualization, Writing – review & editing. MR: Writing – review & editing. PS: Funding acquisition, Writing – review & editing. AS: Funding acquisition, Writing – review & editing.

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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/fmicb.2024.1493272/full#supplementary-material>

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The composite microbial agent controls tomato bacterial wilt by colonizing the root surface and regulating the rhizosphere soil microbial community

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Introduction: Bacterial wilt caused by *Ralstonia solanacearum* seriously affects the healthy growth of tomato seedlings. Biocontrol microbes have been used to manage tomato bacterial wilt. Herein, we aim to investigate the behavior of the *Enterobacter hormaechei* Rs-5 and *Bacillus subtilis* SL-44 composite microbial agent (EB) in the rhizosphere soil, and assess its impact on both the soil microbial community and tomato plant growth in this study.

Methods: The plate confrontation experiment and the pot experiment were respectively used to explore the control ability of EB against *Ralstonia solanacearum* and bacterial wilt disease. The absolute quantitative PCR (AQ-PCR) was employed to investigate the migration ability of EB in the rhizosphere of tomatoes, and the chemotactic response of EB to tomato root exudates was analyzed by the swimming plate method. Scanning electron microscopy was utilized to study the biofilm formation of EB during its colonization on the root surface of tomatoes. Finally, high-throughput sequencing was adopted to analyze the impact of EB on the microbial community in the rhizosphere soil of tomatoes after being infected by *Ralstonia solanacearum*.

Results: The absolute quantitative PCR and scanning electron microscope showed that the EB could migrate and efficiently colonize the elongation zone of tomato roots to form a biofilm. In addition, the EB exhibits a chemotactic response to tomato root exudates like sucrose, leucine, glutamic acid, and aspartic acid. The pot experiment demonstrated that the EB can reduce the incidence of tomato bacterial wilt from 77.78% to 22.22%, and significantly increase the biomass, physicochemical properties, and rhizosphere soil nutrient contents of tomato seedlings. Besides, the relative abundance of beneficial bacteria such as *Massilia*, *Pseudomonas*, *Bacillus*, and *Enterobacter* increased, and the fungi community diversity was improved.

Conclusion: Overall, the EB can reduce the amount of *Ralstonia solanacearum* in rhizosphere soil, and then control tomato bacterial wilt directly. Besides, the EB can migrate to the root under the induction of tomato root exudates and colonize on the root surface efficiently, thereby indirectly regulating the soil microbial community structure and controlling tomato bacterial wilt.

KEYWORDS

tomato seedlings, bacterial wilt, composite microbial agent, soil microbial community, root exudates

1 Introduction

The bacterial pathogen *Ralstonia solanacearum* can invade from the wounds at the root or stem base of the host, and survive in the soil for a long time, which causes the stems and leaves to wilt without a normal supply of water (Su et al., 2022a). The tomato is a vital vegetable crop globally, with an annual global production of about 160 million tons, and assumes a pivotal role in human dietary patterns. The tomato bacterial wilt caused by *R. solanacearum* severely impacts the growth of plants, often resulting in extensive wilting and mortality, leading to significant losses of 35%–90% of tomato yield (Su et al., 2022b). The bacterial wilt, a highly destructive soil-borne disease, represents one of the most extensively prevalent and detrimental plant diseases worldwide (Prokhorchik et al., 2020).

Ralstonia solanacearum exhibits a broad range of hosts and distribution, possesses a complex pathogenic mechanism, and demonstrates strong survival and transmission capabilities (Prior et al., 2016). Therefore, there are no effective methods for completely controlling tomato bacterial wilt (Md Meftaul et al., 2020). Thus, prevention is the main method to prevent tomato bacterial wilt from invading and spreading to plants. At present, many techniques have been used to control tomato bacterial wilt, with pesticides being the most widely used (Qi et al., 2022). However, pesticides can cause environmental pollution and food safety in preventing and controlling bacterial wilt (Su et al., 2022a; Su et al., 2022b).

Fortunately, the biological control of tomato bacterial wilt has become a hot research topic recently. Plant growth-promoting rhizobacteria (PGPR) are highly regarded by researchers as beneficial soil bacteria that colonize the plant rhizosphere and its surrounding environment among a diverse range of soil microorganisms. PGPR facilitate plant growth through both direct and indirect mechanisms, and play a crucial role in managing soil-borne plant diseases (Zhu et al., 2023). The application of *Bacillus velezensis* GL3 demonstrated significant suppression of *R. solanacearum* and enhanced the growth of tomato seedlings in a pot experiment (Agarwal et al., 2020). The results from both pot and field experiments indicated the significant suppression of tomato bacterial wilt by *Bacillus methylotrophicus* DR-08 through the secretion of two antimicrobial metabolites (clostridium difficile and clostridium difficile oxide) (Im et al., 2020). Ling et al. (2020) demonstrated the potential of *Streptomyces* sp. NEAU-HV9, and its secreting actinomycin D as effective biocontrol and antimicrobial agents against tomato bacterial wilt. NEAU-HV9 exhibits potential as biocontrol and novel antimicrobial agents against tomato bacterial wilt. The causes of *R. solanacearum* include the reduction in the number of beneficial bacteria in plant roots, the decline in bacterial diversity, and the accumulation of soil-borne pathogens. Hence, increasing the bacteria with strong beneficial functions in soil is particularly important for the prevention and control of tomato bacterial wilt. The utilization of composite microbial agent has been demonstrated to be more efficacious in the management of plant diseases compared with single microorganism, as indicated by several studies (Lee et al., 2017; Santiago et al., 2017). Zhou et al. (2021) reported that the combined application of *T. virens* Tvien6 and *B. velezensis* X5 was found to exhibit superior efficacy in controlling tomato bacterial wilt compared with the individual bacterial agents. Li et al. (2020) found that the application of the composite microbial agent exhibited enhanced efficacy in managing various soil-borne diseases and promoting the growth of *Avena sativa*,

Medicago sativa, and *Cucumis sativus* seedlings. Besides, the strains *B. velezensis* B63 and *Pseudomonas fluorescens* P142 were observed to significantly suppress the occurrence of tomato bacterial wilt (Elsayed et al., 2019). Previous studies have demonstrated the effective biocontrol capabilities of *Bacillus subtilis* SL-44 against *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria alternata*, and *Botrytis cinerea* and promoted plant growth (Chen et al., 2023a; Chen et al., 2023b). Additionally, *Enterobacter hormaechei* Rs-5 has been found to alleviate salt stress and promote plant growth. Currently, some composite microbial agents have been studied to control tomato bacterial wilt. However, the mode of action of how composite microbial agent control tomato bacterial wilt by colonizing root and regulating the root soil microbial community remains unknown.

Intriguingly, we have not investigated SL-44 and Rs-5 for tomato bacterial wilt control. Therefore, the objective of this study was to prepare Rs-5 and SL-44 composite microbial agent (EB) and then study the control of tomato bacterial wilt by systematically investigating the root chemotaxis, root surface colonization, and mutualistic beneficial interaction between the EB and the tomato seedlings. The primary focus of this study was: (1) to investigate the biological control of tomato bacterial wilt and the ecological behavior of the composite bacterial agent in *R. solanacearum*-infected rhizosphere soil; (2) to evaluate the effect of the EB on the soil microbial community; (3) to evaluate the effect of the EB on soil fertility after *R. solanacearum* infection. These results will provide supplementary data for the development and application of composite PGPR as biological control agent in tomato bacterial wilt.

2 Materials and methods

2.1 Strains and medium

The strains *E. hormaechei* Rs-5, *B. subtilis* SL-44 and *R. solanacearum* GMI1000 were used in this study. The strains *E. hormaechei* Rs-5 and *B. subtilis* SL-44 used were isolated from soil in Xinjiang, China. The strain *R. solanacearum* GMI1000 was provided by Fujian Agriculture and Forestry University. Therefore, we took the first letter “E” of *Enterobacter* and the first letter “B” of *Bacillus*, and combined them into “EB” to represent the composite microbial agent. The strain Rs-5 or SL-44 were cultured in Luria-Bertani (LB) liquid medium (10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, and pH 7.2) with shaking at 30°C and 170 rpm for 36 h, resulting in a cell concentration reaching up to 10¹⁰ CFU/mL. The strain GMI1000 was cultured in the bacteria peptone glucose (BG) liquid medium (Chen et al., 2022) (10 g/L peptone, 1 g/L yeast extract, 1 g/L casamino acid, 10 g/L glucose, and pH 7.4), with shaking at 28°C and 200 rpm for 36 h, resulting in a cell concentration reaching up to 10¹⁰ CFU/mL. Preparation of EB: took equal volume of fermentation broth of the strain Rs-5 and SL-44 and mix well, centrifuged (8,000 g for 10 min at 4°C) and resuspend with sterile distilled water (the density of bacterial suspension was controlled at 10¹⁰ CFU/mL). The preparation of SL-44 bacterial suspension: the cultured bacterial solution was centrifuged (8,000 g for 10 min at 4°C) and then suspended with phosphate buffer solution (pH 7.3) to the OD of 0.5. The preparation of Rs-5 and EB bacterial suspension followed the same procedure as described above.

2.2 The inhibition ability of EB against GMI1000

The inhibition efficacy of EB against GMI1000 was determined by the plate confrontation method. The fermented strain GMI1000 solution (100 μ L) was inoculated onto BG agar plates and spread evenly. Three sterilized round filter papers (φ 0.5 cm) were placed on each LB agar plate, followed by inoculation of 10 μ L of the fermented strain Rs-5, SL-44, or EB solution onto the filter papers. The presence of a bacterial inhibition circle was observed following incubation at 30°C for 48 h. The presence of this inhibition circle indicates the ability of Rs-5, SL-44, or the EB inhibiting GMI1000 growth, and conversely, its absence suggests no inhibitory effect.

2.3 The colonization of EB in tomato root

The tomato seeds were purchased from the agricultural materials store in Lintong, Shaanxi, China and the variety is *Provence*. The tomato seeds were sterilized by immersion in 70% ethanol for 5 min and subsequently in 0.1% HgCl₂ for 10 min, washed several times with sterile water. The soil substrate (vermiculite and perlite in a 3:2 mixture) was added to the plant tissue culture containers (800 mL) and autoclaved (121°C for 20 min). The tomato seedlings were grown in the substrate until they had three true leaves, and then the tomato seedlings were removed and washed in sterile water to remove substrate from the roots. The seedlings were placed in 50 mL centrifuge tubes containing 20 mL bacterial suspension of strains Rs-5, SL-44, and EB. The centrifuge tubes were incubated in a climatic chamber (14/10 h light (350 μ mol m⁻² s⁻¹)/dark period, and a relative humidity of 50%–60%) for 48 h. The tomato seedlings were taken out and their roots were washed with sterile water. Following to that, approximately 1 cm of sections were excised from distinct regions of the tomato roots. At the end, the tomato roots were fixed in 2.5% glutaraldehyde solution and subjected to freeze-drying before observation using scanning electron microscopy (SEM) (Hitachi, Japan).

2.4 Response of EB to simulated tomato root exudates

The specific root exudates selected in this study encompassed glucose, mannitol, sucrose, fructose, leucine, glutamic acid, aspartic acid, alanine, tartaric acid, malic acid, citric acid, and fumaric acid since the predominant constituents of tomato root exudates are sugar, amino acids, and organic acid. The response of Rs-5, SL-44, and EB to simulated tomato root exudates was evaluated by swarm plate assay (Yuan et al., 2015). The different simulated tomato root exudates (10 mM) were added to sterilized semi-solid medium (0.2% agar), shaken well and poured into petri dishes to cool. The concentrated solution was prepared by centrifuging (8,000 g for 10 min at 4°C) 10 mL cultured bacterial solution with 1 mL of phosphate buffer (pH 7.3), and then 20 μ L the concentrated cell solution was taken in the center of the semi-solid medium. The blank control consisted of a semi-solid medium devoid of root exudates. The chemotactic radius was measured following overnight incubation in the above medium. If the composite microbial agent has a chemotactic response to the root exudate, the microbial cells will diffuse outward from the center

of the medium, forming a white circle. Then, a ruler is used to measure the radius of the circle, which is the chemotactic radius.

2.5 Pot experiment

The variety and the disinfection method of the seeds were the same as those mentioned above. The tomato seeds after disinfection were sown in nursery pots containing sterilized soil and transplanted when the tomato seedlings reached three leaves. The soil used for the pot experiments was from the campus of Xi'an Polytechnic University (34°21'N, 109°11'E), Shaanxi, China. The soil electrical conductivity (EC) was 480 μ S·cm⁻¹ and the pH was 7.90. The soil organic matter (SOM), soil alkali-hydrolytic nitrogen (SAN), soil available phosphorus (SAP), and soil available potassium (SAK) contents were 15.2 g·kg⁻¹, 50.62 mg·g⁻¹, 11.46 mg·kg⁻¹ and 180 mg·kg⁻¹, respectively. The pot experiments were divided into four groups as follows: CK, control without any treatment (no EB and pathogen GMI1000); T1, EB treatment; T2, pathogen GMI1000 treatment; and T3, EB and pathogen GMI1000 treatment. There were six pots (φ 23.5 cm $\times$ 19.5 cm) in each treatment and three tomato seedlings with consistent growth in each pot. The tomato seedlings transplanted to the T site in each pot (Figure 1b). The 3 kg of unsterilized soil in each pot. Before transplanting the tomato seedlings, the GMI1000 suspension was sprayed uniformly in the soil with a spray bottle, making the bacterial concentration about 10⁷ CFU g⁻¹ soil for T2 and T3 treatment (5 mL GMI1000). The tomato seedlings were transplanted and inoculated with EB (the concentration about 10⁸ CFU g⁻¹ soil) in the center of each pot. The seedlings were grown under room conditions with temperatures ranging from 26°C to 30°C, 14/10 h light (350 μ mol m⁻² s⁻¹)/dark period, and a relative humidity of 50%–60%. The pot experiment ended after 30 days of tomato transplanted. The tomato plants were watered with deionized water by weighing method every 48 h during growth. The amount of water should be determined according to the weight of the soil and the seedling pot. The plant biomass (fresh weight, dry weight, plant height, root length, and stem diameter), physicochemical properties, and root soil nutrient content were measured at the end of the pot experiment.

Soil sample collection: Two sampling sites were set up to study the migration of the EB in the soil and its effect on the soil microbial community. The site M was located in the middle of the tomato root and the inoculation site of EB (Figure 1b), and the soil around the tomato root (site R). Soil at M and R was taken from three pots for each treatment at 15th and 30th day of the tomato transplanted. It was approximately 1 cm away from the root system of the tomato seedlings when taking the soil sample at the R site. It was approximately 7.5 cm away from the root system of the tomato seedlings when taking the soil sample at the M site. Three pieces of soil (9 g) at M and R were taken from each pot, and then were mixed well. The soil was stored at -80°C after removing impurities such as plant roots from the soil using a 2-mm sieve. The incidence of the tomato bacterial wilt was counted at 15th and 30th day of transplanting. The tomato symptoms were divided into 6 levels according to the proportion of leaf and stem wilt in the whole plant. $n = 0$, no symptoms; $n = 1$, 20% of the whole plant showed wilt symptoms; 2, 40% of the whole plant showed wilt symptoms; 3, 60% of the whole plant showed wilt symptoms; 4, 80% of the whole plant showed wilt symptoms; 5, 100% of the whole plant showed wilt symptoms. The disease index (DI) and relative control efficiency (CE) were calculated by Equations (1, 2).

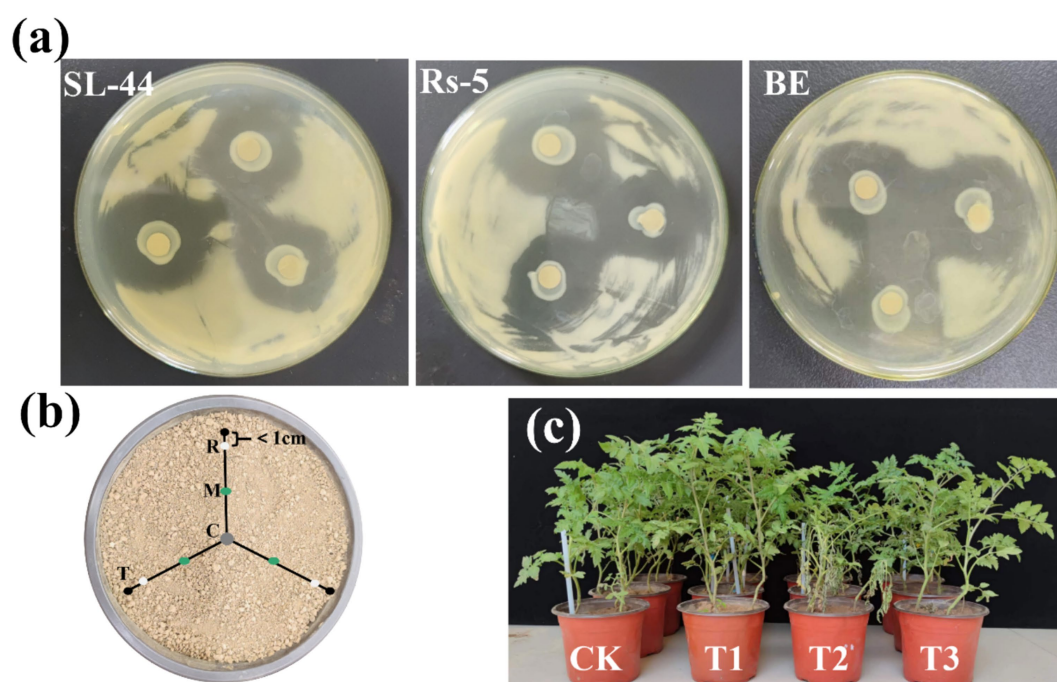


FIGURE 1

The inhibition abilities of the strain SL-44, Rs-5 and the composite microbial agent on GM1000 (a), Soil collection in pot (b) and the growth state of tomato seedlings transplanted for 30 days (c). C, the inoculation site of the composite microbial agent; M, the middle of the tomato root and the composite microbial agent inoculation site; R, the tomato root; T, tomato seedlings.

$$DI = \left(\frac{100 \sum \left(\frac{\text{disease level } n \times \text{number of plants of corresponding level}}{5 \times \text{total number of plants of corresponding treatment}} \right)}{1} \right) \quad (1)$$

$$CE(\%) = \frac{(\text{control disease index} - \text{treatment disease index})}{\text{control disease index}} \times 100 \quad (2)$$

2.6 The biomass and physicochemical properties of seedlings

The biomass of tomato seedlings included fresh weight, dry weight, plant height, root length and stem diameter. Fresh samples were washed with distilled water, then blotted dry with paper and their fresh weight was measured. The fresh samples were subjected to drying at 90°C until a constant weight was achieved to measure their dry weight. Plant height and root length were measured using a straightedge while Vernier calipers were employed for measuring the stem diameter of the seedlings.

The soluble protein was quantified using the Coomassie Brilliant Blue G-250 method and soluble sugars were determined by the sulfuric acid-phenol method (He et al., 2018). The absorbance of the corresponding indicator solutions was measured at wavelengths of 595 and 485 nm, respectively. Subsequently, the contents were calculated using the corresponding standard curves to determine the levels of soluble protein and soluble sugar in plants. Chlorophyll and carotenoid content were determined spectrophotometrically as

described by He et al. (2019). After placing 0.2 g of plant leaves in a solution containing 10 mL of 96% ethanol, shaking well, and protecting from light overnight, the extracts' absorbance was measured at wavelengths of 666, 649, and 470 nm.

Peroxidase (POD) was measured by the guaiacol method, superoxide dismutase (SOD) was measured by NBT photoreduction method, and catalase (CAT) was measured by UV absorption colorimetric method (Wu et al., 2019). The leaves of 0.2 g seedlings were washed and placed in a pre-chilled mortar (containing pre-chilled 0.05 M, pH 7.8 phosphate buffer) to grind into a homogenate, and the supernatant after centrifugation was the enzyme solution. Then the enzyme solution was mixed with its corresponding reaction solution, and the activities of POD, SOD and CAT were quantified at wavelengths of 470, 560, and 240 nm, respectively.

2.7 Root soil nutrient content

Soil organic matter (SOM) content was determined by potassium dichromate oxidation external heating method, soil available potassium (SAK) content was determined by acetic acid-flame photometer, soil available phosphorus (SAP) content was determined by the molybdenum blue method, and soil alkali-hydrolytic nitrogen (SAN) content was determined by alkali-hydrolytic diffusion method (Lu et al., 2020).

2.8 Absolute quantitative PCR

The absolute quantities of EB and GMI1000 in the soil at M or R sites were determined using Absolute Quantitative PCR (AQ-PCR),

with three replicates per treatment. The primers Rs-5 (F) 5'-CACATCCGACTTGACAGACC-3' and Rs-5 (R) 5'-TTACCCGAGAAAGCAC-3' were used to detect Rs-5. The primers SL-44 (F) 5'-GCGTAGAGATGTGGAGGAA-3' and SL-44 (R) 5'-TAGGATTGTCAGAGGATGTCA-3' were used to detect SL-44. The primers GMI1000 (F) 5'-TAACCCAACATCTCACGACA-3' and GMI1000 (R) 5'-ATGCCACTAACGAAGCAGA-3' were used to detect GMI1000.

Soil DNA was extracted from 0.5 g of each sample using the Mag-Bind® Soil DNA Kit M5635 (Omega Bio-tek, United States), followed by standard product preparation. PCR cycling system: DNA template (1 µL), 10 µM Primer F (0.3 µL), 10 µM Primer R (0.3 µL), 2 × SYBR real-time PCR premixture (7.4 µL) and ultrapure water (7 µL). PCR cycling conditions: Pre-denaturation (95°C, 5 min), high-temperature denaturation (95°C, 15 s) and low-temperature annealing (60°C, 30 s), and the above stages were cycled 40 times.

2.9 High-throughput sequencing using NovaSeq PE250

Soil DNA samples were sequenced using the illumina NovaSeq PE250 platform from Shanghai Personalbio Technology Co., Ltd. The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') were used to amplify the V4-V5 variable region of the bacterial 16S rRNA gene. The primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the ITS1-ITS2 variable region of fungi. The raw data generated by Trimmomatic after sequencing was spliced and filtered by flash (flash Version 1.2.11) to obtain clean data to remove the interfering data. Then, Operational Taxonomic Units (OTUs) clustering and species annotation were conducted based on the valid data with a 97% similarity threshold. Additionally, Alpha Diversity and Beta Diversity analyses were performed to explore variations in community structure among different samples or groups.

2.10 Statistics analysis

Statistical analysis of the experimental data was performed using Origin 2021. One-way analysis of variance (ANOVA) in IBM SPSS Statistics 27 was used to evaluate the differences among the four treatments in the pot experiment at $p < 0.05$. OTUs clustering was performed using Uparse (Uparse v7.0). Alpha Diversity and Beta Diversity analyses were performed using Qiime (Version 1.9.1). R (Version 4.0.3, pheatmap package) was used to plot heat maps and R (Version 4.0.3, vcd package) was used to plot ternary heat maps. R (Version 4.0.3, Venn Diagram package) was used to plot Venn diagrams.

3 Results and discussion

3.1 The biocontrol of tomato bacterial wilt by EB

In this study, we investigated the antagonism interaction between the EB and *R. solanacearum* GMI1000, as well as the biocontrol efficacy of EB against tomato bacterial wilt by the plate confrontation

method and pot experiment, respectively. The antagonistic experiment between the EB and GMI1000 exhibited a distinct inhibition zone (Figure 1a), indicating significant inhibitory effects of Rs-5, SL-44, and the EB on GMI1000. The pot experiment demonstrated that the tomato seedlings remained disease-free after the 15th day of transplantation (Supplementary Table S1), which may be related to the relatively long incubation period of bacterial wilt (usually 2–3 weeks) and the absence of mechanical damage (Li et al., 2021). *Ralstonia solanacearum* mainly invades through the wounds of the roots. In this study, no mechanical damage was caused to the tomato seedlings during the transplantation process. Therefore, the colonization rate of GMI1000 may be slowed down, resulting in the disease incidence of bacterial wilt of tomatoes in four treatments being 0 after the 15th day of transplantation. However, the incidence and DI of bacterial wilt in T2 treatment which is only *R. solanacearum* inoculation reached as high as 77.78% and 60, which indicated that *R. solanacearum* seriously affected the healthy growth of tomato seedlings after 30th days of transplantation. The incidence and DI of bacterial wilt in T3 treatment significantly decreased to 22.22% and 13.33%, following the application of EB, indicating that EB significantly inhibited the *R. solanacearum* growth. Furthermore, the CE of the EB against bacterial wilt reached 77.78%. Thus, both the antagonistic experiment and pot experiment of the EB and GMI1000 demonstrated the effective control of bacterial wilt by the EB.

3.2 The migration of EB to tomato root

The migration ability of the EB toward tomato roots was assessed using AQ-PCR in this study, and the corresponding results are presented in Figure 2a. The amount of the EB in the M site for treatments, T1 and T3, was 12.29 and 13.06 (Log_{10} Copies g^{-1} soil), respectively, while in the R site it was 9.85 and 8.60 (Log_{10} Copies g^{-1} soil) on the 15th day after tomato transplanted. The amount of the EB in the M site for treatments, T1 and T3, was 10.68 and 10.12 (Log_{10} Copies g^{-1} soil), respectively, while in the R site, it was 13.45 and 12.98 (Log_{10} Copies g^{-1} soil) at the 30th day after tomato transplanted.

These results demonstrated that the amount of the EB at the R site in T1 and T3 treatments was lower than that of the M site at the 15th day after the tomato transplanted. Conversely, it was significantly increased at the 30th day after the tomato transplanted. AQ-PCR analysis suggested a gradual migration of the EB from its application site (C) toward tomato roots (R) over time. Bacteria have the chemotactic response to the root exudates of plants and can efficiently colonize the root surface, which can promote the migration of the composite microbial agent toward the tomato root in this study. Supplementary Figure S1 demonstrates that both strains Rs-5 and SL-44 exhibit a consistent change pattern at the M and R sites. However, it was observed a slight decrease in the R site in T3 treatment compared with that of T1 treatment at the 30th day after tomato transplanted, potentially attributed to the suppressive effect exerted by GMI1000 on the EB. In addition, the amount of GMI1000 in the rhizosphere soil was quantified for T2 and T3 treatments at the 15th and 30th day after tomato transplanted (Figure 2b). The amount of GMI1000 at the R site in T2 and T3 treatments was 5.92 and 5.42 (Log_{10} Copies g^{-1} soil) at the 15th day after tomato transplanted, respectively. The amount of GMI1000 at the R site in T2 and T3 treatments was 6.11 and 4.72 (Log_{10} Copies g^{-1} soil) at the 30th day after the tomato transplanted, respectively. The study found a progressive increase in the amount of

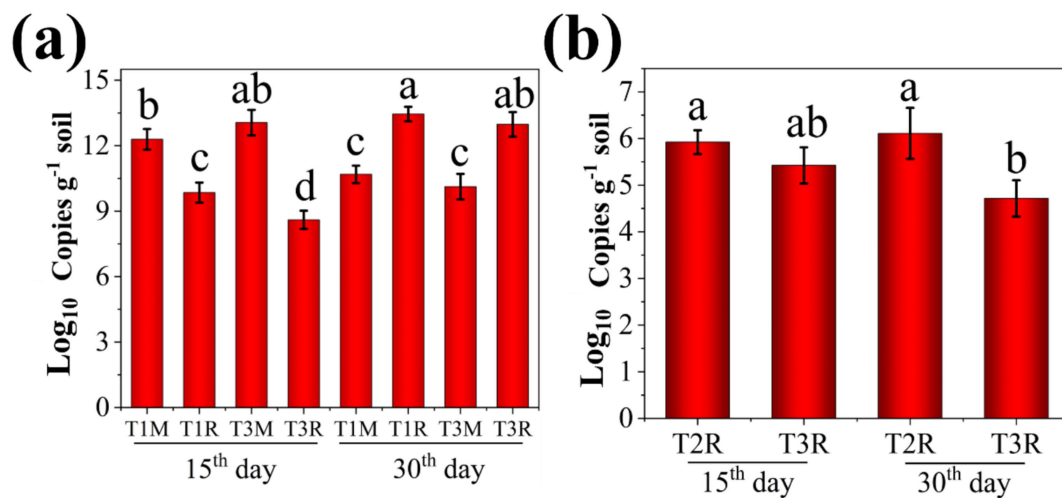


FIGURE 2

The quantities of composite microbial agent and GMI1000 in soil surrounding tomato. (a) The quantities of composite microbial agent at M or R sites; (b) The quantities of GMI1000 at R site. Lowercase letters indicate statistically significant differences between different groups. The same letters (such as a and a) indicate that there is no significant difference between the two groups, while different letters (such as a and b) indicate that there is a significant difference between the two groups ($p < 0.05$).

GMI1000 at tomato root in T2 treatment over time. Conversely, the significant decrease ($p < 0.05$) was observed in the amount of GMI1000 at tomato root in the T3 treatment as time progressed. Overall, the increase of GMI1000 amount observed in the T2 treatment and the corresponding decrease in the T3 treatment with time provide compelling evidence that the EB can inhibit GMI1000 effectively. The GMI1000 may interfere with the colonization of EB through various pathways, such as competing for the carbon sources (amino acids and sugars) secreted by the roots, occupying the binding sites of the mucilage layer on the root surface, and secreting quorum sensing inhibitors to disrupt the formation of the biofilm of beneficial bacteria (Mendes et al., 2013). The migration of soil bacteria to the plant rhizosphere is the premise for its beneficial role in the plant rhizosphere (Liu et al., 2023). Similarly, the EB can migrate to the plant rhizosphere and decrease the amount of GMI1000, thus this is the premise of the EB to be effective in controlling tomato bacterial wilt in our study.

3.3 Chemotaxis toward tomato root exudates and tomato root colonization by EB

Plant root exudates not only provide nutrients for rhizosphere microorganisms but also serve as a driving force for microbial migration and colonization in the plant root system (Gu et al., 2020). The chemotactic movement of functional bacteria in microbial fertilizers toward the root system, induced by root exudates, represents the initial step in root colonization (Webb et al., 2017). The above study found the migration of the EB to the tomato rhizosphere, leading us to hypothesize that root exudates may serve as the chemoattractant for recruiting this EB. Therefore, we studied the chemotactic response of the EB toward various tomato root exudates (glucose, mannitol, sucrose, fructose, leucine, glutamic acid, aspartic acid, alanine, tartaric acid, malic acid, citric acid and fumaric acid) were studied using a swarm plate assay in this study.

The results demonstrated that strain SL-44 exhibited chemotaxis toward sucrose, glutamic acid, and aspartic acid, with respective chemotactic radii of 1.475, 1.975, and 2.325 cm (Table 1). Conversely, strain Rs-5 displayed chemotaxis toward leucine and glutamic acid, with corresponding chemotactic radii of 1.7 and 1.825 cm, respectively. Furthermore, the EB demonstrated chemotaxis toward sucrose, leucine, glutamic acid, and aspartic acid, with corresponding chemotactic radii of 1.125, 1.125, 2.05, and 1.225 cm, respectively. The amino acids (glutamic acid, leucine, and aspartic acid) exhibit a greater capacity to induce the movement of the EB compared to sugar (sucrose). Consequently, amino acids play a pivotal role in this migration process. The study by Feng et al. (2022) demonstrated that rhizosphere chemotaxis was primarily driven by a few key chemotactic factors rather than the cumulative effect of all chemotactic substances. Notably, strains Rs-5 and SL-44 exhibited significant chemotactic responses toward glutamate, with the largest observed chemotactic radius, thus glutamate was judged as the key chemotactic substance in this study. Root exudates encompass a wide array of signaling molecules and chemical inducers, which possess the ability to selectively recruit beneficial microbes to the root system for enrichment (Chen S. et al., 2023). Zhou et al. (2023) demonstrated that root exudates, particularly taxifolin-a flavonoid compound, can effectively induce the migration of *Bacillus* toward tomato roots. Wang et al. (2019) indicated that tomato root exudates (hexanoic acid and lactic acid) possess the ability to mitigate tomato bacterial wilt. Furthermore, it was observed that *Bacillus cereus* AR156 exhibited a chemotactic response toward fructose, lactic acid, sucrose and threonine present in tomato root exudates.

The above results have demonstrated the chemotactic effect of the EB on tomato root secretions, enabling its migration toward tomato roots in response to root exudates. Meanwhile, the colonization and biofilm formation of the EB on tomato roots are also crucial criteria for assessing its biocontrol efficacy. The SEM of the growth and elongation regions of tomato roots under different treatments were presented in Figure 3. The strain Rs-5, SL-44, and the EB exhibited a well colonization

TABLE 1 The chemotactic response of composite microbial toward 12 root exudates of tomato.

Treatments		SL-44	Rs-5	Composite microbial agent
Root exudates				
Sugar	Glucose	-	-	-
	Mannitol	-	-	-
	Sucrose	1.475 ± 0.25 (cm)	-	1.125 ± 0.395 (cm)
	Fructose	-	-	-
Amino acid	Leucine	-	1.7 ± 0.115 (cm)	1.125 ± 0.058 (cm)
	Glutamic acid	1.975 ± 0.171 (cm)	1.825 ± 0.236 (cm)	2.05 ± 0.05 (cm)
	Aspartic acid	2.325 ± 0.15 (cm)	-	1.225 ± 0.171 (cm)
	Alanine	-	-	-
Organic acid	Tartaric acid	-	-	-
	Malic acid	-	-	-
	Citric acid	-	-	-
	Fumaric acid	-	-	-

on the both the meristematic and elongation zones of tomato roots especially on the elongation zones. The biofilm was formed on the tomato root surface due to the large amount of colonization in the elongation zone of tomato root compared with Rs-5 and SL-44 (Figure 3f). Ye et al. (2020) also demonstrated the ability of *Corallococcus* sp. EGB to colonize and form biofilm in the elongated zone of cucumber roots, thereby effectively prevent cucumber wilt invade. Zhang and Shen (2022) proved that biofilm can resist adverse environmental factors, enabling them to grow and reproduce in unstable environment. Therefore, the efficient colonization of the EB in the elongation region enables effective control of tomato bacterial wilt in this study. Sampedro et al. (2020) demonstrated that *Halomonas anticariensis* FP35T performed beneficial function by root chemotaxis, efficient colonization and biofilm formation in the plant root.

3.4 The physicochemical properties and root soil nutrient content of tomato seedlings

The assessment of plant biomass and physicochemical properties provides an intuitive way to validate the efficacy of the EB in managing tomato bacterial wilt. The growth state of tomato seedlings after 30 days of transplanting is depicted in Figure 1c, demonstrating the effective prevention and control of tomato bacterial wilt by the EB, as well as its growth promotion to healthy tomato seedlings. The plant height, fresh weight, dry weight, root length and stem diameter exhibited significant increases of 39.68%, 112.76%, 38.26%, 24.71%, and 29.27%, respectively, in the T3 treatment compared with the T2 treatment for the EB inoculation (Figures 4a–d; Supplementary Figure S2a). The plant height, fresh weight, dry weight,

root length, and stem diameter exhibited significant increases of 19.38%, 35.04%, 37.24%, 27.55%, and 18.60%, respectively, in the T1 treatment compared to the CK treatment. The pot experiment demonstrated that the EB significantly enhanced the biomass of T3 treatment seedlings, which can be attributed to its effective suppression of GMI1000. The application of *B. subtilis* (SSR2I) also significantly enhanced the biomass of tomato seedlings infected with *R. solanacearum* (Jinal and Amaresan, 2020). Besides, the pro-growth properties of the EB such as phosphorus solubilization, nitrogen fixation, IAA, ACC deaminase and ferrophilin production may play a key role (Wu et al., 2019). Li et al. (2023) mentioned that the role of beneficial rhizosphere microbes in regulating root system growth and development, thereby facilitating plant growth.

In our study, the T3 treatment showed a significant increase in soluble protein (142.63%), soluble sugar (72.59%), chlorophyll (196.72%), and carotenoid contents (214.97%) compared with the T2 treatment (Figure 4e; Supplementary Figures S2b–d). Similarly, the T1 treatment showed a significant increase in soluble protein (178.91%), soluble sugar (51.90%), chlorophyll (23.35%), and carotenoid contents (22.17%) compared with the CK treatment. Soluble protein and sugar play a crucial role in regulating the osmotic regulation capacity of plant cells, thereby mitigating damage to the plant cell membrane under stressful conditions (Wang et al., 2022). In addition, the content of chlorophyll and carotenoid in plants are the important physiology index of plant (Zheng et al., 2016). However, it can be seen from the above results that the soluble protein, soluble sugar, chlorophyll and carotenoids content of plant in the T2 treatment are significantly reduced, which may be due to leaf atrophy and growth obstruction of tomato seedlings infected with *R. solanacearum*, resulting in a decline in their synthetic ability (Hahm et al., 2017). Fortunately, the corresponding contents of tomato seedlings were significantly increased after treatment with the EB. The SOD, POD, and CAT activities in the T3 treatment showed a significant increase of 19.99%, 5.22%, and 53.16% respectively, compared with the T2 treatment (Figures 4f–h). Moreover, the SOD, POD, and CAT activities in the T1 treatment showed a significant increase of 53.95%, 78.85%, and 90.21% respectively, compared with CK treatment. The antioxidant enzyme activity of tomato seedlings was significantly enhanced in the T1 treatment compared with the CK treatment, indicating that the EB itself could stimulate plants to increase the antioxidant enzyme activity. Additionally, in the T2 treatment, there was a significant increase antioxidant enzyme activity of tomato, which helped alleviate stress caused by *R. solanacearum*. The highest level of antioxidant enzyme activity was observed in tomato in T3 treatment, which could be attributed to the combined growth promoting effect of EB and alleviation of the biotic stress brought by *R. solanacearum*. The activities of SOD and POD were observed to increase following infection with *R. solanacearum*, while their activities were further elevated upon treatment with the composite microbial agent consisting of *Trichoderma virens* and *B. velezensis* (Zhou et al., 2021). Jinal and Amaresan (2020) demonstrated that *B. subtilis* SSR2I application significantly increased the activities of SOD and POD, thereby enhancing tomato's resistance ability against to bacterial wilt. Besides, studies have revealed that the antioxidant enzyme system in the plant is activated in order to maintain the oxidative balance of the cells, as shown by the increased activity of SOD and the decomposition of superoxide anion into H_2O_2 . At the same time, the POD and CAT activity are subsequently increased, and the generated H_2O_2 is further

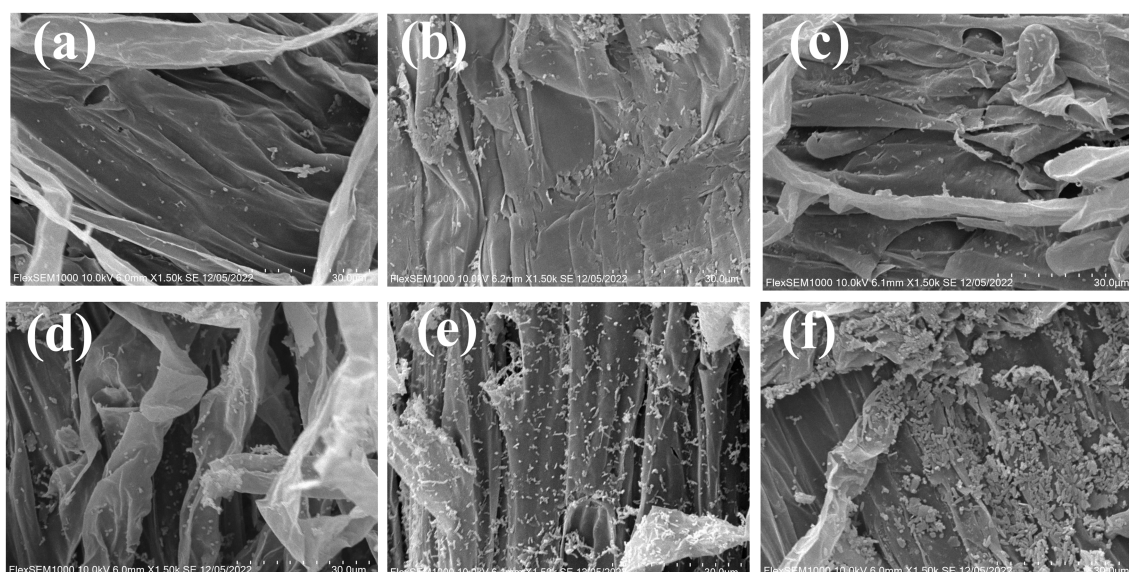


FIGURE 3

The colonization of composite microbial agent in tomato root surface. (a–c) The colonization of strain Rs-5, SL-44 and composite microbial agent in meristematic zone of tomato root; (d–f) The colonization of strain Rs-5, SL-44 and composite microbial agent in elongation zone of tomato root.

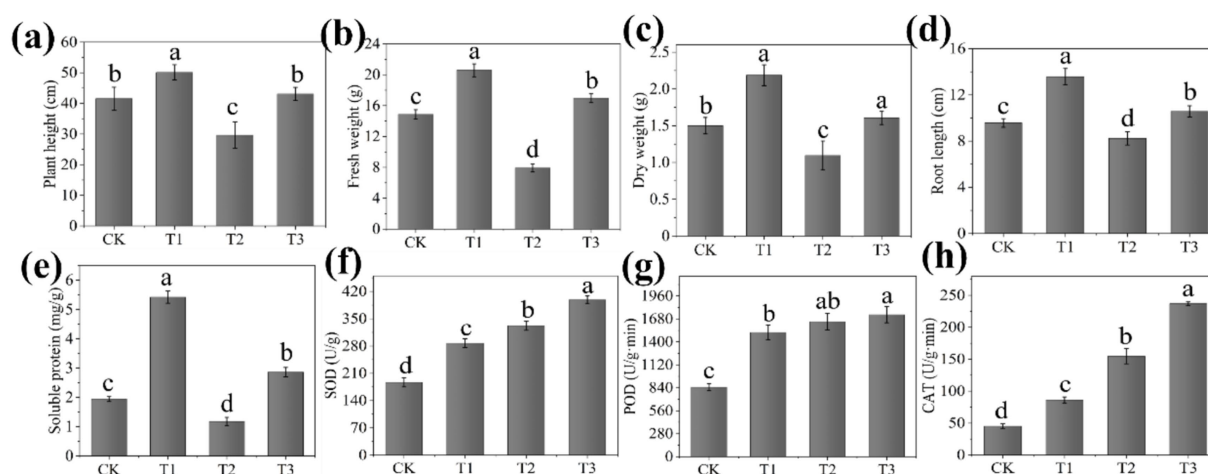


FIGURE 4

Effects of the composite microbial agent on biomass (a–d), the soluble protein content (e), and antioxidant enzyme activities (f–h) of seedlings ($n = 6$). Bars represent means standard error. Different lowercase letters indicate average of six replicates with significant differences at $p < 0.05$ among different treatments.

oxidized and decomposed into water, which eliminates the oxidative stress damage to the cells, thus promoting plant growth under stressful conditions (Qian et al., 2020; Shabaan et al., 2023). Therefore, the above findings align with the outcomes of our study.

The contents of soil organic matter (SOM), soil alkali-hydrolytic nitrogen (SAN), soil available phosphorus (SAP) and soil available potassium (SAK) in the T3 treatment root significantly increased by 11.58%, 14.71%, 27.28%, and 13.92% respectively, compared with the T2 treatment (Supplementary Figure S4). Similarly, the contents of SOM, SAP, and SAK in the T1 treatment showed significant increases of 16.68%, 10.05%, 34.04%, and 19.94% respectively,

compared with the CK treatment. Soil nutrient contents serve as crucial indicators for assessing soil health and nutrient cycling, playing an integral role in evaluating soil fertility (Abdelkrim et al., 2020). The EB as the PGPR in this study, can symbiosis with plant roots, promote the growth and activity of soil microbes, and thus increase the soil organic matter content. Previous studies have found that the compound bacteria had the functions of nitrogen fixation, phosphorus dissolution and potassium dissolution, which will promote the increase of soil alkali-hydrolytic nitrogen, available phosphorus and available potassium in plant rhizosphere (Wu et al., 2019; Yue et al., 2007). Therefore, the increase of rhizosphere soil

nutrient content in T3 treatment was due to the effective inhibition of EB on GMI1000.

In general, the pot experiment revealed a severe impact of bacterial wilt on the healthy growth of tomato seedlings, leading to a significant reduction in biomass, physicochemical properties, and rhizosphere soil nutrient content in plants subjected to T2 treatment. However, the addition of the EB effectively suppressed tomato bacterial wilt and significantly enhanced the physicochemical properties and rhizosphere soil nutrient content of tomato seedlings under T3 treatment.

3.5 Effects of EB on the microbial structure and diversity in tomato rhizosphere soil

The interactions between the inter-root soil microbiome and plants play a pivotal role in facilitating the robust growth and enhanced productivity of plants (Li et al., 2019). Herein, we investigated the impact of EB on the microbial community in the rhizosphere soil of tomato seedlings infected with *R. solanacearum*. We observed a gradual saturation of the number of OTUs in the four groups with increasing sequencing depth. The rarefaction curve for bacteria and fungi exhibited a gradual flattening trend (Supplementary Figure S4), indicating that the amount of sample sequencing data was appropriate and met the requirements of this study. At the 97% similarity level, bacteria and fungi detected a total of 4,119 and 1,810 OTUs, respectively, across all four treatments (Supplementary Figure S5). The Venn diagram revealed that the numbers of shared characteristic bacterial sequences in CK between T1, T2, and T3 treatments were 2,721, 2,581, and 2,681, respectively, (Supplementary Figure S5a). Similarly, the numbers of shared characteristic fungal sequences in CK between T1, T2, and T3 treatments were 538, 518, and 485, respectively, (Supplementary Figure S5b). The comparison between CK and T3 revealed a higher number of bacterial common feature sequences compared to that between CK and T2, indicating a relatively similar composition of bacterial OTUs between CK and T3. We speculated that the aforementioned phenomenon could be attributed to the stress-alleviating effects of the EB on the rhizosphere soil bacterial community in response to *R. solanacearum*. However, the common characteristic sequences of fungi between CK and T3 were relatively fewer, which can be attributed to the synergistic effect of the EB and *R. solanacearum* on the fungal community in rhizosphere soil.

The relative abundance of bacteria and fungi at phylum and genus levels in the rhizosphere soils of different treatments was depicted in Figure 5. In terms of bacterial community composition, 8 phyla of bacteria were detected with abundance more than 1%, namely *Proteobacteria*, *Acidobacteriota*, *Gemmatimonadota*, *Bacteroidota*, *Actinobacteria*, *Verrucomicrobiota*, *Firmicutes* and *Actinobacteriota* (Figure 5a). Among them, the relative abundance of *Proteobacteria* was highest in different treatments, with percentages of 41.94%, 49.06%, 44.88%, and 54.58% for CK, T1, T2, and T3, respectively. Similarly, *Proteobacteria* has been widely identified as the predominant bacterial group in diverse soil ecosystems (Zarraonaindia et al., 2015). Considering that both Rs-5 and GMI1000 belong to *Proteobacteria*, which no wonder that it resulted

in the highest relative abundance of *Proteobacteria* in the T3 treatment. *Ralstonia solanacearum* exerted a significant impact on the relative abundance of bacteria at the phylum level. The abundance of *Acidobacteriota* significantly increased, whereas *Gemmatimonadota* and *Bacteroidota* exhibited a significant decrease in the T2 treatment compared with the CK treatment. However, the relative abundance of some phylum in the T3 treatment did not exhibit a statistically significant difference compared with that of CK treatment following administration of the EB. The 8 of the 10 most abundant bacterial genera found in soil samples were consistent across all four treatments, including *Lysobacter*, *Sphingomonas*, *Massilia*, *RB41*, *Pseudomonas*, *Bacillus*, *Arenimonas*, and *Ramlibacter*. Additionally, *Pseudoxanthomonas* was only detected in CK, *MND1* was present in both CK and T1, *Enterobacter* was present in T1, T2, and T3 while *Ralstonia* was present in T2 and T3 (Figure 5b). The detection of *Enterobacter* in the T1 and T3 treatments was expected; however, it was intriguing to find *Enterobacter* also present in the T2 treatment. We postulate that the *Enterobacter* present in T2 were recruited by the plant root exudates by system-induced resistance strategy to against *R. solanacearum*. Furthermore, both *Enterobacter* and *Ralstonia* were detected in the rhizosphere soil, providing additional evidence supporting our AQ-PCR findings for detecting Rs-5 and GMI1000 in plant rhizosphere soil. Hu et al. (2020) proved that the relative abundance of *Massilia* was higher in healthy soil compared with soil infected with *R. solanacearum*, which aligned with our findings. The relative abundance of *Pseudomonas* was significantly enhanced after EB inoculation when infected with *R. solanacearum*. The *Pseudomonas* sp. strain Y8 was also observed to exhibit significant efficacy in suppressing the growth of *R. solanacearum* (Li et al., 2021). Furthermore, our study revealed that the application of the EB led to a notable increase in the relative abundance of *Bacillus* within the tomato rhizosphere. Shen et al. (2021) also demonstrated the ability of *Streptomyces microflavus* G33 could enhance the relative abundance of *Bacillus* in the rhizosphere, thereby exerting inhibitory effects on tomato bacterial wilt. In general, the EB significantly enhanced the relative abundance of beneficial bacteria *Massilia*, *Pseudomonas*, *Bacillus* and *Enterobacter* in rhizosphere soil infected with *R. solanacearum*, thereby exerting a pivotal role in the management of bacterial wilt and promotion of healthy growth in tomato seedlings. Soil fungi are also integral components of the microbiome and play a pivotal role in maintaining the microecology of the rhizosphere, crucial for plant growth and development (Ali et al., 2020). The impact of *R. solanacearum* on the rhizosphere soil fungal community is evident in Figures 5c,d, particularly in terms of changes in relative abundance at the phylum and genus levels. The top 10 fungal phyla with higher relative abundance were *Ascomycota*, *Basidiomycota*, *Mortierellomycota*, *Zygomycotina*, *Chytridiomycota*, *Blastocladiomycota*, *Zoopagomycota*, *Rozellomycota*, and *Glomeromycota*. *Ascomycota*, *Basidiomycota* and *Mortierellomycota* were the dominant phylum in the fungal community, which was consistent with other studies (Tortora et al., 2011; Shen et al., 2022). The short-term impact of the EB on the relative abundance of fungal community in the original soil sample was found to be statistically insignificant. The relative abundance of *Ascomycota*, *Basidiomycota* and *Chytridiomycota* was significantly decreased, while the relative abundance of *Mortierellomycota*, *Blastocladiomycota* and *Zygomycotina* was increased. On the contrary, the relative abundance

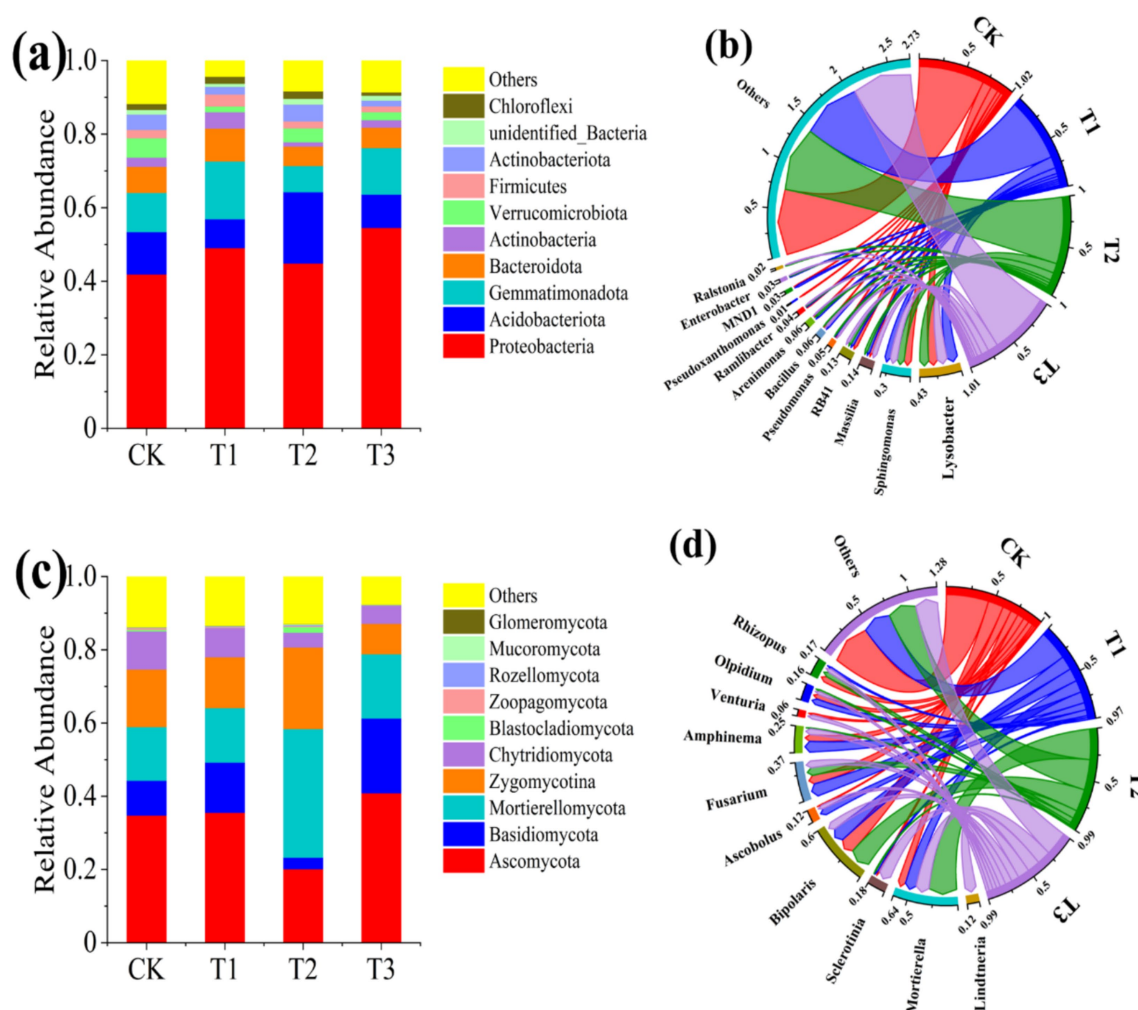


FIGURE 5

Changes in microbial community composition in in tomato rhizosphere soil. (a) Relative abundance of bacteria at the phylum level. (b) Relative abundance of bacteria at the genus level. (c) Relative abundance of fungi at the phylum level. (d) Relative abundance of fungi at the genus level.

of *Ascomycota* and *Basidiomycota*, and the relative abundance of *Mortierellomycota*, *Blastocladiomycota*, and *Zygomycotina* were significantly increased and reduced in tomato rhizosphere soil infected with *R. solanacearum* after application of the EB. The top 10 genera exhibiting high fungal relative abundance in the rhizosphere soil of each treatment were *Lindneria*, *Mortierella*, *Sclerotinia*, *Bipolaris*, *Ascobolus*, *Fusarium*, *Amphinema*, *Venturia*, *Olpidium* and *Rhizopus*.

The diversity and dissimilarity of the rhizosphere soil microbial community were assessed using Alpha Diversity and Beta Diversity analyses. The bacterial Shannon index, Chao1 index, and Simpson index of the T1 treatment exhibited higher values compared to other treatments in [Supplementary Figure S6a](#), while the T2 treatment displayed the lowest values for both bacterial Shannon index and Simpson index. The fungal Shannon index, Chao1 index, and Simpson index were found to be higher in the CK treatment compared to other treatments. Conversely, the fungal Shannon index, Chao1 index and Simpson index exhibited the lowest values in the T3 treatment ([Supplementary Figure S6b](#)). The fungal and

bacterial Goods coverage index of the four treatments were approximately equal to 1. Bacterial and Fungal Beta Diversity were assessed using Weighted unifracs distance and Unweighted unifracs distance, which quantified the dissimilarity in species diversity between the two treatments. Additionally, Principal Component Analysis (PCA) was employed to evaluate the disparity in microbial community structure among the treatments. The results presented in [Figure 6a](#) demonstrate significant disparities in bacterial species diversity between the CK and T2, T1 and T2, while comparatively fewer differences were observed between T2 and T3. In [Figure 6b](#), notable discrepancies in fungal species diversity were observed between CK and T3, T2, and T3. The bacterial community composition and structure exhibited variations among different treatments, as depicted in [Figure 6c](#). The first and second axes represent the contribution values of the first and second principal components to the sample differences, explaining 15.2% and 13.28% of the overall variation in the bacterial community, respectively, with the total explanation of 28.48%. In addition, there were significant differences in the composition and structure

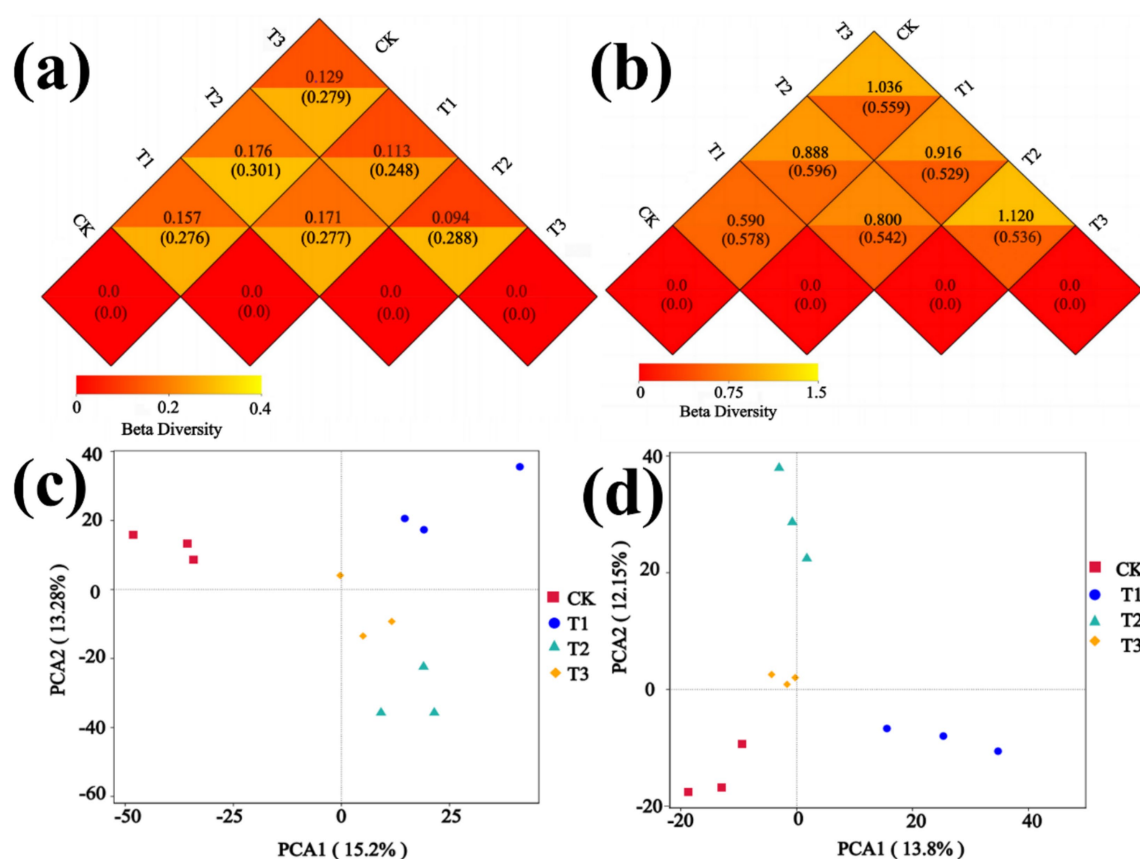


FIGURE 6
Beta diversity analysis of microbial community. (a) Distance Matrix Heatmap of bacteria. (b) Distance Matrix Heatmap of fungi. (c) PCA of bacteria. (d) PCA of fungi.

of the fungal community were observed among different treatments (Figure 6d). The first and second axes represent the contribution values of the first and second principal components to the sample differences, explaining 13.8 and 12.15% of the overall variation in fungal community, respectively, with the total explanation of 25.95%.

4 Conclusion

The EB effectively inhibits tomato bacterial wilt, as evidenced by a significant reduction in the incidence and quantities of *R. solanacearum*, along with notable improvements in plant biomass, physicochemical properties, and rhizosphere soil nutrient contents. The EB was observed to exhibit migration toward the tomato root and colonization in the elongation zone, resulting in the formation of a film induced by root exudates, which serves as a prerequisite for its biocontrol efficacy. Furthermore, the EB can alter the microbial community structure in *R. solanacearum*-infected rhizosphere soil. In summary, the EB achieve their efficient control of tomato bacterial wilt goal through enriching in the rhizosphere, suppressing on *R. solanacearum*, and modulating the rhizosphere soil microbial community. Therefore, the utilization of composite microbial agent holds significant potential as the novel and efficient strategy for the control of tomato bacterial wilt.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

SZ: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. XC: Formal analysis, Resources, Writing – review & editing. NL: Formal analysis, Investigation, Software, Visualization, Writing – review & editing. YH: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. JW: Investigation, Methodology, Writing – review & editing. ZW: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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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/fmicb.2025.1559380/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

The quantities of the stain SL-44 (a) and Rs-5 (b) at M or R sites.

SUPPLEMENTARY FIGURE S2

Effects of the composite microbial agent on stem diameter, soluble sugar, chlorophyll and carotenoid contents biomass of seedlings (n = 6).

SUPPLEMENTARY FIGURE S3

Effects of the composite microbial agent on the nutrient content of the root soil (n = 6).

SUPPLEMENTARY FIGURE S4

The rarefaction curve of bacteria (a) and fungi (b) in tomato rhizosphere soil.

SUPPLEMENTARY FIGURE S5

The 16S rRNA OTU Venn graph of bacteria (a) and fungi (b) in tomato rhizosphere soil.

SUPPLEMENTARY FIGURE S6

Alpha Diversity analysis of microbial community in tomato rhizosphere soil: (a) Shannon index, Chao1 index, Goods_coverage and Simpson index of bacteria community; (b) Shannon index, Chao1 index, Goods_coverage and Simpson index of fungi community.

SUPPLEMENTARY FIGURE S7

The mechanism of the composite microbial agent in the control of tomato bacterial wilt.

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