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Biotechnological applications of endophytes in agriculture, environment and industry, 2nd Edition

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Published in
Frontiers in Microbiology
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-8325-4168-5
DOI 10.3389/978-2-8325-4168-5

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Biotechnological applications of endophytes in agriculture, environment and industry, 2nd Edition

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Citation

Sharma, V. K., Newcombe, G., Li, H., Trivella, D., Soni, R., eds. (2023). *Biotechnological applications of endophytes in agriculture, environment and industry, 2nd Edition*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-4168-5

Publisher's note: In this 2nd edition, the following article has been added: Zahra ST, Tariq M, Abdullah M, Zafar M, Yasmeen T, Shahid MS, Zaki HEM and Ali A (2023) Probing the potential of salinity-tolerant endophytic bacteria to improve the growth of mungbean [*Vigna radiata* (L.) Wilczek]. *Front. Microbiol*. 14:1149004. doi: 10.3389/fmicb.2023.1149004

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frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 15 August 2023
DOI 10.3389/fmicb.2023.1269279

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 29 July 2023
ACCEPTED 03 August 2023
PUBLISHED 15 August 2023

CITATION
Li H, Sharma VK, Newcombe G, Trivella DBB and Soni R (2023) Editorial: Biotechnological applications of endophytes in agriculture, environment and industry.
Front. Microbiol. 14:1269279.
doi: 10.3389/fmicb.2023.1269279

Editorial: Biotechnological applications of endophytes in agriculture, environment and industry

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KEYWORDS

endophytes, agriculture, environment, industry, biotechnological applications

Editorial on the Research Topic
Biotechnological applications of endophytes in agriculture, environment and industry

Endophytes are the plant symbionts that live inside the plant tissue without causing any symptoms of disease for a part of their life-cycle. They are an important untapped reservoir of biological resources. They can promote plant growth by improving the physiological and metabolic functions of host plants via nutrient acquisition, nitrogen fixation, phytohormone production, etc., which can be used to promote agricultural yield and food quality. They also have potential applications in enhanced phytoremediation. In addition, endophytes are known to produce various novel antibiotics that can be used in the pharmaceutical, food, and agricultural industries. Functional genomics studies of endophytes provided more information and a better understanding of the network of complex host-endophyte interactions and other associated microbes to harness the biotechnological potential of endophytes more efficiently and sustainably.

The main aim of this Research Topic was to recover the functional role and application of endophytes for agricultural, medicinal, industrial, and environmental purposes. Within this topic, nine articles have been published that complement our knowledge on the occurrence and diversity of endophytes and the role, mechanism, and biotechnological application of endophytes in these fields.

One of the main causes of the global drop in crop productivity is pathogenic microorganisms. Endophytes diminish the injury triggered by pathogens through synthesizing antibiosis, the production of lytic enzymes, secondary metabolites, hormone activation, etc. (Chaudhary et al.). An et al. isolated an endophytic bacterium *Burkholderia ambifaria* XN08 with antagonistic activity against *Rhizoctonia cerealis*, a wheat (*Triticum aestivum* L.) sharp eyespot pathogenic fungus. The colonization of strain XN08 was accompanied by an enhancement of wheat growth and an induction of wheat sharp eyespot resistance by synthesizing a series of plant growth regulators (indole-3-acetic acid, IAA, etc.), producing antifungal compounds (pyrrolnitrin, etc.), and enhancing the

activities of defense enzymes (polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase). The role of *Trichoderma asperellum* against *Fusarium* wilt disease (FDW) in tomato (*Solanum lycopersicum*) plants was investigated by Sehim et al.. They found that *T. asperellum* exhibited the highest mycelial inhibition rate (53.24%) against *Fusarium oxysporum*. *T. asperellum* enhanced the growth of tomato seeds and controlled the FDW by enhancing the number of leaves, as well as shoot and root length and fresh and dry weights by producing IAA, Phosphate (P) solubilization, and synthesizing bioactive secondary metabolites. Furthermore, *Trichoderma* extract increased shelf-life of tomato fruits by reducing infection by *F. oxysporum* from post-harvest.

Abiotic stress, such as drought and flood stress, heavy metal stress, prevents plants from growing normally and lowers crop output. Endophytes represent safe and effective biological agents that mitigate abiotic stress for plant development. Ou et al. screened out *Klebsiella aerogenes* HGG15 from 28 endophytic bacteria as having superior plant growth promotion (PGP) traits, including P solubilization, IAA, siderophore, and acetoin production, as well as biosafety for silkworms. Flood tolerance of mulberry (*Morus alba* L.) was increased by inoculated *K. aerogenes* HGG15 by synthesizing a series of abiotic stress response factors and growth promotion stimulators such as glycerolipid, sphingolipid, indole, pyridine, and coumarin. Santra and Banerjee isolated a Galactose-Rich Heteropolysaccharide (GRH) from endophytic *Mucor* sp. HELF2. Spraying with 50 ppm GRH has alleviated drought stress in rice seedlings (*Oryza sativa* ssp. indica MTU 7093 swarna) by improving relative water content and fresh weight of the tissues, root length, and shoot length, as well as increasing the soluble sugars, prolines, and chlorophyll contents of rice seedlings and elevating the enzymatic antioxidant parameters. The role of seed endophyte FXZ2 on *Dysphania ambrosioides* Zn/Cd tolerance and accumulation was investigated by Sharma et al.. The study suggests that the Zn/Cd tolerance of the host plant was increased by seed endophyte FXZ2 by altering Zn/Cd speciation in rhizospheric soils and exogenous production of phytohormones to promote growth, lowering oxidative damage while enhancing antioxidant properties. In addition, Zn uptake in inoculated plants was decreased, while Cd accumulation was increased in the inoculated plants that were grown in Zn/Cd contaminated soil. Similarly, Flores-Duarte et al. isolated and selected 4 endophytic rhizobia and non-rhizobia with higher PGP properties and bacterial enzymatic activities from *Medicago* spp., including *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12. Inoculation with combinations of *Ensifer* (rhizobia) and *Pseudomonas* increased plant biomass and nodules ameliorating the physiological state of the plants and helping to regulate plant stress mechanisms, while increasing As, Cd, Cu, and Zn accumulation in plant roots, without significant differences in shoot metal accumulation, on nutrient-poor soils and moderately contaminated with metals/loids. Endophytes provide new insights into agricultural production and environmental health.

In the field of livestock feed production such as silage, microbes with antibacterial and other properties have been extensively researched and used. Zhang et al. assessed the effects of antibacterial peptide-producing *Bacillus subtilis* CP7 on the fermentation quality and bacterial community of different varieties of whole-plant corn silage. The additive *B. subtilis* CP7 enhanced the quantity of dry matter and crude protein, and improved the structure of the bacterial community following silage.

With the development of technology, artificial intelligence (AI) has been extensively used in the biotechnology and applied microbiology sectors. Deep learning, prediction, support vector machines, object detection, feature representation, synthetic biology, amyloid, human microRNA precursors, systems biology, and single cell RNA-Sequencing were the current hot spots, while microRNA and protein-protein interactions (PPIs) are the future trends in this area (Xu et al.). Studying PPIs using AI methods provides a better understanding of the complex network of host-endophyte interactions and other associated microbes to harness the biotechnological potential of endophytes more efficiently and sustainably.

In conclusion, endophytes were developed as an eco-friendly microbial agent for overcoming the tasks faced with conventional farming, the environment, and industry. Coupled with the AI, microbiome, and metabolite analyses, the mechanism of the role of endophytes could possibly be studied effectively and deeply, consequently amplifying the application potential of these beneficial microbes.

Author contributions

HL: Writing—original draft, Writing—review and editing. VS: Writing—review and editing. GN: Writing—review and editing. DT: Writing—review and editing. RS: Writing—review and editing.

Funding

This work was supported by the Natural Science Foundation of China (42267059) and Yunnan International Joint Laboratory of Research and Development of Crop Safety Production on Heavy Metal Pollution Areas.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 28 July 2022
DOI 10.3389/fmicb.2022.906724

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 29 March 2022
ACCEPTED 04 July 2022
PUBLISHED 28 July 2022

CITATION
An C, Ma S, Liu C, Ding H and Xue W (2022) *Burkholderia ambifaria* XN08: A plant growth-promoting endophytic bacterium with biocontrol potential against sharp eyespot in wheat. *Front. Microbiol.* 13:906724. doi: 10.3389/fmicb.2022.906724

Burkholderia ambifaria XN08: A plant growth-promoting endophytic bacterium with biocontrol potential against sharp eyespot in wheat

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Plant growth-promoting bacteria (PGPB) have been considered promising biological agents to increase crop yields for years. However, the successful application of PGPB for biocontrol of sharp eyespot in wheat has been limited, partly by the lack of knowledge of the ecological/environmental factors affecting the colonization, prevalence, and activity of beneficial bacteria on the crop. In this study, an endophytic bacterium XN08 with antagonistic activity against *Rhizoctonia cerealis* (wheat sharp eyespot pathogenic fungus), isolated from healthy wheat plants, was identified as *Burkholderia ambifaria* according to the sequence analysis of 16S rRNA. The antibiotic synthesis gene amplification and ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) analyses were used to characterize the secondary metabolites. The results showed that the known powerful antifungal compound named pyrrolnitrin was produced by the strain XN08. In addition, *B. ambifaria* XN08 also showed the capacity for phosphate solubilization, indole-3-acetic acid (IAA), protease, and siderophore production *in vitro*. In the pot experiments, a derivate strain carrying the green fluorescent protein (*GFP*) gene was used to observe its colonization in wheat plants. The results showed that GFP-tagged *B. ambifaria* could colonize wheat tissues effectively. This significant colonization was accompanied by an enhancement of wheat plants' growth and an induction of immune resistance for wheat seedlings, which was revealed by the higher activities of polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia-lyase (PAL). As far as we know, this is the first report describing the colonization traits of *B. ambifaria* in wheat plants. In addition, our results indicated that *B. ambifaria* XN08 might serve as a new effective biocontrol agent against wheat sharp eyespot disease caused by *R. cerealis*.

KEYWORDS

***Burkholderia ambifaria*, biological control, *Rhizoctonia cerealis*, colonization, antifungal activity**

Introduction

The wheat sharp eyespot, caused predominately by the necrotrophic fungus *Rhizoctonia cerealis*, is one of the most destructive soil-borne fungal diseases in wheat (*Triticum aestivum* L.) and results in yield losses of 10%−40% in the regions of Asia, Oceania, Europe, North America, and Africa (Wang et al., 2018; Zhao et al., 2021). This fungal pathogen can survive in soils or the infected crop residues for a long time, and it reinfects the stems and sheaths of wheat plants in the favorable environmental conditions, blocks the transportation of nutrients, and eventually leads to host death (Su et al., 2020). Traditional agrochemicals, which were still widely used for the effective control of wheat sharp eyespot, had led to an increase in environmental pollution and induced pesticide resistance (Zhang et al., 2017). Therefore, the biological control of wheat sharp eyespot as a green and sustainable agricultural biotechnology has attracted lots of attention (Raymaekers et al., 2020; Xu et al., 2020).

Plant growth-promoting bacteria (PGPB) have been considered promising biological agents for years (Dimkić et al., 2022). They have shown multifunctional plant-promoting ways including the facilitation of nutrient uptake, nitrogen fixation for plant use, the production of plant hormones, direct antagonism against pathogens, and the induction of systemic resistance throughout the plant (Jing et al., 2019). Therefore, many researchers have focused on the exploration of new PGPB with varied beneficial effects in recent years. For example, *Pantoea dispersa*-AA7 and *Enterobacter asburiae*-BY4, which were isolated from sugarcane rhizosphere soils, showed the capacity for nitrogenase and ACC deaminase production (Jing et al., 2019). Saad et al. (2020) isolated 18 strains from the rhizosphere soils of red silk-cotton tree and Chinese banyan and found that *Bacillus thuringiensis* MN419208 exhibited the capacity for plant growth promotion by producing indole-3-acetic acid (IAA) and exopolysaccharides and exerting the capacity of nitrogen fixation, while *Bacillus sonorensis* MN419205, *Bacillus wiedmannii* MN419207, and *Bacillus subtilis* MN419218 showed the antagonistic properties against root rot in *fava beans*. In contrast, a rhizosphere isolated strain of *Pseudomonas* sp. 23S showed antagonistic activity against *Clavibacter michiganensis* subsp. *michiganensis in vitro* and reduced the severity of tomato bacterial canker by inducing systemic resistance (Takishita et al., 2018). However, it is inadequate to excavate the wheat association PGPB, especially in screening the biological control agents against sharp eyespot caused by *R. cerealis*.

On the contrary, although many PGPB have shown excellent antagonistic characteristics under laboratory and greenhouse conditions, the successful application of PGPB under field conditions has been limited by its poor colonization capacity (Rilling et al., 2019). In fact, the effective root colonization of PGPB is considered to be a critical factor in achieving successful plant–microbe interaction (Bo et al., 2022). Compared with the plant rhizobacteria, bacterial endophytes have more opportunities to be in contact with the plant cells, so they could readily exert a direct beneficial effect (Morales-Cedeno et al., 2021).

In our previous study, an endophytic bacterium XN08, which showed great antagonistic activities against varied phytopathogenic fungi including *R. cerealis*, was isolated from healthy wheat plants. The purpose of this study was to evaluate its biocontrol potential against sharp eyespot in wheat. The strain XN08 was identified *via* 16S rRNA analysis, and a known antifungal compound produced by the strain was confirmed by gene amplification and ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) analyses. To observe its colonization in wheat plants, a derivate strain carrying the green fluorescent protein (*GFP*) gene was constructed. The immune resistance of wheat seedlings was also monitored in this study.

Materials and methods

Bacterial strains and growth conditions

The strain XN08 used in this study was obtained from healthy wheat plants in our laboratory. The strain was cultivated in a nutrient broth medium (NB) and maintained at −80°C in a 20% glycerol solution. GFP-tagged *Burkholderia ambifaria* was cultivated in an NB medium containing 100 μg/ml tetracycline (Tc) for the maintenance of plasmids. *Rhizoctonia cerealis*, which was kindly provided by the Center of Biological Pesticide Research, Northwest Agricultural and Forestry University, was maintained on potato dextrose agar (PDA) slants. *Candida albicans*, which was derived from the Center of Microbiological Detection, Shaanxi Institute of Microbiology, was used as indicator fungi to detect the antifungal activity. The pyrrolnitrin was purchased from ChengDu TongChuangYuan Pharmaceutical Co. Ltd. (Chengdu, China). The Xiaoyan 22 (*T. aestivum* L.) seeds were directly purchased from the market.

Phylogenetic analysis

The bacterial genomic DNA was isolated and purified using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (Dalian, China). Genomic DNA was then used as the template for PCR amplification of 16S rRNA gene fragments using the bacterial universal primers (27F-5′-AGAGTTGATCCTGGCTCAG-3′ and 1492R-5′-GGTTACCTTGTTACGACTT-3′). The final amplified reaction volume was 50 μl, containing 5.0 μl of 10× Taq buffers, 4.0 μl of 200 mmol/L dNTPs, 2.0 μl of each primer at 10 μM, 0.5 μl of Ex Taq enzyme (TaKaRa, Dalian), 5.0 μl of genomic DNA, and 31.5 μl of sterilized distilled water. PCR amplification was performed

using the Professional Standard 96 Gradient (Biometra, Jena, Germany) with the following cycling parameters: initial denaturation of DNA for 5 min at 95°C, then 30 cycles of denaturation of DNA for 1 min at 94°C, annealing for 1 min at 53°C, extension for 1.5 min at 72°C, and final incubation for 5 min at 72°C (Vasiee et al., 2018). The PCR products were subsequently purified and sequenced using BGI Biotechnology (Shenzhen, China). DNA sequence alignment was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, phylogenetic trees were constructed using the neighbor-joining (NJ) method implemented in MEGA 5.05 (Arizona State University, Tempe, United States).

Detection of genes associated with antibiotic biosynthesis using the PCR method

The *Burkholderia* spp. have been reported to produce antimicrobial compounds such as siderophore (required *Cep* gene), pyrrolnitrin (required *Prn* gene), and phenazine acid (required *Pca* gene). We designed three sets of low-degeneracy primers for PCR amplification of these genes. These primers are shown in Supplementary material 1. According to the manufacturer's instructions, the amplification was performed in 50 μl reactions with Taq polymerase (TaKaRa Biotechnology, Dalian, China). The PCR products were detected using gel electrophoresis detection. Finally, the PCR products obtained in this study were sequenced by BGI Biotechnology (Shenzhen, China), and the phylogenetic tree was constructed using the neighbor-joining (NJ) method.

Evaluation of antifungal activity *in vitro* and *ex vivo*

Antifungal activity of the *Burkholderia* sp. XN08 was evaluated using both the dual plate confrontation assay and the agar diffusion method. The inhibition ratio was calculated as follows (Cui et al., 2019):

$$Inhibition\ ratio\ (\%) = \frac{(Diameter\ of\ control\ fungus - Diameter\ of\ treated\ fungus)}{(Diameter\ of\ control\ fungus)}. \quad (1)$$

The strain XN08, which was pre-cultured on a nutrient agar medium (NA) plate for 24 h, was inoculated into a 500-ml conical flask containing 200 ml of sterile LB broth and then cultured at 230 rpm for 48 h at 37°C. After cultivation, the cells were removed by centrifugation at 8,000 rpm for 10 min at 4°C. The supernatant was added into preheated PDA medium at 55°C. *Rhizoctonia cerealis* was respectively inoculated on the pure PDA medium plate and PDA medium supplemented with the fermentation broth supernatant of the strain XN08.

Plant leaf tissue was used to determine the antifungal activity of fermentation broth supernatant *ex vivo* as previously described (Fu et al., 2019) with minor modifications. In brief, the top leaves of 14-day-old wheat seedlings with four- to five-leaf stages were used for evaluating the biocontrol efficacy of the fermentation broth supernatant of the strain XN08 against *R. cerealis*. The leaves were wounded at the equator (0.5 mm wide) and inoculated with 5 μl of conidial suspension (2×10^5 spores/ml) of *R. cerealis*. Then, the fermentation broth supernatant of the strain XN08 was sprayed onto the leaf surface. To keep the plant growing, the petioles were wrapped in cotton that contained water to provide nutrients. The leaves were kept in greenhouse system at 23 ± 2°C with a relative humidity (RH) of 95% for 6 days. Daily observations were carried out.

Extraction and identification of antifungal compounds by UPLC-QTOF-MS

The *n*-butanol extracts from the fermentation broth supernatant of XN08 were dissolved with methanol at a concentration of 1 mg/ml, and 50 μl of the solution was added into a 6-mm well in Sabouraud agar medium plates, which were inoculated with *C. albicans* and cultured overnight at 37°C. Then, the plates were incubated at 28°C for 72 h, and the inhibition zones around the wells were observed to determine the antimicrobial activity. The redissolved sample was further purified using reverse-phase high-performance liquid chromatography (Waters 2695, PDA detector 2998) with a C18 column (YMC-Pack Pro C18, 250 × 4.6 mm S-5 μm, 12 nm; YMC CO., LTD, Japan) and eluted with a methanol–water mixture (methanol:water = 8:2) at a flow rate of 1.0 ml/min. The OD at 254 nm was monitored. The MS was operated in negative ion mode and was set to total ion chromatogram mode with the following parameter settings: capillary voltage, 1.0 kV; low collision energy, 6V; source temperature, 100°C; desolvation temperature, 500°C; and desolvation gas flow, 800 L/h. Data acquisition and processing were conducted using Masslynx version 4.1 (Waters, Manchester, United Kingdom).

Detection of plant growth-promoting traits *in vitro*

IAA detection

The strain XN08 was propagated overnight in 100 ml of LB medium and then supplemented with 1 ml of L-tryptophan solution with a concentration of 50 μg/ml. After incubation for 42 h, 1 ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4,

250 ml of distilled H_2O, and 7.5 ml of 0.5 M $FeCl_3 \bullet 6H_2O$) and allowed to stand at room temperature for 20 min, and then the color changes were observed (Patten and Glick, 2002).

Phosphate solubilization detection

A single colony of strain XN08 was spot inoculated onto Pikovskaya's agar plate at 28°C and incubated at 28°C for 48 h. The formation of a halo-zone around the colony was observed (Patten and Glick, 2002).

Siderophore detection

A single colony of strain XN08 was spot inoculated on CAS medium [medium component (1L): chrome azurol S (CAS), 60.5 mg; hexadecyltrimetyl ammonium bromide (HDTMA), 72.9 mg; piperazine-1, 4-bis (2-ethanesulfonic acid; PIPES), 30.24 g; and 1 mM $FeCl_3 \bullet 6H_2O$ in 10 mM HCl 10 ml agarose (0.9%, w/v)], and then color changes around the colonies were observed visually (Shahid et al., 2012).

Proteinase detection

A single colony of the strain XN08 was spot inoculated on a modified tryptic soy broth medium and then the zones of proteolysis around the colonies were observed visually (Shahid et al., 2012).

Pot experiments

Wheat seeds were surface-sterilized in 2.5% sodium hypochlorite for 5 min and in 75% ethanol for 2 min and then soaked in sterile-distilled water for 24 h. The sterilized wheat seeds were put into a sterile 10-ml glass bottle and then cultured for 7 days in the artificial climate box with a temperature of 28°C and 90% RH. The seedlings with consistent growth were selected for the pot experiments. All experiments have been conducted in a 10-ml glass bottle, and each experiment group consisted of four bottles, each of which contains three seedlings.

- **Group 1:** The seedlings were poured with a 2-ml suspension of GFP-tagged *B. ambifaria* (10^7 CFU/ml).
- **Group 2:** The control group consisted of seedlings soaked in 2 ml of sterile water.
- **Group 3:** The seedlings soaked in a 2-ml of sterile water were scratched and inoculated with 500 μl of the suspension of *R. cerealis* containing 1×10^8 CFU/ml spores.
- **Group 4:** The seedlings were poured with 2 ml suspension of GFP-tagged *B. ambifaria* (10^7 CFU/mL) and inoculated with 500 μl of suspension of *R. cerealis* containing 1×10^8 CFU/ml spores.

All of the treated seedlings were further cultured in an artificial climate box (RH 90% and temperature 28°C) and then tested for plant growth-promoting and biocontrol properties. Three seedlings were sampled for each treatment at different growth stages. The samples were washed and wiped dry, and then their fresh weight and shoot height were measured.

Colonization of XN08 in wheat tissues

The seedlings of group 1 were scanned and imaged by CLSM (Laser Scanning Confocal Microscopy; DM6000, Leica Microsystems). The STED beam was generated by a 592-nm depletion beam. All images were detected using hybrid (HyD) detectors controlled by LAS-AX imaging software. All STED images were deconvolved using Huygens software (Scientific Volume Imaging) and analyzed offline using LAS AF Lite (Leica).

The activities of defense enzymes in wheat

The activity levels of polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia-lyase (PAL) were determined according to the instructions provided by the manufacturer with the respective enzyme activity assay kits (Nanjing Jiancheng Bioengineering Institute, China). The article numbers of the kits are A136-1-1 (PPO), A084-3-1 (POD), and A137-1-1 (PAL).

Results

Identification of the strain XN08

As shown in Figures 1A,B, the strain XN08 exhibited off-white colony morphology and secreted viscoelastic substances on the NA plate. The cells appeared as short rod shapes under the light microscope (Figure 1C). A 1,438 bp region of the 16S rDNA gene was amplified from the genomic DNA of the strain XN08, and the sequence analysis indicated that the strain XN08 shared 99.85% identity with *B. ambifaria* AMMD (Genebank Accessions: CP00040) in the NCBI nr database. The phylogenetic tree was constructed (Figure 1D) using the neighbor-joining method. The result also showed that the strain XN08 had a close relationship with *B.*

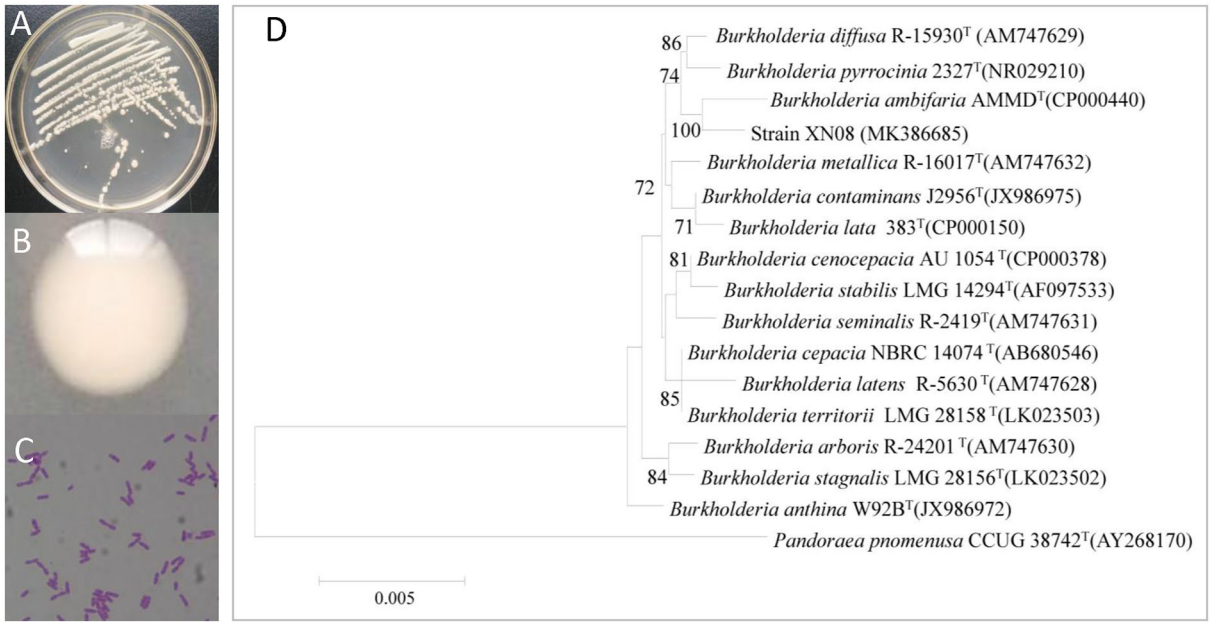

FIGURE 1
Morphological and molecular identification of the endophytic bacterium XN08. The colonies **(A)**, colony characters **(B)** of the strain XN08 on LB medium and microscopic characters **(C)** under a light microscope (×400), phylogenetic tree of the strain XN08 based on 16S rRNA sequence **(D)**.

ambifaria AMMD. Therefore, the strain XN08 was identified as *B. ambifaria*.

Biocontrol potential of the strain XN08 against *R. cerealis*

Compared with the control group (Figure 2A), *B. ambifaria* XN08 showed dramatically a high antagonistic activity against *R. cerealis* under the co-cultural condition (Figure 2B). In addition, no obvious *R. cerealis* growth was observed on the PDA medium added with the fermentation supernatants of the strain XN08 (Figure 2C). Moreover, the plant leaves sprayed with the supernatant of strain XN08 maintained a healthy green color, and no obvious disease symptoms were observed after inoculation with *R. cerealis* (Figure 2D). Usually, the leaves would become yellow after pathogen infection. The above results demonstrated that the strain XN08 may produce extracellular antifungal compounds.

Identification of potential antifungal compounds

To predict the potential antifungal substances produced by *B. ambifaria* XN08, PCR was used to detect the biosynthetic genes of antifungal compounds. Three genes, namely, *Cep* R, *Prn*, and *Pca*, were amplified with predesigned three primers pairs, respectively. A key gene fragment sequence (*Prn* 3, 503 bp) involved in pyrrolnitrin synthesis was successfully amplified (Figures 2E,F). A phylogenetic tree was constructed by sequence alignment using the neighbor-joining method. The sequence had a close relationship with tryptophan halogenase from the *prn* A gene of *B. ambifaria* AMMD (CP009799; Figure 2G). The result indicated that *B. ambifaria* XN08 had the potential to synthesize pyrrolnitrin. An antifungal activity assay was performed to identify the fraction containing the antifungal compound. The *n*-butanol-extracted fraction of *B. ambifaria* XN08 fermentation broth (Figures 2H,I) exhibited obvious antifungal activity against *C. albicans* (Figure 2J). Subsequently, pyrrolnitrin was detected in the *n*-butanol-extracted fraction using the UPLC-QTOF-MS method (Figure 2K).

In vitro plant growth-promoting traits of the strain XN08

As shown in Figures 2L–O, the strain XN08 exhibited a series of potential plant growth-promoting traits including protease, IAA, and siderophore production. Also, the strain could solubilize phosphate.

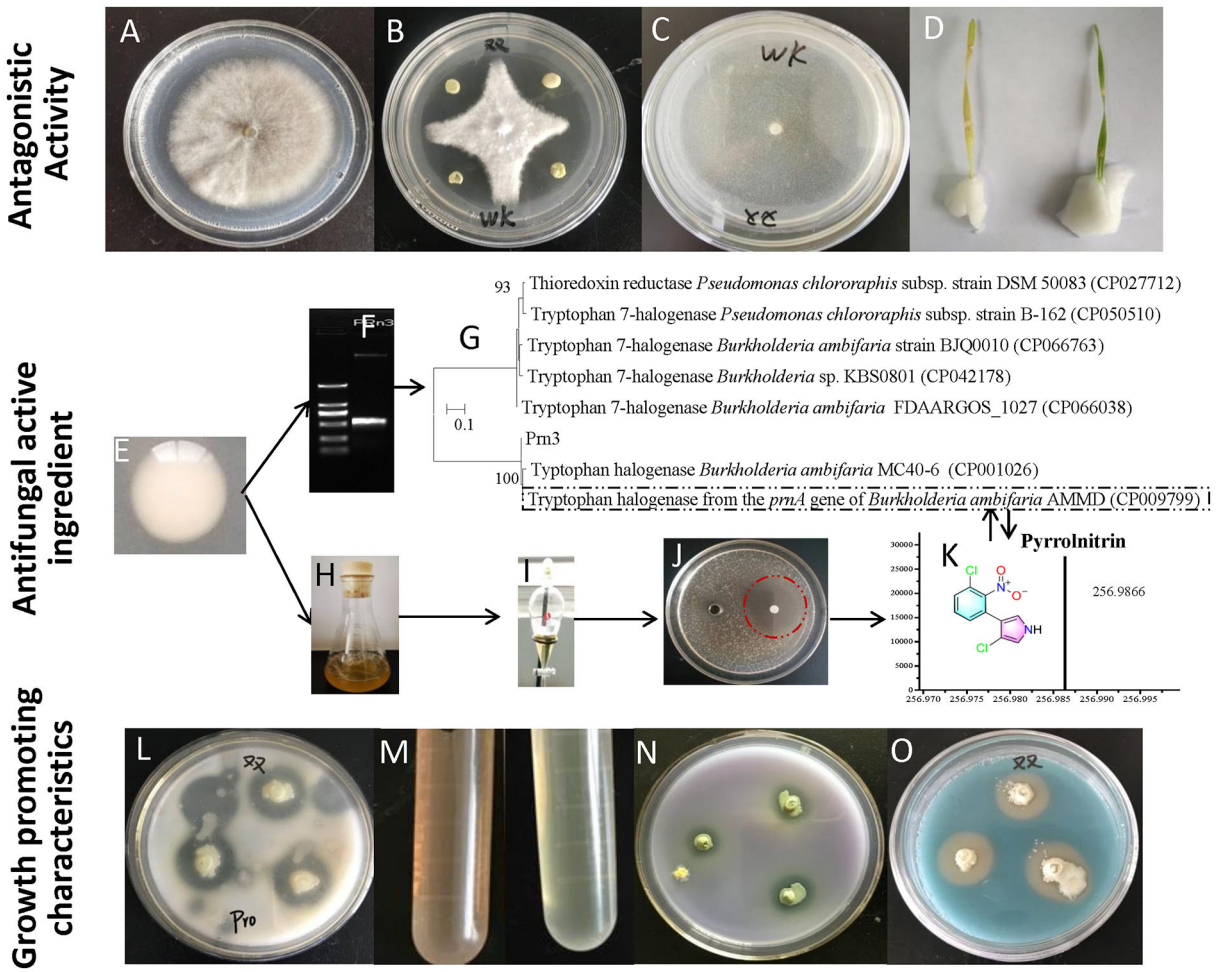

FIGURE 2
Antagonistic activities and plant growth-promoting characteristics of the strain XN08. *R. cerealis* grown on PDA medium **(A)**, *in vitro* antagonism of *B. ambifaria* XN08 against *R. cerealis* **(B)**, *R. cerealis* grown on PDA medium supplemented with the fermentation broth supernatant of *B. ambifaria* XN08 **(C)**, inhibition of the fermentation broth supernatant of the strain XN08 against *R. cerealis* on detached leaves of wheat plants **(D)**, amplification and sequence comparison of the antibiotic synthesis genes **(E–G)**, fermentation of the strain XN08 **(H)**, extraction of antifungal compounds **(I)**, detection of antifungal activities **(J)**, identification of antifungal compounds by UPLC-QTOF-MS method **(K)**, and plant growth-promoting traits of *B. ambifaria* XN08 *in vitro* [**(L)**, proteinase production; **(M)**, IAA production; **(N)**, the capacity of phosphate solubilization; **(O)**, siderophore production].

Evaluation of plant colonization of GFP-tagged *B. ambifaria*

A derivative strain carrying the *GFP* gene was successfully constructed, and CLSM was used to observe the cells with green fluorescence as shown in Figure 3A. The GFP-tagged *B. ambifaria* showed a slightly weaker antagonistic activity (inhibition ratio of 70.14%) against *R. cerealis* compared with the wild strain XN08 (inhibition ratio of 75.45%; Figures 3B,C). In the pot experiment (Figure 3D), a small number of GFP-tagged *B. ambifaria* cells were found to colonize the root of wheat when the strain was inoculated into the rhizosphere soil of plants for 2 days (Figures 3E,F). At 7 days after inoculation, large numbers of bacterial cells were observed in the root tips (Figures 3G,H).

Growth-promoting effects of *B. ambifaria* XN08 on wheat in the pot experiments

After 7 days of inoculation, the biological characteristics of the plant changed significantly as shown in Figure 4A. First, shoot height and fresh weight of the seedlings inoculated with both *R. cerealis* and GFP-tagged *B. ambifaria* reached 15.18 ± 2.54 cm and 0.28 ± 0.07 g for each seedling, which were higher than those of the seedlings inoculated with equal volumes of water (14.35 ± 2.78 cm, 0.26 ± 0.06 g) and the seedlings inoculated with *R. cerealis* (6.98 ± 1.34 cm and 0.12 ± 0.03 g) (Figures 4B,C). No significant difference was observed between the wheat seedlings with or without *R. cerealis* when inoculated with GFP-tagged *B. ambifaria*.

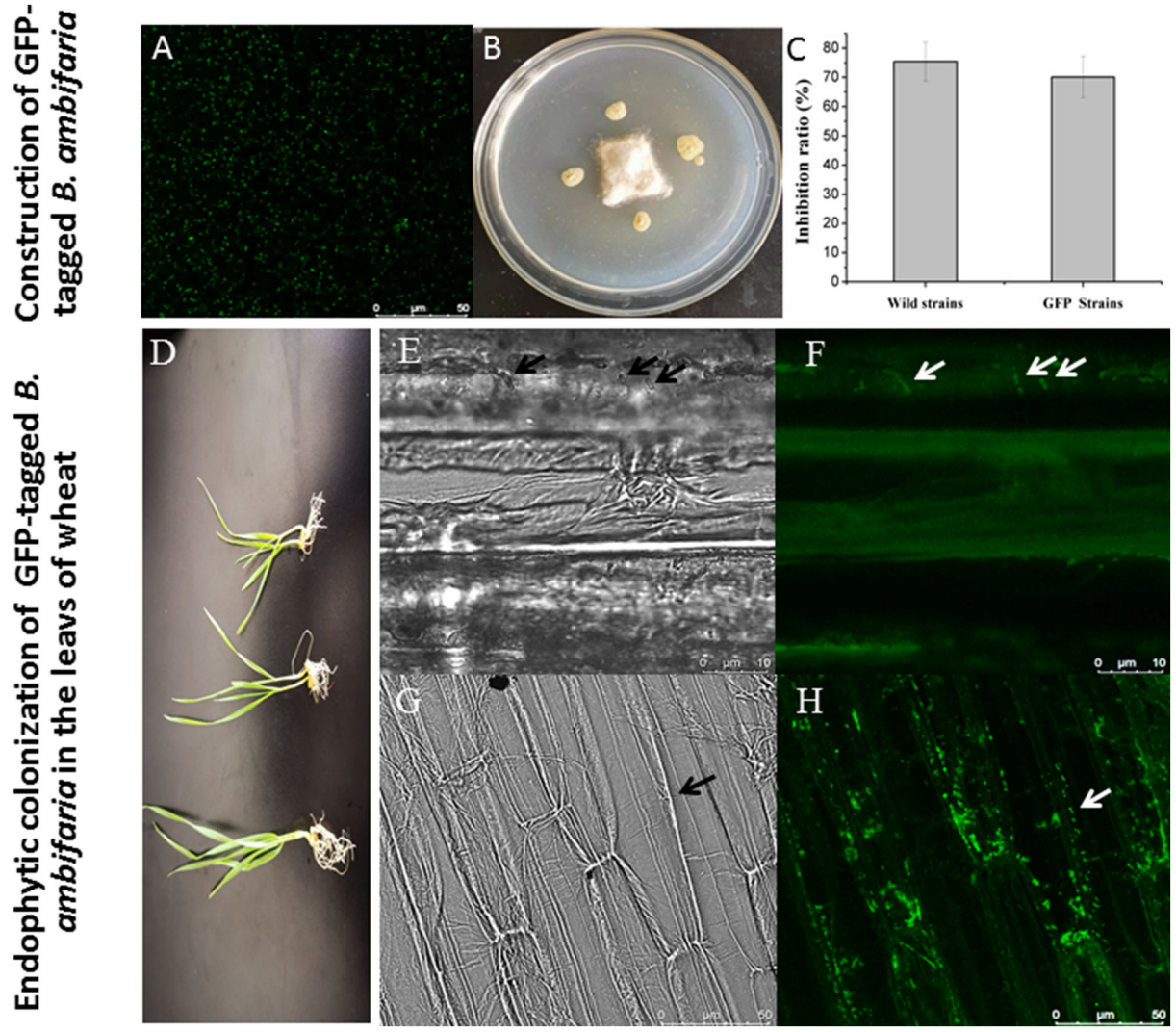

FIGURE 3
Colonization of GFP-tagged *B. ambifaria* in the seedlings of wheat. The observation of GFP-tagged *B. ambifaria* by CLSM **(A)**, antagonistic activities against *R. cerealis* **(B,C)**, the wheat seedlings inoculated GFP-tagged *B. ambifaria* at different growing stages **(D)**, colonization *of* GFP-tagged *B. ambifaria* XN08 at 2 days after inoculation (photographs under dark **(E)** and bright **(F)** fields) and 7 days after inoculation (photographs under dark **(G)** and bright **(H)** fields).

In addition, the seedlings inoculated with *R. cerealis* had obvious sharp eyespot symptoms, while those plants inoculated with both *R. cerealis* and GFP-tagged *B. ambifaria* had no obvious symptoms.

The PPO, POD, and PAL enzyme activities of wheat seedlings at different growth stages for different treatment groups were tested. As shown in Figures 4D–F, the activities of PPO, POD, and PAL in the roots of wheat inoculated with both GFP-tagged *B. ambifaria* and *R. cerealis* were higher than those of the other groups and showed peaks at 5, 3, and 3 days after inoculation, respectively.

Discussion

Wheat hosts a high diversity of endophytic bacteria, including different genera such as *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chitinophaga*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Klebsiella*, *Leifsonia*, *Microbispora*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Roseomonas*, *Staphylococcus*, *Streptomyces*, and *Xanthomonas* (Rana et al., 2020). Several studies have exploited wheat-associated PGPB. It was reported that 13 endophytic bacteria isolated from wheat showed multifarious plant beneficial traits

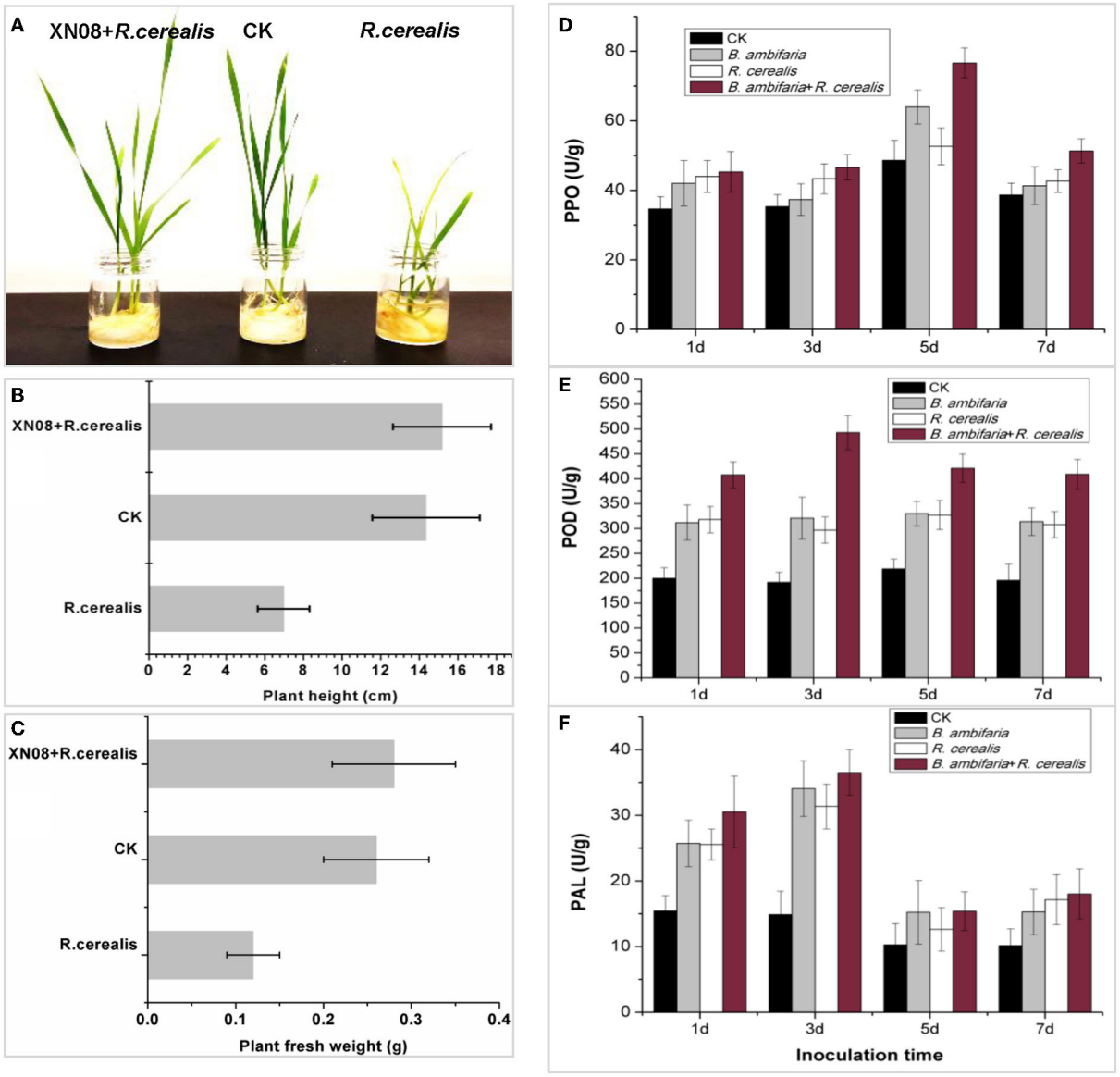

FIGURE 4
Antagonistic activities and plant growth-promoting characteristics of the strain XN08 in the pot experiments. Morphological changes in wheat seedlings **(A)**, plant heights **(B)**, plant fresh weights **(C)**, and the activities of defense enzymes, including PPO **(D)**, POD **(E)**, and PAL **(F)** under different treatments.

with the capacity of P-solubilization, nitrogenase, and IAA production (Rana et al., 2020). Larran et al. (2016) isolated the endophytes from wheat cultivars and found that the endophytes of *Penicillium* sp., *Bacillus* sp., and *Paecilomyces lilacinus* significantly suppressed the growth of pathogens *in vitro* and have the potential to be developed as new biological agents against the tan spot of wheat. In this study, the endophytic bacterium *B. ambifaria* XN08, which was isolated from healthy wheat plants, showed potential as a novel biological control agent against the wheat sharp eyespot. After all, only *Bacillus* spp. were exploited as biocontrol agents against *R. cerealis* in the previous reports (Ji et al., 2019; Yi et al., 2022).

Burkholderia is a genus of gram-negative bacteria with a wide environmental and geographic distribution (Estrada-De los Santos et al., 2001). Over the last years, there was an increasing interest in the genus *Burkholderia* due to its great potential value in plant growth promotion, biocontrol of plant pathogens, and phytoremediation (Bach et al., 2021). It was reported that bacterial endophytes belonging to *Bacillus* and *Burkholderia* genera were the most effective isolates in controlling bacterial and fungal pathogens *in vitro* (Morales-Cedeno et al., 2021). In fact, *Burkholderia* genus has rich antibiotic synthesis genes (Kim et al., 2021), and it has been reported to produce a large number of antifungal substances such as pyrrolnitrin,

siderophores, and phenazines (Mullins et al., 2019), all of which play important roles in controlling fungal diseases in plants. In this study, a known powerful antifungal compound named pyrrolnitrin, production by XN08, was found, proving that the strain is valuable in controlling diseases including the wheat sharp eyespot. In addition, it is quite interesting that pyrrolnitrin production is quorum-sensing regulated, which indicates that efficient environmental colonization is crucial for the effective control of the wheat sharp eyespot disease (Chapalain et al., 2013).

Plant growth promotion and environmental colonization characteristics also determine the great potential of the *Burkholderia* genus as biocontrol agents (Paungfoo-Lonhienne et al., 2016). To observe the bacterial colonization directly and conveniently, a derivate strain carrying *GFP* gene was constructed in this study. The GFP-tagged *B. ambifaria* showed a similar inhibition ratio against *R. cerealis* as the wild-type strain, which indicated that the derivate strain could be used to efficiently observe its colonization in wheat plant tissue. Fluorescent labeling technology was the most intuitive way of representing the colonization of strain and was widely used in studying the plants and microorganisms interaction (Rilling et al., 2019; Sa et al., 2021). A number of potential biocontrol strains, including *Pseudomonas fluorescens*, *Paenibacillus glycanilyticus*, *Burkholderia tropica*, and *Bacillus velezensis*, labeled with a green fluorescent protein have been used to study their colonization capacity in plant tissue (Bernabeu et al., 2015; Kang et al., 2018; Li et al., 2019; Elsayed et al., 2020). However, as far as we know, this is the first time that *B. ambifaria* was labeled with green fluorescent protein to study the colonization characterized in this study.

It was reported that PGPB induced plants defense-related genes expression such as phenylalanine ammonia-lyase (PAL), catalase (CAT), polyphenol oxidase (PPO), peroxidase (POD), and superoxide dismutase (SOD), which might assist the plant to protect from or reduce the impact of pathogen attacks (Jiang et al., 2019; Kamou et al., 2019; Wu et al., 2019; Zhu et al., 2019; Ashajyothi et al., 2020; Singh et al., 2021). Our results showed that the significant colonization of XN08, visually observed with CLSM, was accompanied by the higher activities of PPO, POD, and PAL, which indicated that *B. ambifaria* XN08 was involved in the immune-induced resistance of wheat seedlings. In addition, the results also showed that the endophytic bacterial strain XN08 could significantly improve the growth of wheat inoculated with *R. cerealis*. The fresh weight and shoot height of the seedlings inoculated with both *R. cerealis* and GFP-tagged *B. ambifaria* were similar to those of the control group. Meanwhile, no obvious sharp eyespot symptom was observed for the group seedlings inoculated with both *R. cerealis* and GFP-tagged *B. ambifaria*. It is obvious that XN08 may provide an adequate protection against wheat disease.

On a whole, the results obtained in this study revealed that XN08 had great potential for biological control against the wheat sharp eyespot. However, it is worth noting that the successful application of XN08 for biocontrol of sharp eyespot in wheat needs knowledge of the ecological/environmental factors affecting the colonization of crops. Therefore, further observation using GFP-tagged *B. ambifaria* under different environmental conditions should be performed.

Conclusion

Taken together, it was concluded that *B. ambifaria* XN08 was able to efficiently inhibit the growth of *R. cerealis* by producing antifungal compounds and showed the capacity to enhance plant growth by synthesizing a series of plant growth regulators. In addition, this strain exhibited significant colonization in wheat plants, which was accompanied by an enhancement of wheat plants' growth and an induction of immune resistance for wheat seedlings. These data indicated that the strain XN08 might be used as a new biocontrol agent against wheat sharp eyespot.

Data availability statement

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

Author contributions

CA performed the experiments, wrote, and edited the manuscript. WX designed and supervised the project. All experiments were also performed by SM, CL, and HD. All authors contributed to the article and approved the submitted version.

Funding

This study was simultaneously supported by the National Natural Science Foundation of China (21576160) and the Scientific and Technologic Research Program of Shaanxi Academy of Sciences, China (No. 2018K-09).

Acknowledgments

The authors thank the National Natural Science Foundation of China for financial support. They also thank Shaanxi Province Academy of Sciences for financial support, authors acknowledge technical support of Dr. Chenlin for the construction of a derivate *Burkholderia ambifaria* strain carrying GFP gene used in this study.

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.2022.906724/full#supplementary-material

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TYPE Original Research
PUBLISHED 12 August 2022
DOI 10.3389/fmicb.2022.978550

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 26 June 2022
ACCEPTED 29 July 2022
PUBLISHED 12 August 2022

CITATION
Ou T, Gao H, Jiang K, Yu J, Zhao R, Liu X, Zhou Z, Xiang Z and Xie J (2022) Endophytic *Klebsiella aerogenes* HGG15 stimulates mulberry growth in hydro-fluctuation belt and the potential mechanisms as revealed by microbiome and metabolomics. *Front. Microbiol.* 13:978550. doi: 10.3389/fmicb.2022.978550

Endophytic *Klebsiella aerogenes* HGG15 stimulates mulberry growth in hydro-fluctuation belt and the potential mechanisms as revealed by microbiome and metabolomics

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Growth promotion and stress tolerance induced by endophytes have been observed in various plants, but their effects on mulberry regularly suffering flood in the hydro-fluctuation belt are less understood. In the present study, endophytic *Klebsiella aerogenes* HGG15 was screened out from 28 plant growth promotion (PGP) bacteria as having superior PGP traits *in vitro* and *in planta* as well as biosafety for silkworms. *K. aerogenes* HGG15 could actively colonize into roots of mulberry and subsequently transferred to stems and leaves. The 16S ribosomal RNA (V3–V4 variable regions) amplicon sequencing revealed that exogenous application of *K. aerogenes* HGG15 altered the bacterial community structures of mulberry roots and stems. Moreover, the genus of *Klebsiella* was particularly enriched in inoculated mulberry roots and was positively correlated with mulberry development and soil potassium content. Untargeted metabolic profiles uncovered 201 differentially abundant metabolites (DEMs) between inoculated and control mulberry, with lipids and organo-heterocyclic compounds being particularly abundant DEMs. In addition, a high abundance of abiotic stress response factors and promotion growth stimulators such as glycerolipid, sphingolipid, indole, pyridine, and coumarin were observed in inoculated mulberry. Collectively, the knowledge gained from this study sheds light on potential strategies to enhance mulberry growth in hydro-fluctuation belt, and microbiome and metabolite analyses provide new insights into the growth promotion mechanisms used by plant-associated bacteria.

KEYWORDS

endophyte, plant growth promotion, flood, microbiome, metabolite

Introduction

The Three Gorges Reservoir (TGR), the largest reservoir ever built in China, was constructed for the purposes of flood control, hydropower generation, and navigation, where the water level is periodically maintained 145 m above sea level in summer (May–September) but increasing to 175 m in winter (October–April) (Wang et al., 2018; Yin et al., 2020). As a result of thus artificial water control, a 30 m water level fluctuation occurs on the banks, called a hydro-fluctuation belt. This annual reverse-seasonal flood maintaining nearly half a year in the TGR has caused serious degradation of the vegetation in the hydro-fluctuation belt, which has led to considerable soil erosion, habitat loss, biodiversity decline, and environmental pollution (Liu and Willison, 2013). Mulberry (*Morus* L.), a woody perennial, is a plant species with potential for revegetating the hydro-fluctuation belt of the TGR due to its strong resistance to flood stress and ability to grow well in this limited-nutrient environment (Huang et al., 2013). However, while initially vigorous, mulberry trees ("Guisangyou 62," *Morus alba* L.) planted in 2012 at the hydro-fluctuation belt for revegetation were partially dead and their growth status varied considerably, after several years of fluctuating water levels. The well-growing newly planted trees were deeply rooted and had strong trunks and healthy leaves, while poorly-growing newly planted trees had underdeveloped root systems. Moreover, many wild mulberry trees grew exuberantly and had great vitality in the same field, which were planted by local residents and presented prior to the establishment of the TGR, but their genetic background was unclear (Xie et al., 2021). Given that the development of mulberry trees in TGR was often adversely affected by flood stress, there is a need to promote its growth and increase flood tolerance so that it can be used in revegetation efforts.

Exposure of plants to adverse environmental conditions disrupts their metabolism, ultimately leading to reduced fitness and productivity (Montesinos-Navarro et al., 2020). In the course of evolution, plants have evolved an array of protective mechanisms allowing them to adapt and survive in such stressful environments. A crucial step in plant responses is their timely perception of the stress to respond in a rapid and efficient manner. Responses typically involve activation of specific ion channels and kinase cascades and the accumulation of reactive oxygen species and phytohormones, all of which result in appropriate defense reactions and thus increase plant tolerance. Most commonly plants adjust their metabolic pathways to produce a series of anti-stress substances when encountering stress challenges. For instance, the concentrations of secondary metabolites (glycosides, alkaloids, phenolics, terpenoids) are highly accumulated in *Catharanthus roseus* (Saravanan, 2021) and *Elaeis guineensis* (Ibrahim and Jaafar, 2012) when treated with high levels of carbon dioxide. Moreover, maize specifically produced iron-benzoxazinoid complexes to defend against herbivores and further improve their growth (Hu et al., 2018).

Apart from their intrinsic mechanisms, plants also can alleviate the burden of environmental stresses by associating with particular endophytic microbes. Plant endophytes are typically non-pathogenic microbes that colonize in the interior space of plant tissues at some period in their life cycle, including roots, stems, leaves, flowers, fruits, and seeds (Compant et al., 2010; Zhang et al., 2019). They have been shown to play a crucial role in maintaining terrestrial ecosystems since their beneficial functions including defending hosts from biotic stress, alleviating harm from abiotic stress, and supporting host plant nutrition by increasing phosphorus, nitrogen, and iron levels (Bacon and White, 2015). Increased biomass in inoculated plant has been reported as a result of their colonization by a variety of endophytic genera such as *Bacillus* (Xu et al., 2019), *Streptomyces* (Jaemsaeng et al., 2018), *Klebsiella* (Zhang et al., 2017), *Pantoea* (Xie et al., 2017), and *Pseudomonas* (Han et al., 2015). In addition, the improvement of stress resistance in crop plants using endophytic microorganism is cost-effective and ecofriendly for the environment (Olanrewaju et al., 2017; Nascimento et al., 2018). For example, inoculation of endophytic *Bacillus cereus* PE31 could promote the phytoremediation efficiency of *Phytolacca acinosa* in Cadmium contaminated soils (Liu et al., 2022). Moreover, introduction of exogenous endophytes can induce host plants to recruit beneficial taxa. The process alters associated-microbiome composition of host, thus indirectly attributes to plant development as well (Welmillage et al., 2021). The application of endophytic *Rhizobium* sp. RF67 isolated from *Vaccinium angustifolium* resulted in root-associated bacteria variation, which boosted cooperation of plant-growth-promoting endophytes (Yurgel et al., 2022). Moreover, *Piriformospora indica* recruited the member of Firmicutes and decreased Proteobacteria in rice roots, resulting in enhancement of rhizosheath and improvement of drought tolerance (Xu F. Y. et al., 2022). Beneficial interactions between endophyte and plant have gradually become a focus of many studies addressing ecological restoration such as deserts (Jain et al., 2021), saline-alkaline lands (Lu et al., 2021), and oil or heavy metal contaminated lands (Liu et al., 2022). However, the utilization of endophyte and plant to restore ecosystems subjected to repeated flood stress such as the hydro-fluctuation belt of the TGR has not received much attention.

The results of 16S ribosomal amplicon sequencing in our previous study revealed that the complexity of endophytic bacterial interactions in well-growing mulberry trees, including newly planted mulberry and wild mulberry, was higher than it was in poorly-growing mulberry tree. Additionally, well-growing trees were found to have recruited similar endophytic bacteria that enabled them to flourish in hydro-fluctuation belt (Xie et al., 2021). Thus, we hypothesized that endophytic bacteria of well-growing mulberry trees could be a potential resource to promote mulberry growth in such settings. Therefore, objectives of this work were to (i) obtain endosphere-derived bacteria from well-growing mulberry trees and detect their potentials to benefiting the

growth of mulberry *in vitro* and *in planta*; (ii) test plant colonization capability of plant growth promoting bacteria by using of a green fluorescence protein marker; and (iii) further decipher the underlying preliminary mechanisms how bacteria promote mulberry growth by comprehensively interrogating the mulberry associated microbiome and metabolome.

Materials and methods

Sample collection and isolation of endophytic bacteria

Samples were collected from well-growing mulberry trees including wild mulberry trees (WM) and newly planted mulberry trees (NM) from the hydro-fluctuation belt in Longjiao Town, Yunyang County, Chongqing Municipality, China (30°49′26″ N, 108°52′14″ E) in May, 2020. A total of four types of sample were obtained including two compartments (stem and root) of WM and NM. Three biological replicates were performed for each sample. Characteristics of mulberry trees were shown in **Supplementary Table 1**. These mulberry trees were suffered flood stress lasting for about 6 months. The elevation of this sampling area was approximately at 172 m above sea level and the water level of TGR was about 152 m above sea level at that time. Mulberry stem segments were collected at approximately 25 cm in length where leaves and small side branches had been removed. The sampling depth of root was about 15 cm below soil surface and the length of root segments was about 20 cm. All samples were immediately transported back to the laboratory and stored at 4°C until further processing.

Surface sterilization of the mulberry stem and root was performed according to a previously described procedure (Xu et al., 2019), and the endophytic bacteria were isolated using the fragmentation technique (Liotti et al., 2018). Samples were washed with tap water to remove soil and other debris before being cut into pieces with 3.0–5.0 cm in length. The samples were then thoroughly soaked in 75% ethanol and rapidly flame-sterilized. Afterward, samples were peeled to obtain smaller fragments and placed on nutrient agar (NA), Gause's agar (GA), Luria-Bertani agar (LB), and Trypticase Soya agar (TSA) medium at 28°C for 5 days. Isolates with different morphological characteristics were purified and stored with 50% glycerol at −80°C.

Screening of plant growth promoting bacteria and molecular characterization analysis

The plant growth promotion (PGP) traits of endophytes, including phosphate (P) solubilization, indole-3-acetic acid (IAA), siderophore, and acetoin production, and antagonistic activity against phytopathogen *Sclerotinia sclerotiorum*, which could seriously infect mulberry flowers and branches, were qualitatively determined by following procedures. Specifically, isolates were inoculated in LB medium and incubated at 30°C with 180 rpm for 16 h, and then 10 μL of each bacterial culture was inoculated on different medium to detect PGP traits, respectively. The ability of isolates to P solubilization was evaluated in Pikovskaya's agar medium containing tricalcium phosphate (Gaind, 2016) and the siderophore activity was tested in chrome azurol-s agar medium (Jasim et al., 2013). The detections of IAA and acetoin activities were conducted in LB medium as described by Patten and Glick (1996) and Vivijs et al. (2014), respectively. The antifungal bioactivities were evaluated on PDA medium by the dual culture technique as described by Xu et al. (2019).

Molecular characterization of plant growth promoting bacteria (PGPB) was tested based on 16S rRNA gene sequence. The genomic DNA of PGPB was extracted using a PrepMan Ultra Sample Preparation Reagent kit (Applied Biosystems, Palo Alto, CA, United States) according to the manufacturer's instructions and amplified by PCR using universal 16S rRNA gene primers 27F/1492R (Ou et al., 2022). Each 25 μL PCR mixture contained 12.5 μL of 2 × Rapid Taq Mater Mix (Vazyme, Nanjing, China), 1 μL of each primer (10 μM), and 10 ng of template DNA. The PCR reaction was carried out using the following conditions: 1 cycle of 95°C for 4 min; followed by 30 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min; and a final extension at 72°C for 8 min. The PCR amplified products were purified with the DNA Clean & Concentrator^{TM}-5 Kit (Zymo Research, United States) and then sequenced by the Sanger method at Sangon Biotechnology Co., Ltd., Shanghai, China. The nucleotide sequences of PGPB were compared using Basic Local Alignment Search Tool (BLAST) method and deposited in NCBI GenBank database. In present study, the isolates with high level of identity (97–100%) were selected as the closest match, and all isolates were classified to the genus level.

Evaluation of the activity of the bacteria in mulberry

The isolates exhibiting high PGP potentials *in vitro* were selected and their effects on mulberry seedling growth were further evaluated *in planta*. Fresh colony of PGPB was inoculated into LB medium and incubated at 30°C with 180 rpm for 18 h. The cultures were centrifuged at 8000 rpm for 10 min and precipitate was adjusted at 1×10^7 CFU/mL using the sterile water (Ou et al., 2022).

"Guisangyou 62," a variety of white mulberry, is a potential for phytoremediation since the quick-growth and

ecological-adaptation characterizations (Zhao et al., 2013; Jiang et al., 2019). This mulberry cultivar thus was applied in the present study. Mulberry seeds were surface sterilized with 75% ethanol for 3 min and 10% sodium hypochlorite for 3 min. After five times rinse with sterile distilled water, seeds were placed in a 9-cm-diameter petri dish containing sterilized moistened filter paper and maintained in growth chamber at 25°C with a photoperiod of 12 h at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 70% humidity. At two-leaf stage, seedlings were transferred to pot which was filled with mixtures of humus and filed soil (4:1; *v*/*v*) collected from Southwest University experimental farm (29°49′1″ N, 106°24′57″ E). The mixed soils were sterilized four times by autoclaving at 121°C for 2 h (Li et al., 2012; Cao et al., 2013). Each pot contained three mulberry seedlings with similar size and height. After 2 weeks, mulberry seedlings with three-leaf were inoculated with PGPBs through irrigating 30 mL of bacterial suspension into soils in each pot, and the mulberry seedlings treated with sterile water were served as control. Each treatment was replicated twenty times. After 30-day inoculation, eight seedlings were randomly selected in each treatment to measure length of root and shoot, fresh and dry weight of root and shoot, and number of root tip.

To assess the effects of PGPBs on mulberry growth under flood stress condition, the remaining mulberry seedlings were completely submerged wherein the water level was 30 cm above soil surface. After 40 days, the degree of cell death of mulberry leaf was determined using Evan's blue staining (Taylor and West, 1980). Finally, nine seedlings were randomly selected and the root fresh weight was calculated, and the total length and fork of roots were analyzed using the GXY-B root analysis system (Hangzhou Lvbo instrument Co., Ltd, Hangzhou, China).

Bio-safety assessment of plant growth promoting bacteria on silkworms

In order to lay a good foundation for utilization of PGPB to promote mulberry growth in the field, the biosafety was studied through feeding silkworms with PGPBs-infected mulberry leaves. One milliliter of 1×10^7 CFU/mL bacterial suspension was sprayed on the surface of each leaf (Xie et al., 2017). The sterile water was used as control. Mulberry leaves dried naturally were applied to feed healthy silkworm larvae (871 × 872 strain) at the 4th instar with two times each day. Afterward, all silkworms were fed with clean mulberry leaves until cocoon formation. Each treatment consisted of three replicates and each replicate contained forty silkworms. The growth status of silkworm was observed every day. The survival rate and rate of cocoon formation of three replicates were determined. Forty silkworms were randomly selected to measure cocoon weight, cocoon shell rate and weight, and pupal weight.

Identification of HGG15 strain

Based on plant growth promoting activity in mulberry seedlings and bio-safety on silkworm of PGPBs, the HGG15 strain was finally screened out for further study. The morphological characteristics of HGG15 in LB incubated at 37°C for 24 h were recorded and further observed in scanning electron microscope (Hitachi, SU3500, Japan). Gram staining was performed and observed under an optical microscope (Becerra et al., 2016). Series of biochemical tests such as Voges-Proskauer and motility test were conducted using an HK-MID-66 kit (HUANKAI, China) following the protocol provided by the manufacturer. Phylogenetic tree of HGG15 based on 16S rRNA gene sequence was constructed by applying the neighbor-joining method using MEGA version 6.0 with 1,000 replicates of bootstrap values (Tamura et al., 2011).

Colonization of mulberry by HGG15 strain

To explore colonization characteristics of HGG15 strain in mulberry, the plasmid pGFP4412 containing green fluorescence protein (*gfp*) and kanamycin resistance gene was transformed into the wild type HGG15 strain by electroporation technique. The competent cells were prepared in 10% glycerol and electroporated at 2.5 kv for 5 ms. Transformed colonies that emitted green fluorescence stably under fluorescence microscopy (Leica, DM3000, Germany) were obtained and designated HGG15/gfp strain.

The cultivation of mulberry seedlings was shown as described above and roots of mulberry at two-leaf-stage were immersed in 1×10^7 CFU/mL bacterial suspension for 4 h and then re-planted into the sterile soil. The sterile distilled water was served as control group. Afterward, seedlings were removed from pots, surface disinfected using 75% ethanol for 1 min, and separated into roots, stems and leaves at different time after inoculation. And then, these tissues of mulberry were cut into small pieces to observe the colonization using fluorescence microscopy. In addition, these tissues were weighed and ground in 1 mL sterile distilled water, and serially diluted and plated on LB plates supplemented with 50 μg/mL kanamycin to count the number of colony. Three replicates were used for each treatment.

Detection of mulberry associated bacterial communities after HGG15 inoculation

To explore the potential mechanism of HGG15 strain to promote mulberry growth whether attributed to variation of host-associated microbiome, the rhizosphere soil, root, and stem

of mulberry were collected after 30-day inoculation. A total of nine individual seedlings were randomly selected and three seedlings mixed as one replicate. The rhizosphere soils adhering to the mulberry root approximately 1 mm were obtained using a sterile scalpel. And then, approximately 5 cm roots and stems were collected for each mulberry seedling, and washed with tap water to remove soil and other debris. The root and stem samples were thoroughly soaked in 75% ethanol and rapidly flame-sterilized and stored at −80°C for further processed.

The DNA of all samples was extracted using FastDNA® Spin Kit (MP Bio, Santa Ana, CA, United States) (Xu F. Y. et al., 2022) according to manufacturer's instructions with the moderated modification. Briefly, approximately 0.5 g of stems and roots were homogenized in liquid nitrogen. The prepared stem and root tissues and rhizosphere soil were transferred into Lysing Matrix E Tubes. And then, sodium phosphate buffer and MT buffer were added and supernatants were collected by centrifugation (12,000 rpm, 10 min). Then, 250 μL of PPS was added in tube and centrifuged at 12,000 rpm with 5 min. The upper phase was then collected and transferred to a new tube and 1 mL binging matrix suspension was added. Afterward, 600 μL mixtures were transferred into SPIN™ Filter tubes and subsequently centrifuged. Finally, 500 μL SEMW-M was added into SPIN™ Filter tubes and centrifuged (12,000 rpm, 2 min), and DNA was eluted using 100 μL DES. The DNA was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC, United States). The bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Ou et al., 2019) by an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, United States). Each 20 μL PCR mixture contained 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each Primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The PCR reactions were conducted using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. Sterile water was served as negative control sample to avoid potential microbial contaminants in the DNA extraction and amplification processes. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to manufacturer's instructions. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Pairs of reads were spliced into a sequence according to the direct overlap relationship of paired-end reads. At the same time, the quality of reads and splicing effect were controlled and filtered, and correction of the sequence direction was made according to the end of the box sequence. Finally, the high-quality sequences obtained after filtering were assigned to samples according to barcodes. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

Operational taxonomic units (OTUs) with 97% similarity cutoff (Edgar, 2013) were clustered using UPARSE (version 7.0.1090), and chimeric sequences were identified and removed by VSEARCH (version 1.0.10). The taxonomy of each OTU representative sequence was analyzed using the BLAST algorithm with Silva database (release138[1]) against the bacterial 16S rRNA genes (Pruesse et al., 2007; Quast et al., 2013). Each sample was rarefied to 2000 reads (Beckers et al., 2017). The sequences classified as cyanobacteria and mitochondria were removed from the OTU table. The filtered table was used for further analyses. The α-diversity including Shannon and Sobs indices was calculated using Mothur software (version 1.30.2) at a 97% identity level (Schloss et al., 2009). The different significances of α-diversity among samples were compared based one-way analysis of variance (ANOVA). Bacterial community structures were analyzed at different classification levels using R software (version R-3.3.1). The Venn diagram was generated using R script and principal coordinates analysis (PCoA) was conducted based on Bray–Curtis distances at OTU level. The comparison of endophytic bacterial abundance was analyzed using Wilcoxon rank-sum test based on genus level. Moreover, Spearman correlation coefficient of the top 20 abundant bacterial genera and environmental factors including mulberry growth parameters and soil properties was calculated and displayed on the heat map (Zhou et al., 2017).

Assessments of soil biochemical properties

Approximately 50 g soil samples were collected from each pot containing mulberry for detection of the biochemical properties after 30-day inoculation, and each treatment consisted of three replicates. Specifically, soils were dried at 105°C and filtered by a 2 mm sieve. Afterward, the soil sample was subjected to analyze organic carbon (OC), organic matter (OM), available phosphorus (AP), total potassium (TK), available potassium (AK), and available iron (Fe). Soil properties were analyzed using standard soil test methods as described by the agriculture protocols (Lu, 2000; Zhou et al., 2017). Soil OM, TP, and TK were determined by the dichromate oxidation process, Mo-Sb anti spectrophotometric method, and atomic absorption spectrophotometry, respectively. AP was extracted with NH_4F-HCl solution, and then determined by ultraviolet visible spectrophotometer. AK was extracted with 1 M NH_4OAc, and then determined by flame absorption

1 http://www.arb-silva.de

spectroscopy. Fe was extracted with DTPA-$CaCl_2$-TEA, and then determined by atomic absorption spectrophotometry.

Analysis of mulberry root metabolites

To understand if variations in host metabolism were correlated with HGG15 strain, metabolomics of mulberry root were employed after 30-day inoculation. A total of 18 individual mulberry seedlings were collected in each treatment and three seedlings were randomly mixed as one replicate. Six biological replicates were performed for each treatment. The entire roots were washed with tap water to remove soils and 50 mg of roots were accurately weighed, and then the metabolites were extracted using 400 μL of 80% methanol solution containing 2 mg/L L-2-chlorophenylalanine. The mixture was settled at −20°C and processed by a high-throughput tissue crusher Wonbio-96c (Wonbio Biotechnology, Shanghai, China) at 50 Hz for 6 min, then vortexed for 30 s and ultrasonically treated at 40 Hz for 30 min at 5°C, followed by precipitation for 30 min at −20°C and centrifugation at 13,000 rpm at 4°C for 15 min. The supernatants were then transferred into vials for subsequent ultra-performance liquid chromatography-tandem mass (UHPLC-MS/MS) spectrometry. A quality-control sample was prepared by mixing 20 μL of each sample in order to control the accuracy and stability of the method.

Samples were separated on Thermo UHPLC plat equipped with an Acquity Beh C18 column (2.1 mm × 100 mm i.d.; 1.7 μm; Waters, Milford, DE, United States) by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Mobile phase A was 0.1% formic acid in water, and phase B was 0.1% formic acid in acetonitrile: isopropanol (1: 1, *v*/*v*). The sample injection volume was 2 μL, and the flow rate was set to 0.4 mL/min with a column temperature of 40°C. The gradient condition is as follows: from 0 to 3 min, 5–20% (B); from 3 to 9 min, 20–95% (B); from 9 to 13 min, 95% (B); from 13 to 13.1 min, 95–5% (B), from 13.1 to 16 min, 5% (B) for equilibrating the systems. The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive Mass Spectrometer equipped with an electrospray ionization source operating in either positive or negative ion mode. The optimal parameters were set as follows: scan type: 70–1050 m/z; sheath gas flow rate: 40 psi; aux gas flow rate: 30 psi; aus gas heater temperature: 400°C; capillary temperature: 320°C; ion-spray voltage floating, −2.8 kV in negative mode and 3.5 kV in positive mode, respectively; resolution: 17500 (MS^2). Data acquisition was performed with the Data Dependent Acquisition mode.

The raw data were imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, United States) for peak detection and alignment. The preprocessing results generated a data matrix that consisted of the retention time, mass-to-charge ratio values, and peak intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. Then, peaks were searched in human metabolome database (HMDB)[2] and Metlin database[3] for metabolite identification. Orthogonal partial least squares discriminate analysis (OPLS-DA) and principal components analysis (PCA) analysis were used to determine global metabolic changes. The *P*-values were estimated with paired Student's *T*-test on single dimensional statistical analysis. Variable importance in projection (VIP) of metabolites represented their contributions to the global metabolic changes and the metabolites with VIP > 1 and *P*-value < 0.05 were considered to be differential metabolites. The functions of these metabolites and metabolic pathways were studied using scipy (Python packages)[4] based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The heatmap of correlation between top 40 metabolites of mulberry root and bacterial genera was generated using the Pearson correlation coefficient.

Statistical analysis

All data were performed using SPSS Statistics (Version 17.0. Chicago, IL, United States). Differences between treatments in the mulberry seedling and mulberry associated-bacterial diversity were analyzed using Tukey's one-way ANOVA. The statistical significances of silkworm growth and soil properties were analyzed using *T*-test. ***, **, and * indicated significant difference at $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

Results

Isolation of endophyte and screening of plant growth promoting bacteria

A total of 343 endophytic bacteria were isolated from mulberry plants. 119 and 88 isolates were obtained from stems and roots of wild mulberry, respectively, while 68 and 68 isolates were obtained from stems and roots of newly planted mulberry, respectively (**Supplementary Table 2**). Among them, 28 isolates exhibited phenotypes commonly observed bacteria capable of plant growth promotion, including the ability to solubilize P, produce IAA, siderophores, and acetoin, and exhibit antagonism against *Sclerotinia sclerotiorum* as revealed by the presence of an inhibition zone (**Table 1**), and their near full-length 16S rRNA gene sequences were deposited in the GenBank under accession numbers ON786677-ON786703 and ON090422. A total of 25 and 27 isolates possessed

2 http://www.hmdb.ca/
3 https://metlin.scripps.edu/
4 https://docs.scipy.org/doc/scipy/

P-solubilization and IAA-producing capability, respectively. Moreover, 16 and 25 isolates had the ability to produce siderophores and acetoin, respectively, and fifteen PGPBs had anti-fungal capability *in vitro*. Ultimately, *Enterobacter* sp. HLG5, *Lelliottia* sp. HTJ13, *Pantoea* sp. HLJ21, and *Klebsiella* sp. HGG15 strains were screened as those strains with the highest potential for plant growth promotion based on these traits *in vitro*.

Effect of plant growth promoting bacteria on mulberry growth and their bio-safety for silkworms

To determine the effect of these four bacteria on mulberry growth, bacterial suspensions were inoculated into mulberry seedlings. Co-cultivation experiments revealed that application of these strains had a large positive impact on mulberry shoot (**Figure 1A**) and root growth (**Figure 1B**). The four strains significantly promoted length of mulberry leaf (**Figure 1C**) and shoot (**Figure 1D**) ($P < 0.05$) compared with control plants. A corresponding significant increase of fresh weight of mulberry shoots was also observed in endophyte-treated plants (*Enterobacter* sp. HLG5: 81.51%; *Lelliottia* sp. HTJ13: 100.37%; *Klebsiella* sp. HGG15: 158.83%; *Pantoea* sp. HLJ21: 115.89%) (**Figure 1E**). In addition, mulberry seedlings treated with PGPBs had a higher dry weight of mulberry shoots than controls, increasing by 117.15, 147.82, 184.19, and 122.68% in *Enterobacter* sp. HLG5, *Lelliottia* sp. HTJ13, *Klebsiella* sp. HGG15, and *Pantoea* sp. HLJ21 group, respectively (**Figure 1F**). All of the bacterial isolates enhanced shoot growth, but differed in their stimulation of the roots of mulberry. The number of root tips of mulberry inoculated with *Enterobacter* sp. HLG5 (64.87%), *Klebsiella* sp. HGG15 (235.21%), and *Pantoea* sp. HLJ21 (165.92%) strains was greatly increased compared to control ($P < 0.05$) (**Figure 1G**). Moreover, *Klebsiella* sp. HGG15 significantly increased root length (63.28%) (**Figure 1H**), fresh weight (195.92%) (**Figure 1I**), and dry weight (348.79%) (**Figure 1J**).

Additionally, the mulberry roots inoculated with these four strains exhibited greater vigor after 40 days of flood stress (**Figure 2A**). Specifically, root biomass (30.48 and 71.43%) (**Figure 2B**), total root length (52.61 and 53.74%) (**Figure 2C**), and the number of root fork (74.23 and 82.55%) (**Figure 2D**) were significantly higher in plants treated with *Klebsiella* sp. HGG15 and *Lelliottia* sp. HTJ13 under flood condition, respectively. Moreover, Evan's blue staining to reveal dead tissues indicated leave cell death in mulberry treated with *Klebsiella* sp. HGG15, *Lelliottia* sp. HTJ13, and *Pantoea* sp. HLJ21 were slighter than in control plants or plants treated with *Enterobacter* sp. HLG5 (**Figure 2E**). Together, these results suggested that both *Klebsiella* sp. HGG15 and *Lelliottia* sp. HTJ13 had the greatly potential ability to relieve the negative impacts of flood stress on mulberry.

The potential use of PGPBs in the hydro-fluctuation belt of the TGR would require demonstration that they would not interfere with the down-stream uses of mulberry, such as in silk production. The impacts of PGPBs were thus evaluated by feeding them to silkworms. The growth of silkworms fed at the fifth instar and pupal stages presented did not differ from that of control insects (**Supplementary Figure 1A**). The PGPBs had no negative impacts on whole cocoon weight, pupal weight, cocoon shell weight, and rate of cocoon shell development in silkworms (**Supplementary Figures 1B–E**). Although the *Enterobacter* sp. HLG5 significantly enhanced whole cocoon weight (16.21%), pupal weight (16.23%), and cocoon shell weight (20.33%) of the survival silkworms (**Supplementary Figures 1B–E**), the survival rate (53.3%) (**Supplementary Figure 1F**) and cocoon production rate (65.0%) (**Supplementary Figure 1G**) of silkworms were significantly decreased in the insects fed *Enterobacter* sp. HLG5, and bodies of the dead silkworms were black and soft. Overall, these results revealed that *Enterobacter* sp. HLG5 strain was harmful to silkworms, while *Lelliottia* sp. HTJ13, *Klebsiella* sp. HGG15, and *Pantoea* sp. HLJ21 all exhibited no toxicity to silkworms.

Characterization of HGG15 strain

Klebsiella sp. HGG15 strain was ultimately selected for further study since it exhibited the plant growth promoting traits *in vitro* and *in planta* as well as posing no bio-safety concerns for silkworms. Colonies of *Klebsiella* sp. HGG15 strain were white on LB medium (**Figure 3A**). Gram staining and scanning electron microscope results showed it was a short rod and gram-negative bacterium (**Figures 3B,C**). Meanwhile, the strain produced hydrogen sulfide and nitrate reductase, but was negative for utilization of urea and lactose (**Supplementary Table 3**). Moreover, phylogenetic analysis based on full length 16S rDNA indicated that it was identical to *Klebsiella aerogenes* (**Figure 3D**), being consistent with its morphological characteristics, biochemical, and molecule characteristics.

Colonization and population dynamics of *Klebsiella aerogenes* HGG15/gfp in mulberry

To examine the colonization characteristics of *K. aerogenes* HGG15 in mulberry seedlings, plasmid pGFP4412 containing a *gfp* marker gene was transferred into wild type strain to generate *K. aerogenes* HGG15/gfp strain (**Supplementary Figures 2A,B**), which exhibited green fluorescence (**Supplementary Figure 2C**). This strain could be detected in various tissues of mulberry seedling by fluorescence microscopy after inoculation.

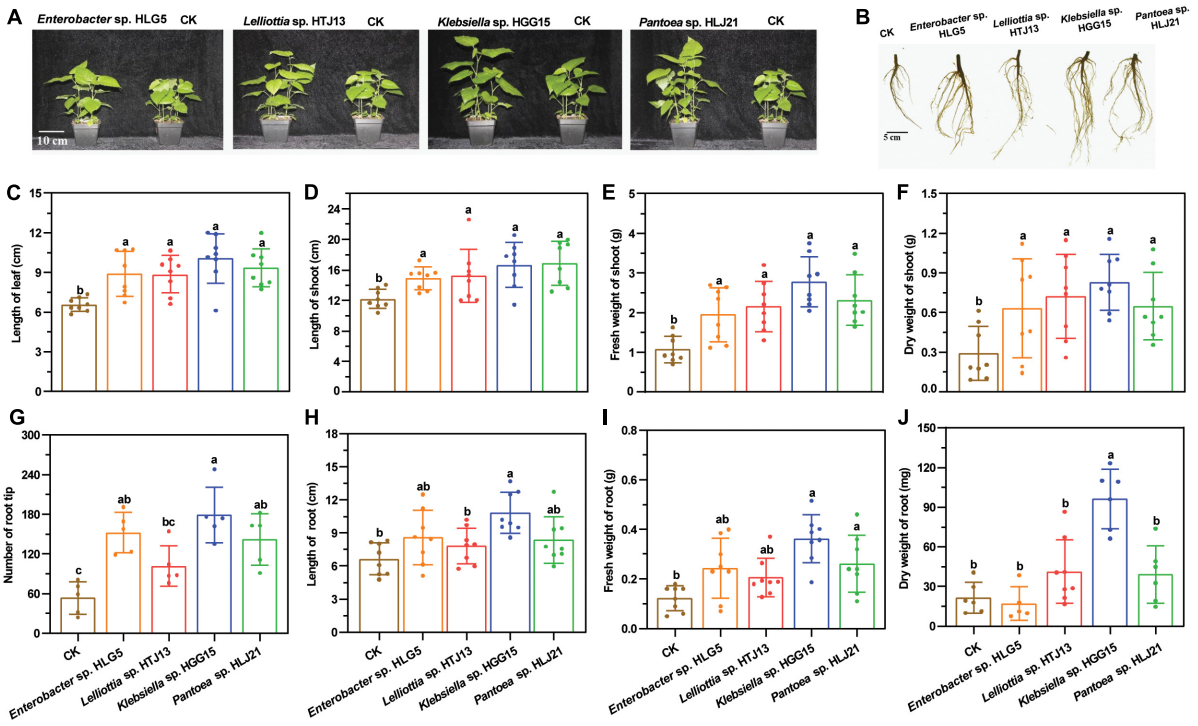

FIGURE 1
The growth promotion ability of potential four PGPBs on mulberry seedlings after 30-day inoculation. **(A)** The growth status of mulberry applied with the 1×10^7 CFU/mL bacterial suspensions. **(B)** The representative photograph of mulberry root architectures. **(C)** Length of mulberry leaf. **(D)** Length of mulberry shoot. **(E)** Fresh weight of mulberry shoot. **(F)** Dry weight of mulberry shoot. **(G)** Number of mulberry root tip. **(H)** Length of mulberry root. **(I)** Fresh weight of mulberry root. **(J)** Dry weight of mulberry root. Values represented the mean ± standard deviation of replicates (n = 8). Different letters indicated statistical differences using Tukey's one-way ANOVA ($P < 0.05$).

K. aerogenes HGG15/gfp first formed bacterial aggregates at the sites of root hair emergence (Figure 4A1) and colonized on the plant lateral roots (Figure 4A2). It subsequently proceeded to enter the plant root epidermis (Figure 4A3) after about 1–2 day post infection (dpi) where many gfp-tagged bacterial cells could be observed in the cortex of stem tissue by 3–5 dpi (Figures 4A4–6). As time progressed, a few colonies could be seen in the petiole and leaf (Figures 4A7–9). As expected, no gfp-labeled cells were ever observed in mulberry seedlings treated with sterile water (Figures 4Ar,s,I).

The quantification of *K. aerogenes* HGG15/gfp within plant tissues was performed by selective culturing of plant macerates since the strain had the capability to grow on the LB plates containing 50 μg/mL kanamycin (Figure 4B) and exhibited green fluorescence (Figure 4C). The numbers of *K. aerogenes* HGG15/gfp decreased progressively from mulberry roots to the stems and leaves. The localization and distribution of *K. aerogenes* HGG15/gfp within the plants changed with time (Figure 4D). The amount of bacteria in roots gradually decreased after initially multiplying rapidly there. Population size of fresh root tissue was 8.37 and 5.42 lg CFU/g at 3 and 21 dpi, respectively. A similar temporal pattern of colonization of stems and leaves was also seen. Total of 7.65 lg CFU/g in stems and 7.21 lg CFU/g in leaves were initially seen and dropped to 4.15 lg CFU/g in stems and 3.94 lg CFU/g in leaves by 21 dpi, respectively.

Mulberry-associated microbiome is influenced by *Klebsiella aerogenes* HGG15

Bacterial community composition of mulberry treated with *K. aerogenes* HGG15 was compared to that of control plants by 16S ribosomal RNA amplicon analysis. Complete data sets were submitted to the NCBI SRA database (Accession Number: SRP367158). All rarefaction curves tended to reach a plateau (Supplementary Figure 3), indicating that the depth of sequencing was sufficient. Changes in α and β diversity were assessed to detect the effects of *K. aerogenes* HGG15 on mulberry-associated bacterial communities. Alpha diversity assessed by Sob and Shannon indexes was not significantly different between control and *K. aerogenes* HGG15-treated plants, whereas α diversity strongly varied between different tissues (Supplementary Figure 4A). As expected, the α diversity was highest in rhizosphere soils and lowest in stems. Moreover,

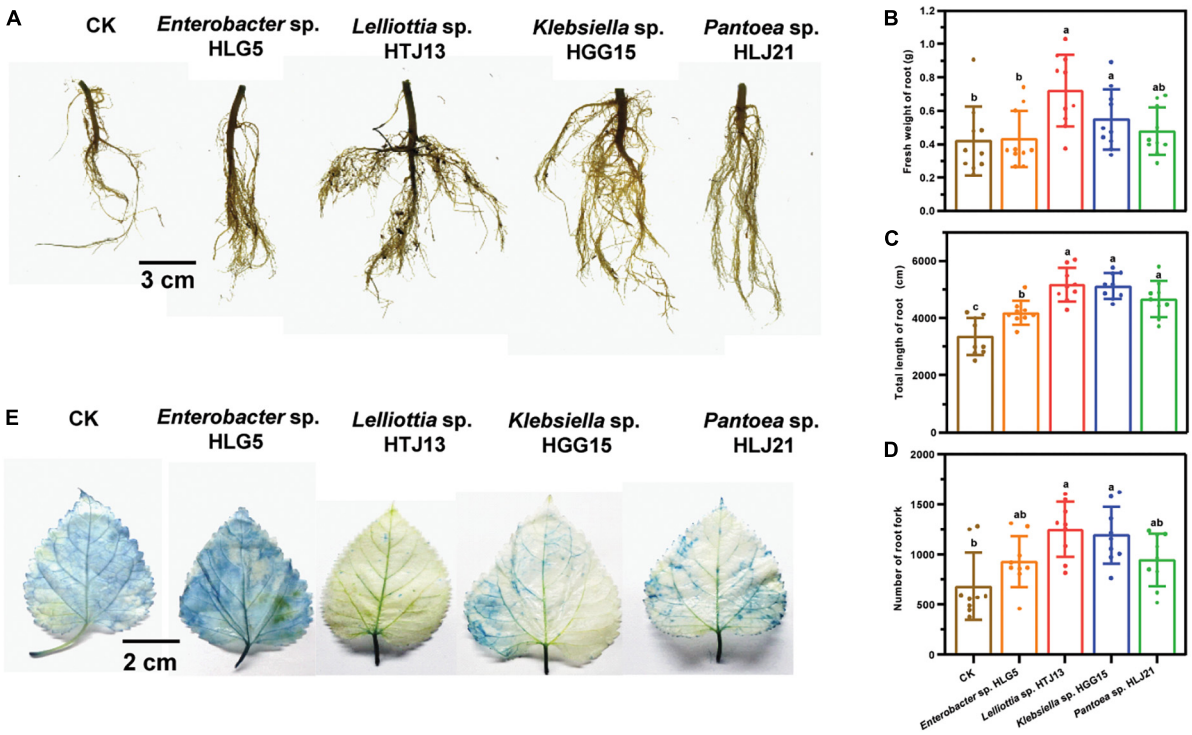

FIGURE 2
Effect of potential four PGPBs on mulberry flood tolerance. **(A)** Root architecture of mulberry after suffering flood stress. **(B)** Fresh weight of mulberry root. **(C)** Total length of mulberry root. **(D)** Number of mulberry root fork. **(E)** Representative photograph of the damage of mulberry leaf suffering flood stress by Evan's blue staining. Values represented the mean ± standard deviation of replicates (n = 9). Different letters indicated statistical differences using Tukey's one-way ANOVA ($P < 0.05$).

the number of shared OTUs and unique OTUs showed a trend of gradual decline from rhizosphere soil to stem (Supplementary Figure 4B). In addition, the results of β diversity analysis as revealed by PCoA showed that bacterial communities mainly formed two separate clusters with the distance between inoculated samples being smaller than between controls (Figure 5A), although the statistical differences of groups were not significant according to PERMANOVA analyses (rhizosphere soil: P = 0.2, R^2 = 0.3; root: P = 0.1, R^2 = 0.4; stem: P = 0.2, R^2 = 0.3). These results indicated that *K. aerogenes* HGG15 strain might impact the bacterial community regardless of whether it was the rhizosphere or endosphere.

To further understand the influence of *K. aerogenes* HGG15 on the shifts of microbial communities, the relative abundance of various bacterial taxa was analyzed. *K. aerogenes* HGG15 altered the composition of bacterial communities in root and stem, however, little differences between the control and inoculation groups were seen in the rhizosphere soil (Figure 5B). At the order level, the relative abundance of Sphingomonadales (HGG15: 29.53%; CK: 17.56%) and Cytophagales (HGG15: 12.15%; CK: 4.75%) were increased in inoculated roots, while the relative abundance of Rhizobiales (HGG15: 17.09%; CK: 34.50%) was decreased (Figure 5B). Moreover, Rhizobiales (HGG15: 42.88%; CK: 11.24%) and Saccharimonadales (HGG15: 15.02%; CK: 0.67%) were more abundant in stems of inoculated plants. In contrast, Pseudomonadales (HGG15: 0.13%; CK: 1.57%), Burkholderiales (HGG15: 4.24%; CK: 27.87%), Propionibacteriales (HGG15: 1.41%; CK: 7.42%), and Corynebacteriales (HGG15: 16.17%; CK: 26.29%) were enriched in stems of control plants. At the genus level, the roots of inoculated plants harbored significantly more *Ohtaekwangia* (HGG15: 11.09%; CK: 3.89%) and *Rhizorhapis* (HGG15: 28.25%; CK: 16.60%) than control plants (Figure 5B). *Rhizobium* (HGG15: 9.88%; CK: 6.52%), *Devosia* (HGG15: 5.52%; CK: 0.67%), *Brevundimonas* (HGG15: 3.72%; CK: 1.12%), *Methylorubrum* (HGG15: 26.70%; CK: 3.60%), and *TM7a* (HGG15: 13.22%; CK: 0.22%) were the predominant genera in the stems of *K. aerogenes* HGG15 inoculated plants, while the relative abundance of *Oxalicibacterium* (HGG15: 0%; CK: 5.39%), *norank_f_Oxalobacteraceae* (HGG15: 0%; CK: 10.11%), *Ralstonia* (HGG15: 2.31%; CK: 10.56%), *Aeromicrobium* (HGG15: 1.28%; CK: 6.97%), and *Rhodococcus* (HGG15: 0.51%; CK: 4.04%) were lower than in control plants (Figure 5B). These results revealed that *K. aerogenes* HGG15 substantially shifted endophytic bacterial composition

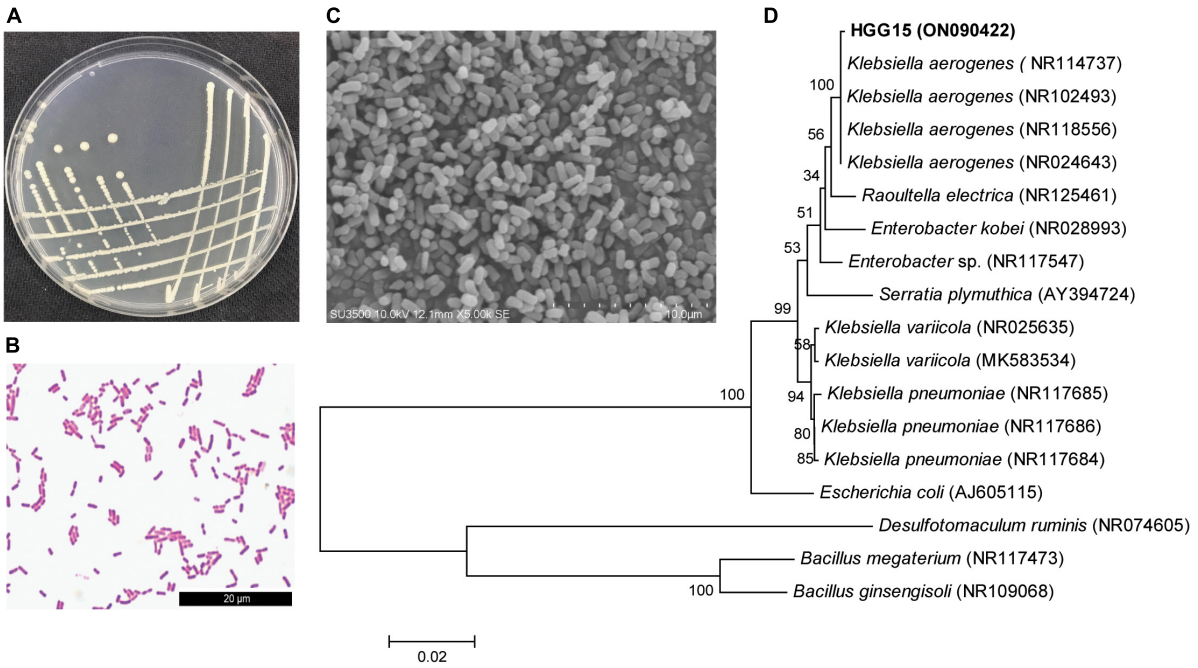

FIGURE 3
Characterization of *Klebsiella* sp. HGG15 strain. **(A)** Colony feature on LB medium after 24 h. **(B)** Gram-positive staining. **(C)** Scanning electron microscopy of bacterial cells. **(D)** Phylogenetic tree based on 16S rDNA. The tree was constructed by MEGA 6.0 using neighbor-joining analysis of 1,000 bootstrap replications.

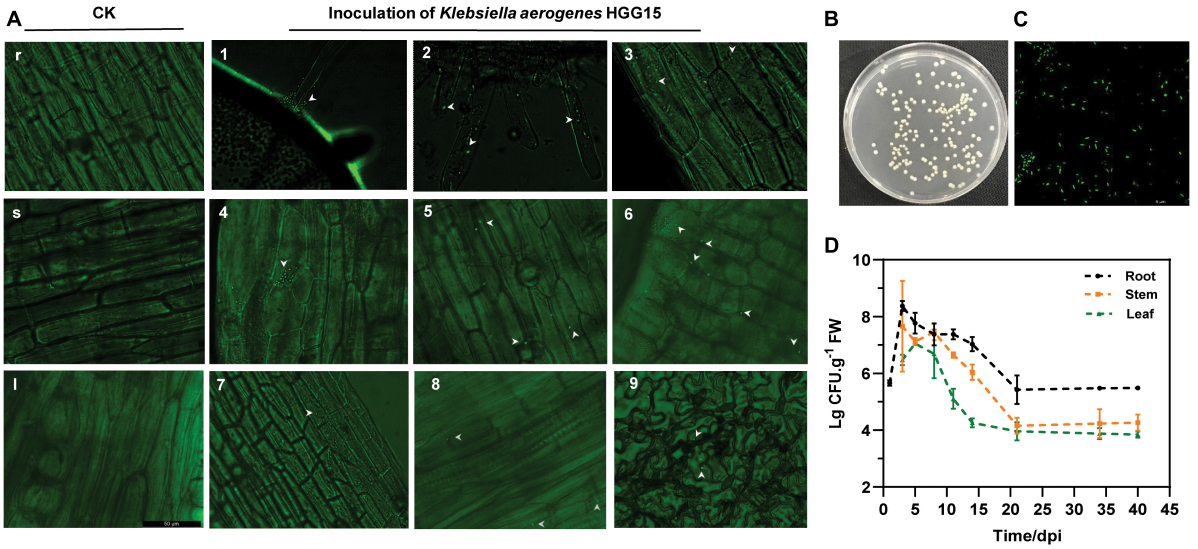

FIGURE 4
Colonization and population dynamics of gfp-tagged *K. aerogenes* HGG15 in mulberry seedlings. **(A)** Infection and colonization of gfp-tagged *K. aerogenes* HGG15 in mulberry. r, mulberry root of control group in 1 dpi; s, mulberry stem of control group in 3 dpi; l, mulberry petiole of control group in 7 dpi; 1, mulberry root hair of inoculation group in 1 dpi; 2, mulberry lateral root of inoculation group in 1 dpi; 3, mulberry root epidermis of inoculation group in 2 dpi; 4–6, mulberry cortex of inoculation group in 3–5 dpi; 7–8, mulberry petiole of inoculation group in 7 dpi; 9, leaf epidermis of inoculation group in 12 dpi. **(B)** Cultural feature of gfp-tagged *K. aerogenes* HGG15 re-isolated from mulberry on LB medium containing 50 μg/mL kanamycin. **(C)** Observation of re-isolated gfp-tagged *K. aerogenes* HGG15 strain under green fluorescence at 488 nm. **(D)** Population dynamics of gfp-tagged *K. aerogenes* HGG15 in mulberry seedling.

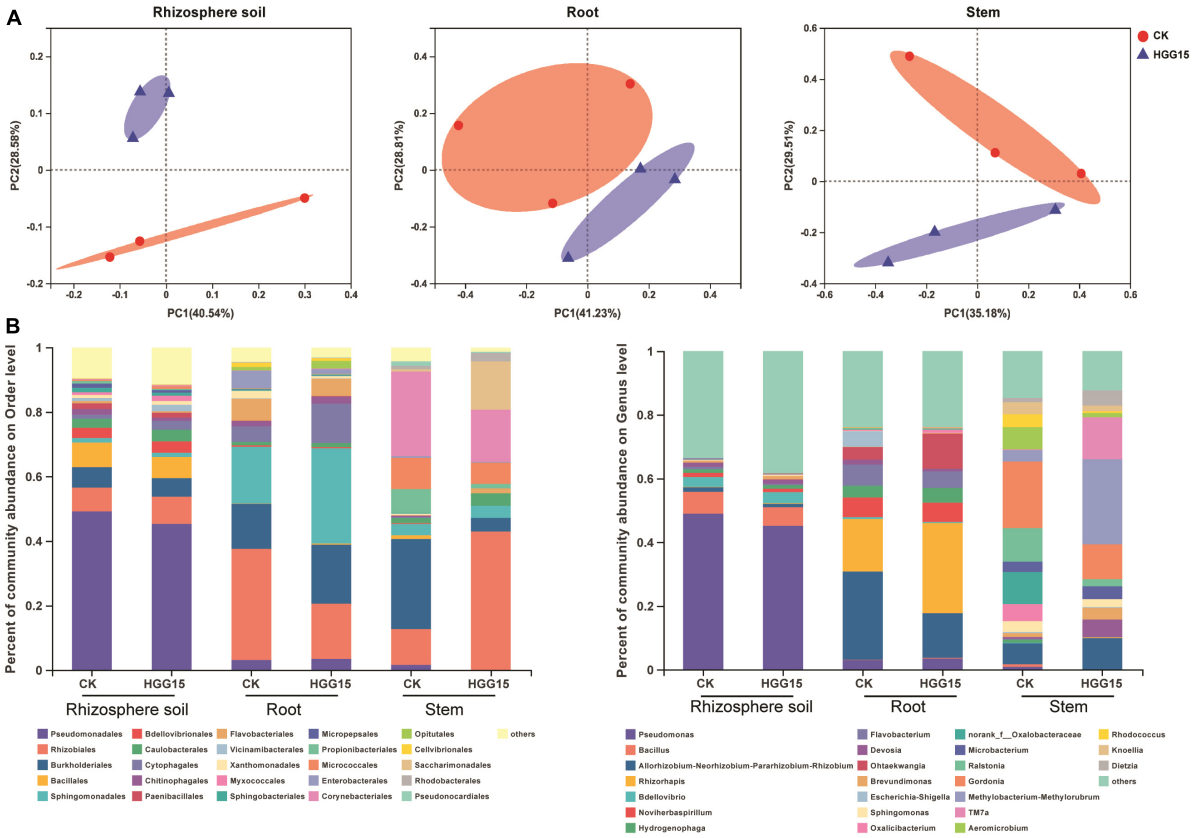

FIGURE 5
Effect of *K. aerogenes* HGG15 strain on mulberry associated bacterial communities. **(A)** Principal coordinate analysis of bacterial communities in the rhizosphere soil, root and stem of mulberry based on Bray–Curtis distances. **(B)** Composition of bacterial communities in the rhizosphere soil, root and stem of mulberry at order (left) and genus (right) level. Taxa with an abundance < 0.01 were included in "others." Each column represented the mean of three biological replicates.

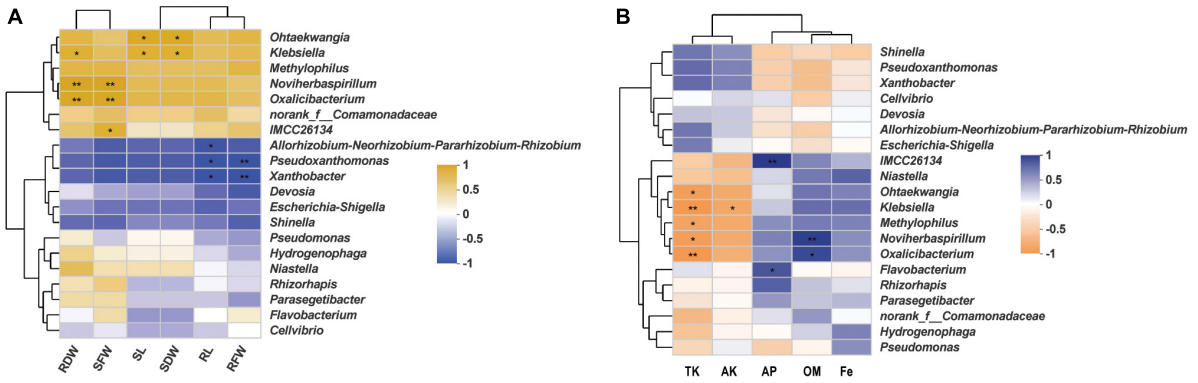

FIGURE 6
The relationship of root bacterial genera and mulberry development and soil properties. **(A)** Heatmap of correlation between mulberry growth parameters and top 20 bacterial genera using the Spearman correlation coefficient. RDW and SDW represented the dry weight of mulberry root and shoot, respectively. RFW and SFW represented the fresh weight of mulberry root and shoot, respectively. SL and RL represented mulberry shoot length and main root length, respectively. **(B)** Heatmap of correlation between soil properties and top 20 bacterial genera using the Spearman correlation coefficient. TK, AK, AP, OM, and Fe represented total potassium, available potassium, available phosphorus, organic matter, and available iron, respectively.

of mulberry seedlings, while it had little effect on bacterial communities in rhizosphere soil.

In order to uncover any significant differences in endophytic communities between *K. aerogenes* HGG15-treated and control plants, the Wilcoxon rank-sum test of bacterial community composition was investigated at the genus level. The relative frequency of *Xanthobacter* and *o_norank_c__Alphaproteobacteria* was significantly lower ($P < 0.05$) in roots of *K. aerogenes* HGG15 treated plants than in controls, while the proportion of *Methyloversatilis* and *MND1* was higher ($P < 0.05$) in treated plants (**Supplementary Figure 5A**). Notably, the genus of *Klebsiella* was relatively abundant in mulberry roots after inoculating *K. aerogenes* HGG15 ($P < 0.01$), while it was hardly detected in the rhizosphere soil and stem tissues (**Supplementary Figure 5B**), suggesting *Klebsiella* specifically colonized on mulberry roots. The Spearman correlation of the top 20 bacterial genera in root with mulberry growth indexes (**Supplementary Table 4**) was further investigated to uncover role of the *Klebsiella* genus. Results revealed that there was a positive relationship between *Klebsiella* and mulberry development, especially in root dry weight, shoot dry weight, and shoot length ($P < 0.05$) (**Figure 6A**), indicating that *Klebsiella* played a crucial role in promoting mulberry growth. In addition, soil property analysis showed that the content of organic matter was significantly higher in *K. aerogenes* HGG15 group ($P < 0.01$) (**Supplementary Figure 6B**), and potassium content was lower compared to that in the control group (**Supplementary Figures 6C,D**) ($P < 0.01$), but the contents of organic carbon, available phosphorus, and iron elements did not show dramatic variation (**Supplementary Figures 6A,E,F**). Furthermore, a heatmap constructed using the Spearman correlation coefficients was used to determine how the various microbial communities correlated with soil factors, revealing that *Klebsiella* was positively linked to total potassium and available potassium (**Figure 6B**). Altogether, these results suggested that *K. aerogenes* HGG15 strain significantly affected mulberry endophytic bacterial community, especially in root, and that the abundance of *Klebsiella* genus in root exhibited positive correlations with mulberry development and potassium content of soil, suggesting *Klebsiella* might contribute to mulberry seedling growth.

Klebsiella aerogenes HGG15 affects the mulberry metabolite profile

Since *K. aerogenes* HGG15 colonized on the interior of mulberry roots (**Figure 4D**) and promoted plant growth, and the genus *Klebsiella* is also a dominant colonizer in mulberry roots (**Supplementary Figure 5B**), untargeted metabolomics of roots were analyzed to determine if and how this strain affected mulberry metabolites. PCA of metabolites demonstrated that inoculated and control plants differed in their metabolome regardless of negative mode or positive mode of metabolite analysis (**Supplementary Figure 7A**). The metabolite profiles of mulberry were then subjected to OPLS-DA, which showed difference between the control and *K. aerogenes* HGG15-treated group in both negative and positive analytic modes (**Figure 7A**). The score plots of PCA and OPLS-DA exhibited an obvious separation between the control and treatment groups, indicating *K. aerogenes* HGG15 was responsible for the distinction in categories and quantities of metabolites in mulberry. In addition, little variation among the biological replicates of each group was observed (**Figure 7A** and **Supplementary Figure 7A**), which illustrated the sufficient reproducibility and reliability of the experiment.

Metabolites with a threshold *P*-value < 0.05 and VIP > 1 were considered as differentially accumulated metabolites (DAMs). In total, there were 201 different identifiable metabolites, consisting of 125 and 76 metabolites from the positive and negative ionization modes, where 131 and 70 metabolites were upregulated and downregulated, respectively (**Supplementary Figure 7B**). According to their molecular structure and function based on the HMDB, the DAMs were classified into eight categories at the superclass level. The most dominant group of DAMs was lipids and lipid-like molecules which contained glycerophospholipids, fatty acyls, glycerolipids, sphingolipids, and prenol lipids. The other DAMs were classified into organoheterocyclic compounds, organic acids and derivatives, organic oxygen compounds, phenylpropanoids and polyketides, nucleosides, nucleotides, and analogues, benzenoids, and organosulfur compounds (**Table 2**). At the class level, the content of 25 category substances (78.13%, occupying 32 categories), such as glycerolipid, sphingolipid, indole, and pyridine, were significantly upregulated in mulberry after *K. aerogenes* HGG15 treatment (**Table 2**).

To further understand the DAMs function and the related biological processes they participated in, the top 20 pathways enrichment analysis of the DAMs was conducted using KEGG. The results showed that the ABC transporter, glycerophospholipid metabolism, purine metabolism, arginine biosynthesis, and tryptophan metabolism were significantly enriched (**Figure 7B**). Most of these pathways are involved in basic biological functions of plants, indicating that primary metabolism in mulberry was significantly affected by *K. aerogenes* HGG15. Furthermore, the VIP values of DEMs were calculated to explore the specifically different metabolites in mulberry. As shown in **Figure 7C**, the metabolites 2-hydroxy-3-methoxyestrone, PC [18:4(6Z,9Z,12Z,15Z)/0:0], N5-acetyl-N2-gamma-L-glutamyl-L-ornithine, and polyoxyethylene (600) monoricinoleate were significantly decreased in roots after *K. aerogenes* HGG15 treatment. In contrast, some compounds, e.g., octadecanedioic acid, 4-(2-aminophenyl)-2,4-dioxobutanoic acid, pratenol A, flumioxazin, hippurate, 3-methyldioxyindole,

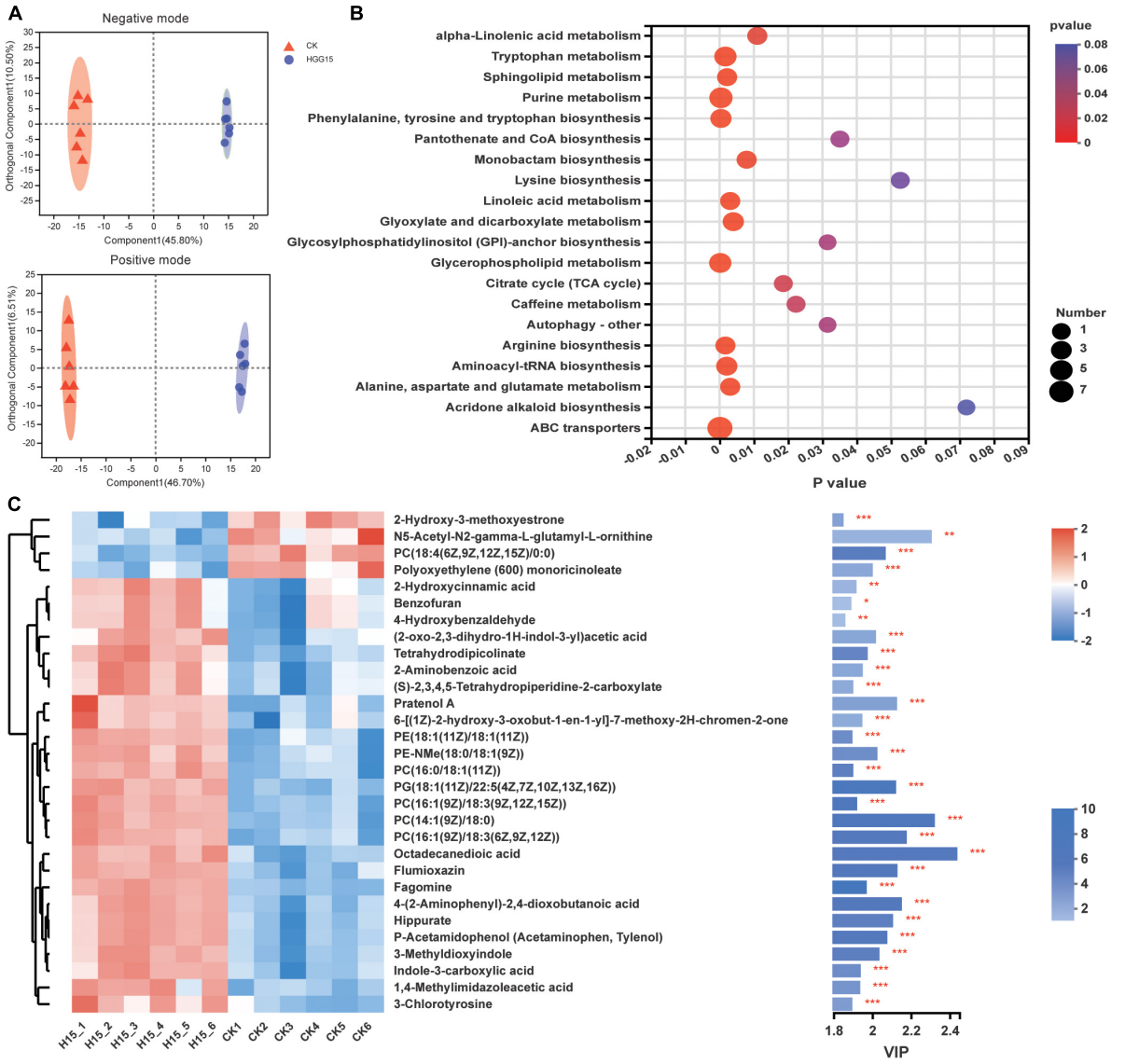

FIGURE 7
Effects of *K. aerogenes* HGG15 strain on metabolites of mulberry root. **(A)** Orthogonal partial least squares discriminant analysis of mulberry metabolites using positive and negative modes. **(B)** KEGG pathway enrichment analysis of differentially accumulated metabolites. The *x*-axis and *y*-axis represented the *P*-value of enrichment and enrichment pathway, respectively. The size of node represented the amount of metabolite enriched in the pathway. **(C)** Variable importance in projection scores of top 30 different metabolites between control and inoculated group. Left, heatmap of the differentially accumulated metabolites. The correlation of color and value was shown in the upper right bar. Right, bar chart of differentially accumulated metabolites. The length of bar indicated the contribution degree of this metabolite. The color of bar showed the difference degree of metabolite and the darker color represented the larger value of $-\log_{10}$(*P*-value) as shown in lower right bar. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

tetrahydrodipicolinate, fagomine, 2-aminobenzoic acid, and indole-3-carboxylic acid exhibited strong positive correlations with *K. aerogenes* HGG15 treatment. These results illustrated that the metabolites of mulberry root were strongly influenced by *K. aerogenes* HGG15, likely explaining its large effect on mulberry growth. In addition, a heatmap of correlation was generated to explore the potential relationship between differential bacteria altered by *K. aerogenes* HGG15 and metabolites of mulberry root. As expected, the metabolites were closely associated with differential bacterial microbiome of mulberry roots (**Supplementary Figure 8**). In particular, a greatly significant correlation has appeared between DAMs and bacterial taxa such as *o_norank_c_Alphaproteobacteria*, *Klebsiella*, *Methyloversatilis*, *Reyranella*, *Ammoniphilus*, *Conexibacter*, and *Ohtaekwangia*. Thus, differential bacteria altered by *K. aerogenes* HGG15 could further influence

TABLE 1 Characterization of endophytic bacteria for potential plant growth-promoting traits.

Strains	P-solubilization (cm)	IAA (mg/mL)	Siderophores (cm)	Inhibition zone (cm)	Acetoin (μg/mL)
Enterobacter sp. HLJ6-2	0.90	7.15	—	0.23	—
Enterobacter sp. HGJ7-2	0.67	19.75	—	—	101.23
***Enterobacter* sp. HLG5**	0.80	2.76	0.29	0.30	33.87
Atlantibacter sp. HLJ20	—	6.25	0.12	—	223.44
Atlantibacter sp. HLJ10	0.90	2.66	0.36	—	200.35
Pantoea sp. HLJ16-1	0.70	6.11	—	0.31	200.35
Kosakonia sp. HLJ19-2	0.60	6.01	—	—	370.67
Leclercia sp. HNG10-2	0.27	7.37	—	—	164.74
***Lelliottia* sp. HTJ13**	0.17	3.53	0.24	0.13	363.93
Rahnella sp. HNJ30	0.13	18.49	0.44	—	254.23
Pantoea sp. HGJ8	0.80	13.92	—	—	33.87
Pantoea sp. HGJ11	—	11.74	0.36	—	—
Pantoea sp. HTJ6	0.33	5.91	0.51	0.43	32.91
***Pantoea* sp. HLJ21**	0.60	10.96	0.17	1.33	76.21
Pantoea sp. HTJ25	0.73	5.86	0.40	—	87.76
Pantoea sp. HNJ8	1.03	8.64	0.25	1.20	165.70
Pantoea sp. HNJ21	0.20	6.01	0.50	0.36	65.63
Pantoea sp. HNJ32	0.37	13.05	—	—	32.91
Pantoea sp. HNG24	0.93	4.8	—	0.63	85.84
Klebsiella sp. HNG29	—	3.39	0.20	0.30	411.09
Klebsiella sp. HLG11	0.67	4.8	—	0.87	69.48
Klebsiella sp. HLG12	0.97	2.51	—	—	19.44
Klebsiella sp. HLG15	0.67	5.38	0.10	0.10	223.44
Klebsiella sp. HLG29	0.97	37.56	0.23	—	157.04
***Klebsiella* sp. HGG15**	0.90	6.69	0.38	0.40	363.93
Klebsiella sp. HGG18	1.10	3.44	—	0.80	273.48
Klebsiella sp. HGG20	1.33	—	—	—	—
Klebsiella sp. HTG12	0.60	3.1	0.14	0.60	61.78
Total	25	27	16	15	25

The bold isolates were selected as potential PGPBs since their great growth-promoting traits.

mulberry metabolites, where the synergistic linkage of microbiome and metabolites might together contribute to the development of host plant.

Discussion

Abiotic stresses are the foremost limiting factors for agricultural productivity. Various strategies including transgenic technology and molecular breeding have been considered to assist plant to relieve the stresses of such adverse conditions created by environmental extremes. Such approaches are often time consuming and might meet societal pressures. The modulation of endophytic colonization of plants is gaining wide popularity as an alternate strategy for improving stress tolerance of plants (Glick, 2014; Tiwari et al., 2016), since endophytes could provide sustainable benefits of both improving plant tolerance to biotic or abiotic stresses as well as stimulating plant growth (Bacon and White, 2015; Zhang et al., 2019). Therefore, the application of endophytes could be a potential method for enhancement of mulberry growth in the hydro-fluctuation belt of the TGR. Bacteria in the genera of *Enterobacter*, *Pantoea*, and *Klebsiella* have been the most extensively studied taxa and have been shown to benefit plant development (Ullah et al., 2019; Xu S. D. et al., 2022). Earlier studies reported that *Enterobacter* sp. E5 increased resistance of banana to *Fusarium* wilt disease (Liu et al., 2019). Zhang et al. (2017) also revealed that the endophyte *Klebsiella* sp. LTGPAF-6F could contribute to both growth promotion and improvement of drought tolerance of wheat in greenhouse studies. In the present study, a total of 28 isolates from mulberry were screened as PGPBs and *Enterobacter* spp., *Pantoea* spp., and *Klebsiella* spp. were the dominant genera with traits, which might play crucial roles in endowing mulberry beneficial traits in hydro-fluctuation belt (**Table 1**). Among them, four isolates were selected for assessment their PGP traits *in planta* and showed excellent growth stimulating effects to mulberry seedlings regardless of whether they experienced flood stress conditions or not

(**Figures 1**, **2**), and strain *Klebsiella aerogenes* HGG15 was found to be much superior to these other strains.

The plant growth promoting effect of bacteria is correlated with its colonization of host plants. It makes sense that successful colonization is critical for impacts of endophytes and their host plants as has been seen in many studies (Jha and Kumar, 2007; Pavlova et al., 2017; Ullah et al., 2019). In this study, *K. aerogenes* HGG15/gfp initially attached to emerging root hairs and lateral roots, indicating that it likely invades plants through these natural sites. Hallmann and Berg (2006) showed that, like *K. aerogenes* HGG15/gfp, endophytic bacterial population were lower within above-ground tissues, e.g., stem and leaves than in roots. Interestingly, we also found through amplicon sequencing analysis that other *Klebsiella* also preferentially colonized on mulberry roots (**Supplementary Figure 5B**), which might be the reason why *K. aerogenes* HGG15 greatly influenced root biomass and architecture of mulberry (**Figures 1**, **2**). It has been reported that some *Klebsiella*, such as *K. pneumonia*, are opportunistic pathogens of animals (Li and Huang, 2017; Álvarez-Marín et al., 2021), while major species of *Klebsiella* usually did not cause disease in plants. More importantly, most of *Klebsiella* such as *Klebsiella* sp. D5A (Liu et al., 2016) and *Klebsiella* sp. SBP-8 (Singh et al., 2015) could improve plant utilization of nitrogen, phosphorus, and iron, and contribute to biological control or environmental tolerance. This taxon thus has attracted attentions as a plant growth stimulator in agricultural systems. Notably, the results of our safety evaluation of *K. aerogenes* HGG15 revealed that it had no side effects on the silkworm. These results suggested that the endophytic bacterium *K. aerogenes* HGG15 might be a potential growth promoter for crop plants, but its biosafety will need further evaluation by determining haemolysis, cytotoxicity, antibiotic resistance and genotoxicity.

Inoculation of exogenous bacteria not only influences the microbiome structure of different plant niches (Li et al., 2021), but also can attract certain beneficial bacteria, such as *Rhizobium* (Guo and Chi, 2014), *Methylorubrum* (Alessa et al., 2021), and *Microbacterium* (Vilchez et al., 2018), to promote plant growth and inhibit disease occurrence (Zhuang et al., 2021). We explored the bacterial community in different compartments of mulberry and found that bacterial β diversity in rhizosphere soil, root, and stem of mulberry was influenced by *K. aerogenes* HGG15 (**Figure 5A**), while there was no obvious effect on α diversity (**Supplementary Figure 4A**). The taxonomic composition of endophytic bacteria exhibited differences between the control and inoculated plants, especially those inhabiting in root and stem, but there was little effect of inoculation on rhizosphere soil communities (**Figure 5B**). *Methylorubrum* was the preponderant bacterial endophyte appearing in *K. aerogenes*-inoculated mulberry. Similar findings were found in soybean (Hara et al., 2019) and pine seeds (Koskimäki et al., 2022), which were dominantly colonized by *Methylorubrum* and exhibited higher biomass of shoots and roots. This taxon may thus contribute to mulberry growth. Notably, *Ralstonia*, one of the most destructive plant bacterial pathogens (Pan et al., 2013), was markedly decreased in mulberry after inoculation. In addition, compared with control

TABLE 2 The classification of significantly different metabolites of mulberry root.

Superclass	Class	Regulate	Number
Lipids and lipid-like molecules	Glycerophospholipids	Up/down	52
	Fatty acyls	Up/down	21
	Steroids and steroid derivatives	Up/down	4
	Glycerolipids	Up	4
	Sphingolipids	Up	2
	Prenol lipids	Down	1
Organoheterocyclic compounds	Pyridines and derivatives	Up	4
	Indoles and derivatives	Up	3
	Piperidines	Up	3
	Tetrahydrofurans	Up/down	2
	Benzofurans	Up	2
	Imidazopyrimidines	Up	2
	Azacyclic compounds	Up	1
	Furans	Up	1
	Diazines	Up	1
	Benzoxazines	Up	1
	Benzothiophenes	Down	1
	Benzopyrans	Up	1
Organic acids and derivatives	Carboxylic acids and derivatives	Up/down	19
	Keto acids and derivatives	Down	1
Organic oxygen compounds	Organooxygen compounds	Up/down	11
Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Up	2
	Flavonoids	Down	2
	Stilbenes	Down	1
	Isocoumarins and derivatives	Up	1
	Coumarins and derivatives	Up	1
Nucleosides, nucleotides, and analogues	Purine nucleotides	Up/down	2
	Purine nucleosides	Up	2
Benzenoids	Phenols	Up	2
	Benzene and substituted derivatives	Up	1
Organosulfur compounds	Thioethers	Down	1

plants, roots of mulberry treated with *K. aerogenes* HGG15 accumulated significantly less *Xanthobacter* (**Supplementary Figure 5A**), a potential phytopathogen causing serious disease in cruciferous plants (Mansfield et al., 2012). These results indicated that *K. aerogenes* HGG15 may play a crucial role in antagonizing against pathogens as well as is a good candidate strain as bio-fertilizer, but its antagonistic efficiency should be further studied.

In the process of bacteria-assisted plant growth promotion, some material exchange, energy transformation, and information communication continuously take place among microbes, plant roots, and the soil environments (Sun L. J. et al., 2020; Sun et al., 2022). In our study, the increased content of organic matter and decreased content of potassium in soils of mulberry inoculated with *K. aerogenes* HGG15 were found (**Supplementary Figure 6**), indicating a feedback effect of this strain on soil processes. This finding is in accordance with previous study that physicochemical properties of soils of *Brassica juncea* were strongly influenced by endophytic bacterial *Serratia marcescens* PRE01 and *Arthrobacter ginsengisoli* PRE05 (Wang et al., 2020). Interestingly, we found that *K. aerogenes* HGG15 strain had a capability to dissolve unavailable potassium *in vitro* (**Supplementary Figure 9**), suggesting that this strain could transform the unavailable potassium to available form, and thus assisted mulberry trees uptake useful potassium from soil to benefit their growth. Metabolites in plant roots usually exert a variety of functions, such as facilitating primary metabolism and root growth, rhizosphere communication, and plant defense (Doell et al., 2021). Mutualistic interactions of plant with endophyte can facilitate plant growth promotion through the accumulation of beneficial plant metabolites (Kundu et al., 2021), as in the case of fungus *Piriformospora indica* that colonize in Chinese cabbage, which stimulated plant synthesis of metabolites involved in the tryptophan and phenylalanine metabolism as well as γ-aminobutyrate (Hua et al., 2017). Furthermore, *Bacillus amyloliquefaciens* enhanced maize growth by improving nutrient uptake and influencing plant primary metabolism, especially glucose, fructose, alanine and γ-aminobutyric acid metabolism (Vinci et al., 2018). Most differentially abundant compounds found in our study were annotated as lipids and lipid-like molecules (**Table 2**), especially glycerolipids and sphingolipids, which not only contributed to abiotic and biotic stress resistance but also regulated basically cellular processes (Luttgeharm et al., 2016). It was worth noting that we found the production of indole derivatives affiliated with organoheterocyclic compounds in mulberry was elevated by *K. aerogenes*. These compounds have been shown to enhance lateral root growth (Sun X. et al., 2020). Additionally, the indole-3-carboxylic acid, one of the indole derivatives, is the best known of the auxins, which is involved in camalexin biosynthesis (Wang et al., 2012) and plays an important role in plant pathogen defense (Magnus et al., 1980). *K. aerogenes* HGG15 enhanced the content of indole-3-carboxylic acid in mulberry roots, suggesting that this strain might induce resistance of host plant to biotic or abiotic stress. Notably, cinnamic acids, isocoumarins, coumarins, and their derivatives, as important phenylpropanoid and polyketide compounds, were significantly upregulated in mulberry after *K. aerogenes* HGG15 inoculation (**Table 2**). Among them, the downstream substance of cinnamic acid has been described as a potential antioxidant compound in mulberry (Park et al., 2017), which might be beneficial for mulberry development under abiotic stress conditions. Coumarins are known to play a key role in the transport of iron-mobilization in plant and are recognized as plant signals shaping host microbiomes (Stringlis et al., 2018). Moreover, coumarins and their derivatives have been extensively studied for their potential to help plants cope with biotic and abiotic environmental stress (Stringlis et al., 2019). Therefore, the above-mentioned metabolites might be crucial drivers of mulberry growth promotion and its enhanced stress resistance when inoculated with *K. aerogenes* HGG15.

Conclusion

The growth of mulberry trees is greatly hindered by flood stress and limited-nutrition in the hydro-fluctuation belt of the TGR region. In the current study, *Klebsiella aerogenes* HGG15 was ultimately screened out as having superior plant growth promotion proprieties *in vitro* and strongly stimulated growth and enhanced flood tolerance of mulberry *in planta*. Moreover, it was not harmful to silkworm and could extensively and persistently colonize on mulberry. The inoculation of *K. aerogenes* HGG15 greatly altered the establishment of endophytic bacterial communities in mulberry, especially in roots and stems. In addition, the large change in abundance of abiotic stress response factors and compounds that promote plant growth such as glycerolipid, sphingolipid, indole, pyridine, and coumarin were observed in inoculated mulberry. Taken together, these results of this study help us to understand the interactions between this endophyte and plants and mechanisms driving these interactions, and provide innovative approaches for revegetation in the hydro-fluctuation belt through enhancing microbe-assisted plant growth.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, SRP367158; ON786677–ON786703; ON090422.

Author contributions

TO and JX designed the experiments. TO, HG, KJ, JY, and RZ performed the experiments. TO analyzed and wrote the manuscript. JX and XL revised the manuscript. ZZ and ZX conceived the study and contributed resources. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by JX of the National Natural Science Foundation of China (31870518), Natural Science Foundation of Chongqing (cstc2019jcyj-msxmX0396), and Fundamental Research Funds for the Central Universities (XDJK2019B047).

Acknowledgments

We are sincerely grateful to Prof. Steven Lindow (University of California, Berkeley, United States) for his critical review and thorough editing of this manuscript. We also thank for agronomist Changyu Qiu (Promotion Station of Sericulture Technology, Guangxi Zhuang Autonomous Region, China) and Chuanshu Huang (Institute of Sericulture Science and Technology Research, Chongqing, China) for providing mulberry seeds and silkworm eggs, respectively.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.978550/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 21 September 2022
DOI 10.3389/fmicb.2022.995830

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 16 July 2022
ACCEPTED 29 August 2022
PUBLISHED 21 September 2022

CITATION
Sharma VK, Parmar S, Tang W, Hu H, White JF Jr and Li H (2022) Effects of fungal seed endophyte FXZ2 on *Dysphania ambrosioides* Zn/Cd tolerance and accumulation. *Front. Microbiol.* 13:995830. doi: 10.3389/fmicb.2022.995830

Effects of fungal seed endophyte FXZ2 on *Dysphania ambrosioides* Zn/Cd tolerance and accumulation

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Metal-induced oxidative stress in contaminated soils affects plant growth. In the present study, we evaluated the role of seed endophyte FXZ2 on *Dysphania ambrosioides* Zn/Cd tolerance and accumulation. A series of pot experiments were conducted under variable Zn (500, 1,000, and 1,500 mg kg^{-1}) and Cd (5, 15, 30, and 60 mg kg^{-1}). The results demonstrated that FXZ2-inoculation significantly enhanced the growth of *D. ambrosioides* and improved its chlorophyll and GSH content. In the rhizosphere, FXZ2 inoculation changed the chemical speciation of Zn/Cd and thus affected their uptake and accumulation in host plants. The exchangeable and carbonate-bound fractions (F1 + F2) of Zn decreased in the rhizosphere of FXZ2-inoculated plants (E+) as compared to non-inoculated plants (E-) under Zn stress (500 and 1,000 mg kg^{-1}), correspondingly, Zn in the shoots of E+ decreased ($p < 0.05$). However, at Cd stress (30 and 60 mg kg^{-1}), the F1 + F2 fractions of Cd in E+ rhizospheric soils increased; subsequently, Cd in the shoots of E+ increased ($p < 0.05$). FXZ2 could exogenously secrete phytohormones IAA, GA, and JA. The study suggests that seed endophyte FXZ2 can increase Zn/Cd tolerance of host plant by altering Zn/Cd speciation in rhizospheric soils, as well as exogenous production of phytohormones to promote growth, lowering oxidative damage while enhancing antioxidant properties. For Zn/Cd accumulation, it has opposite effects: Zn uptake in E+ plants was significantly ($p < 0.05$) decreased, while Cd accumulation in E+ plants was significantly ($p < 0.05$) increased. Thus, FXZ2 has excellent application prospects in Cd phytoextraction and decreasing Zn toxicity in agriculturally important crops.

KEYWORDS

seed endophytes, *Dysphania ambrosioides*, metal stress, phytoremediation, mechanism, chemical speciation

Introduction

Plants rely on various metals for normal physiology, but higher or excess metals in the soil not only deteriorate the soil health and change the native microbial community but also adversely affect the physiology and metabolism of plants (Kidd et al., 2012; Chen et al., 2014; Parmar and Singh, 2015; Etesami, 2018). Zinc (Zn) is an essential element for plants, but a higher concentration of Zn in the soil adversely affects plant growth via root growth inhibition, mitotic efficiency, chromosomal aberrations as well as oxidative stress (Jain et al., 2010). Cadmium (Cd) is a non-essential trace element that can cause toxicity even at lower concentrations (Wagner, 1993; Nan et al., 2002; Kuriakose and Prasad, 2008), accumulates readily in the soil and enters the food chain via enrichment in food crops (Wang et al., 2022). The bioavailability, mobility, and toxicity of these metals to plants depend on their chemical forms rather than the total contents (Liu et al., 2007). Therefore, the chemical speciation of metals in the soil may have an important impact on plants (Tüzen, 2003; Ahlf et al., 2009).

It is well known that metal-contaminated soils cause various problems to the surrounding environments, such as plants survival, agricultural production, food safety, and human health; therefore, the remediation of these metal-contaminated soils is of utmost importance (Hussain et al., 2022). Some plants growing in highly metal-contaminated environments evolved to tolerate metal stress; they have potential applications in phytoremediation. Previous studies have demonstrated that plant-associated microbes, i.e., endophytes can increase host plants' metal tolerance properties, enhance their growth, and influence their metal accumulation (Sharma et al., 2019; Rattanapolsan et al., 2021; Ważny et al., 2021; Hussain et al., 2022). It is believed that endophytes induced tolerance and growth improvement of host plants to metal stress by detoxification through chelation and compartmentalization of metal ions, increasing nutrient absorption and root growth, changing the distribution of metal in plant cells, modulating the antioxidative system, and secretion of phytohormones (Bilal et al., 2018; White et al., 2019; Chang et al., 2021; Akhtar et al., 2022).

FXZ2 is a fungal seed endophyte that has been isolated from *Arabis alpina,* and it has been identified to be *Epicoccum nigrum* (GenBank accession number is ON209455) (Chu et al., 2017). Our previous studies have demonstrated that FXZ2 has high tolerance and adsorption capacity for lead (Pb) and Cd, and it can significantly enhance host plants' growth under Zn/Cd stress. Seed endophytes are attributed to providing beneficial traits such as improving nutrient uptake, reducing susceptibility to drought and temperature stress, and improving the growth of host plants. However, the role of seed endophytes on the plants' metal tolerance and accumulation as well as its mechanisms are still unknown. For the beneficial characteristics that the seed endophyte can be transferred to the next generation through vertical transmission (Li et al., 2019), therefore, in practice, it has more advantages than the other symbiotic microbes. For example, the seed endophyte RE3-3 *Herbaspirillum frisingense* was successfully transmitted to the next generation seeds of *Phragmites australis* and, consequently, enhanced seedling development and growth under Cd stress (Gao and Shi, 2018).

Dysphania ambrosioides (L.) Mosyakin and Clemants is a dominant plant in Pb-Zn mining sites of Huize County, Yunnan Province, China. It has been reported as a Cd-accumulator and a Pb-hyperaccumulator, which showed potential application in phytoremediation of multi-metal-contaminated sites (Wu et al., 2004; Li et al., 2012; Li X. et al., 2016). The present study aimed to investigate the role of fungal seed endophyte FXZ2 on *D. ambrosioides* Zn/Cd tolerance under variable Zn (500, 1,000, and 1,500 mg kg^{-1} soil) and Cd (5, 15, 30, and 60 mg kg^{-1} soil) stress. Further, the speciation of Zn/Cd in rhizospheric soils of *D. ambrosioides* was tested by Tessier sequential extraction methods. The objective of this study is to elucidate how the seed endophyte FXZ2 altered the metals' chemical speciation in rhizospheric soils and thus affected their absorption, translocation, and accumulation in host plants. The novelty of this work is that it gives important information about the function of seed endophytes in increasing the survival and growth of host plants under metal stress conditions.

Materials and methods

Fungal seed endophyte FXZ2

The fungal seed endophyte FXZ2 was previously isolated from the seeds of *Arabis alpina*, which were collected from the Pb-Zn mining sites of Huize County, Yunnan Province, Southwest China (25°28′17″ N, 103°37′34″ E) (Chu et al., 2017). FXZ2 was identified to be *Epicoccum nigrum* based on its morphological features and molecular analysis (Chu et al., 2017), and its GenBank database accession number is ON209455[1]. The isolate showed better Pb and Cd tolerance and adsorption capacity, and has been authorized by the Patent Office of the People's Republic of China (ZL 2017 1 0028569. 2). It was submitted to the Chinese General Microbiological Culture Collection Center (CGMCC NO.13573).

Phytohormone production

To assess for phytohormones jasmonic acid (JA), indole-3-acetic acid (IAA), and gibberellic acid (GA) production, the

1 http://www.ncbi.nlm.nih.gov/

isolate FXZ2 was grown in PDB (potato dextrose broth) at 28 ± 2°C for 21 days in a shaker. After that, the culture was filtered and the broth was collected and extracted three times with ethyl acetate, followed by concentration using a vacuum rotary evaporator. Finally, the extract was dissolved in methanol for phytohormone tests according to the manufacturer of plant hormone kits (MLBIO Biotechnology Co., Ltd., Shanghai). A change in the color of the reaction mixture was measured by a spectrophotometer at a wavelength of 450 nm. And the concentrations of IAA, GA, and JA in the extracts were calculated by comparing the OD of the extracts to the standard curve of the IAA, GA, and JA. Three replicates were performed.

Pot experiments

The mature seeds were collected from naturally growing *D. ambrosioides* and surface sterilized as Li et al. (2012). Subsequently, the seeds were germinated on a plastic tray that contained a fixed soil substrate (perlite: peat moss, 3:7, vol:vol) in a light incubator (25 ± 1/18 ± 1°C, 16/8 h day/night cycle, 60% relative humidity). Twenty-one days later, the germinated seedlings with equal size were transplanted to the pots (1 seedling/pot), which contained 150 gm of sterilized soil substrate mixed with the overages of $ZnSo_4.7H_2O$ or $CdCl_2.2.5H_2O$ to the final concentration of 0, 500, 1,000, and 1,500 mg Zn kg^{-1} and 0, 5, 15, and 30 mg Cd kg^{-1}, respectively. The pots were kept in a random configuration and exposed to artificial plant lighting (16/8 h day/night cycle). Every 2–3 days, the plants were irrigated with autoclaved water, and once a week Peter's General Purpose 20-20-20 fertilizer (Grace Sierra Horticultural Products, Milpitas, CA, USA) was given.

For the inoculation, FXZ2 was grown on PDA plates at 25°C for 7 days. Then, the mycelia were scraped off and suspended in autoclaved distilled water and divided equally into two portions (A and B). Suspension B was autoclaved at 121°C for 20 min. The pots were randomly divided into two groups (I and II). Further, the plants of group-I were sprayed with suspension A (E+) and group II with autoclaved suspension B (E-) at different time intervals 7, 15, 30, and 45 days of the transplant. The plants were harvested after growing for 60 days, and the fresh leaves were collected from E+ and E- and flash frozen right away with liquid nitrogen, preserved at −80°C, and used within 2 weeks for biochemical analysis. Simultaneously, the rhizospheric soil from each pot was collected, air-dried, and kept in poly-bags with proper labels for subsequent analysis.

Plant growth parameters

Shoot, root length, and the dry biomass

The harvested plants were washed under tap water and finally rinsed with deionized water. After that, the plants were divided into shoots (all aboveground parts) and roots (all belowground parts), and the length was measured. Finally, the shoots and roots were oven-dried at 50–60°C to constant weight, and then the dry biomass was recorded. The dried plant samples were used for metal content analysis.

Total chlorophyll content

Ten plants were selected randomly from each group before harvesting, and the total chlorophyll content of the youngest fully developed leaves of each plant was analyzed using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Inc., Tokyo, Japan). And the final chlorophyll content of each group was an average of 10 plants.

Lipid peroxidation

A chemical assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure the lipid peroxidation extent, which was expressed in nanomoles of malondialdehyde (MDA) formation per gram of tissue. Three replicates were made. To do this, the frozen leaves' tissue was crushed in a chilled phosphate buffer (50 mM, pH 7.2). Then, the homogenate was centrifuged for 10 min at 3,500 rpm and 4°C. After that, the supernatant was transferred to a new tube and the MDA was measured spectrophotometrically (MAPADA UV-1800 PC).

Glutathione content

The total glutathione (T-GSH) and oxidized glutathione (GSSG) assay kits were used for GSH analysis (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). To do this, the frozen leaves were homogenized in an extraction buffer (1:4 ratio, wt/vol). Then, the homogenate was centrifuged for 10 min at 3,500 rpm and 4°C. After that, the supernatant was used for GSH analysis (Rahman et al., 2006).

The absorbance of the assay mixture was measured according to the manufacturer's protocol, and the T-GSH and GSSG content was calculated using the given formulas. The GSH content was expressed in micromoles per gram of fresh leaves, which was the calculated difference of GSSG content from the T-GSH content according to the formula mentioned in the kit.

Cd/Zn accumulation in the plants

The dried root/shoot samples were homogenized into fine powders, respectively. Then, 0.2 g powders were digested in 5 ml HNO_3 (65% w/w) at 110°C for 2 h. After cooling 1 ml H_2O_2 (30% w/w) was added and the mixture was heated for 1 h. The digests were then diluted to 50 ml with triple-distilled water (Shen et al., 2013). Finally, the

concentrations of Cd/Zn were estimated by flame atomic absorption spectrometry (Li et al., 2014). The test was performed in triplicate.

Chemical speciation of Cd/Zn in rhizospheric soils

The chemical speciation of Zn/Cd in rhizospheric soils was tested according to the method of Tessier et al. (1979). The method consists of five steps that give rise to five fractions operationally defined as F1 (exchangeable), F2 (carbonate bound), F3 (Fe-Mn oxides bound), F4 (organic bound), and F5 (residual). Briefly, 1 gm fine powder of the soil was taken into a 50-ml polycarbonate centrifuge tube. First fraction was extracted with 20 ml 1.0 M $MgCl_2$ (pH 7.0) for 1 h with continuous agitation. The second fraction was extracted with 10 ml 1.0 M sodium acetate (pH adjusted to 5.0 with acetic acid) for 5 h with continuous agitation. The third fraction was extracted with 20 ml 0.04 M $NH_2OH.HCl$ in 25% sodium acetate (pH 2.0) for 6 h at 96°C in a water bath with occasional agitation. The fourth fraction was extracted with 3 ml 0.02 M HNO_3 and 5 ml 30% H_2O_2 (pH adjusted to 2.0 with HNO_3) for 2 h at 96°C in a water bath with occasional agitation; after that, 3 ml 30% H_2O_2 (pH 2.0 with HNO_3) was added and extracted for 2 h at 96°C in a water bath with occasional agitation; subsequently, after cooling, 5 ml 3.2 M ammonium acetate in 20% (v/v) HNO_3 was added, and the samples were diluted to 20 ml and agitated continuously for 30 min. The fifth fraction was the residue left from the organic fraction. It was digested with 4 ml HCl-HNO_3 (3:1, v/v) mixture at 80°C for 30 min, then 100°C for 30 min, and finally 120°C for 1 h. After that, cooled and 1 ml $HClO_4$ was added to continue digestion at 100°C for 20 min, followed by 120°C for 1 h. The concentrations of Zn/Cd were determined by flame atomic absorption spectrometry in different fractions (Li et al., 2014). Triplicates were made. The effect of FXZ2 inoculation (FE) was introduced to evaluate the influence on the chemical speciation of Zn/Cd in the rhizospheric zone. Here, FE = $(F^{E+} - F^{E-})/F^{E-}$, where F^{E+} and F^{E-} represent the corresponding fractions of metals in the E+ and E- treatments, respectively. The FE data were represented as heatmap drawn using Heatmap function of R version 4.1.1 (2021).

Statistical analysis

Boxplots were drawn using the ggboxplot function of the ggpubr package (version "0.4.0.999") in R version 4.1.1 (Core TeamR, 2021) and RStudio 2021.09.0 (R Studio Team, 2021). The difference between E+ and E- was determined using Student's *t*-test significant at the level of <0.05% performed in RStudio and one-way ANOVA and Duncan test ($p < 0.05$).

Results and discussion

The effect of FXZ2 on *Dysphania ambrosioides* growth

No matter at Zn or Cd stress, FXZ2 significantly improved the shoot length of *D. ambrosioides* ($p < 0.05$) (**Figures 1**, **2**). However, it had different effects on the root length and dry biomass of *D. ambrosioides* under Zn stress and Cd stress. At all Zn concentrations, FXZ2 decreased the root length of *D. ambrosioides*, but the difference was only significant ($p < 0.05$) at 1,500 mg kg^{-1} Zn stress (**Figure 1**). Both the dry biomass of shoots and roots of E+ were significantly ($p < 0.05$) higher than those of E- at all Zn concentrations. Contrary to this, at all Cd concentrations, FXZ2 improved the root length of *D. ambrosioides* ($p > 0.05$) except at 30 mg kg^{-1} Cd stress ($p < 0.05$) (**Figure 2**). The dry biomass of E+ shoots was significantly ($p < 0.05$) higher than that of E- shoots. However, the dry biomass of E+ roots was more than that of E- roots at all Cd concentrations, but the difference was only significant ($p < 0.05$) at 15 and 60 mg kg^{-1} Cd stress.

Although Zn is an essential element required for plant growth, its high concentration in the soil could affect essential plant metabolic functions and cause retarded growth and senescence (Yadav, 2010). High Cd concentration negatively affects mineral nutrition and carbohydrate metabolism and consequently decreases plant biomass production (John et al., 2009). Increased Cd also alters the activity of antioxidant enzymes, including superoxide dismutase, peroxidase, etc. (Sun et al., 2007). In the present study, it was found that with the increase of Zn/Cd concentration in the soil, both the dry biomass of E+ and E- shoots and roots decreased (**Figures 1**, **2**). But still, the dry biomass of E+ was better than E-. The finding suggests that fungal seed endophyte FXZ2 improved *D. ambrosioides* growth under different Zn/Cd stress. These results are similar to previous studies that microbial inoculation positively affected the plant biomass under Zn and/or Cd stress (He et al., 2013; Bilal et al., 2018; Singh et al., 2018; Zhu et al., 2018; Zhai et al., 2022). In addition, the present study showed that the plant exposure to Cd stress affects the biomass in a dose-dependent manner; similar observations were also reported by other authors (Sun et al., 2007; Kamran et al., 2015; Khan et al., 2015; Shahid et al., 2019; Zhang et al., 2019).

In general, FXZ2 induced enhancement of plant growth indicators such as shoot and root lengths. Their dry weight indicates a plant's ability to tolerate Zn and Cd stress and has shown positive growth (Kamran et al., 2015).

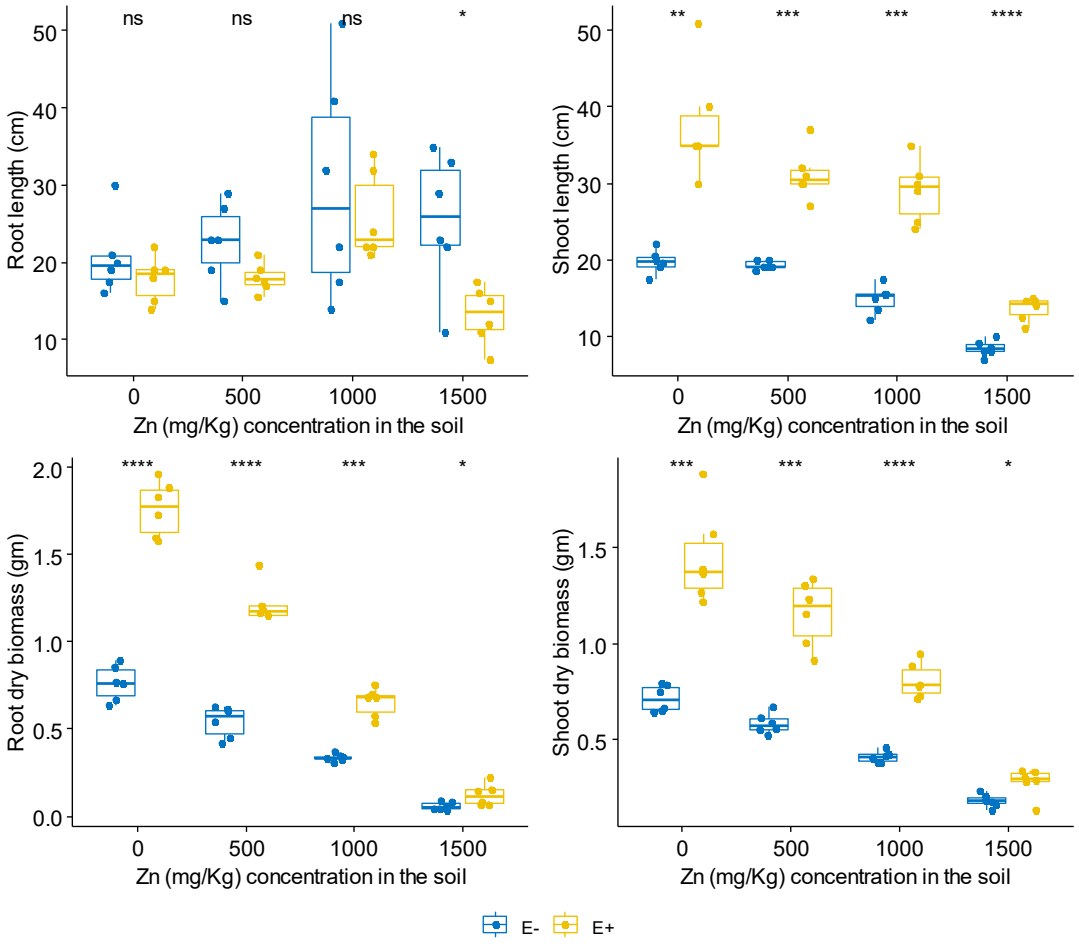

FIGURE 1
The effect of FXZ2 on the growth of *Dysphania ambrosioides* under Zn stress (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.00005$, ns $p > 0.05$ *t*-test).

Both bacterial and fungal endophytes have been linked to the improved plant growth-related characteristics of the host plants under metal stress (Bilal et al., 2018; Zhu et al., 2018; Shahid et al., 2019; Rattanapolsan et al., 2021; Hussain et al., 2022).

The effect of FXZ2 on *Dysphania ambrosioides* Zn/Cd accumulation

The uptake and accumulation of Zn/Cd in the shoots and roots of E+ and E- are shown in **Table 1**. Generally, the Zn concentrations in E+ and E- plants differed from the Zn concentration in the soil (**Table 1**). At 0 mg kg^{-1} Zn stress, the Zn content in the shoots of E+ plants was significantly ($p < 0.05$) high than that of E- plants, however, this was only slightly more ($p > 0.05$) in the roots of E+ plants. Contrary to this, at 500 and 1,000 mg kg^{-1} Zn stress, the shoot Zn content in E+ plants was significantly ($p < 0.05$) lower than that in E- plants, while only slightly more ($p > 0.05$) in E+ plants at 1,500 mg kg^{-1} Zn treatment. Similarly, the root Zn content was more ($p > 0.05$) in the E- plants than E+ plants at 500 and 1,000 mg kg^{-1} Zn treatment, while less ($p > 0.05$) in E- plants at 0 and 1,500 mg kg^{-1} Zn treatments.

The results suggest that the effect of FXZ2 on Zn uptake and accumulation was variable with the Zn content in the soil. Bilal et al. (2018) reported that the consortia endophytic microbes decreased Al and Zn content in the shoots and roots of *Glycine max* L. under 2.5 mM Al and Zn stress. Garg and Singh (2018) found that *Rhizophagus irregularis* combined with silicon amended soil and individually also decreased leaves and roots Zn content under Zn stress (600 and 1,000 mg kg^{-1}). While the other studies showed different results; for example, the endophytic bacterium *Sphingomonas* sp. increased Zn uptake in *Sedum alfredii* (Chen et al., 2014). Similarly, dark septate endophyte *Exophiala pisciphila* increased Pb,

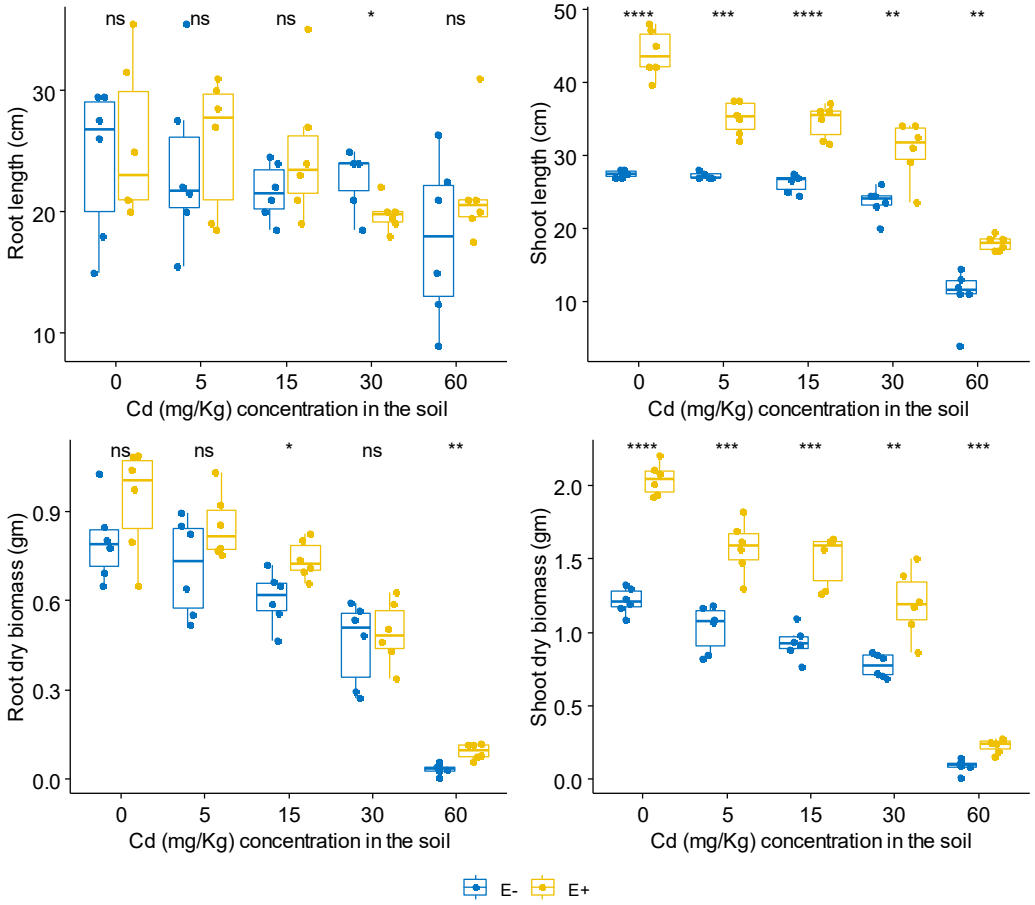

FIGURE 2
The effect of FXZ2 on the growth of *Dysphania ambrosioides* under Cd stress (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.00005$, ns $p > 0.05$ *t*-test).

Zn, and Cd content in the roots and decreased in the shoots of *Zea mays* L. (Li et al., 2011); rhizobacterium *Enterobacter ludwigii* increased the Zn content in wheat under metal stress (Singh et al., 2018). This indicates that different microbes have different effects on host plant metal accumulation. Therefore, artificial manipulation of these microbes can be exploited to achieve the desired beneficial response.

At 0 mg kg^{-1} Cd stress, the Cd content was more ($p > 0.05$) in the shoots and roots of E- than E+ plants. However, the shoot and root Cd contents were higher in E+ plants at all Cd treatments than those in E- plants. The difference was significant ($p < 0.05$) at 60 mg kg^{-1} Cd stress, while the difference was non-significant ($p > 0.05$) at 5, 15, and 30 mg kg^{-1} Cd (**Figures 1**, **2** and **Table 1**). FXZ2-induced Cd content increase in the shoots and roots was consistent with other studies (Ren et al., 2006; Soleimani et al., 2010; Wan et al., 2012; Deng et al., 2013; He et al., 2013). Besides, plant growth-promoting bacteria such as *Rhizobium sullae* and *Pseudomonas* sp. (Chiboub et al., 2019), arbuscular mycorrhizal fungi (Berthelot et al., 2018; Rafique et al., 2019), and arbuscular mycorrhiza and silicon amended soil in combination as well as alone (Garg and Singh, 2018) were also found to increase Cd accumulation in host plants. However, the finding was opposite to some previous studies that reported relatively lower Cd content in the roots and shoots and roots of the endophyte inoculated plants under Cd stress (Wang et al., 2016; He et al., 2017; Zhan et al., 2017; Shahid et al., 2019). Nevertheless, it is interesting to note that in both cases, growth-promoting endophyte inoculation has potential applications: If the endophyte can increase metal accumulation in host plants, it can be potentially used in phytoextraction. On the other hand, if the endophyte can decrease metal accumulation in host plants, it can be potentially used to reduce the metal content of agriculturally important crops to safe levels of consumption. Generally, metal contents in plant samples depend on the bioavailability of metals in soil (Kim et al., 2015), but this study provides sufficient evidence that endophytes can affect metal accumulation and growth under metal stress (**Figures 1**, **2** and **Table 1**).

TABLE 1 Zn/Cd accumulation in the shoots and roots of FXZ2 inoculated plants (E+) and non-inoculated plants (E-).

The original concentration of Zn/Cd in the soils (mg kg^{-1})		The treatment of FXZ2	The concentration of Zn/Cd in the plants (mg kg^{-1})*	
			Shoots	Roots
Zn	0	E-	253.83 ± 4.83a	142.03 ± 19.15a
		E+	500.55 ± 69.23b	197.67 ± 110.19a
	500	E-	2,113.00 ± 113.86d	660.50 ± 65.30ab
		E+	1,850.00 ± 36.72c	591.20 ± 17.41ab
	1,000	E-	2,646.33 ± 79.10e	1,766.33 ± 453.99c
		E+	2,184.67 ± 190.17d	1,281.33 ± 90.98bc
	1,500	E-	3,180.33 ± 48.79f	3,074.33 ± 947.53d
		E+	3,370.33 ± 236.05f	3,576.33 ± 345.30d
Cd	0	E-	0.16 ± 0.08a	0.32 ± 0.11a
		E+	0.14 ± 0.02a	0.27 ± 0.13a
	5	E-	5.10 ± 0.07ab	23.75 ± 1.56ab
		E+	8.39 ± 1.30ab	24.91 ± 8.18ab
	15	E-	12.28 ± 0.98abc	43.23 ± 6.63ab
		E+	16.97 ± 0.88bc	59.50 ± 6.73ab
	30	E-	23.48 ± 0.75cd	97.28 ± 24.17bc
		E+	34.48 ± 3.98d	167.08 ± 24.79c
	60	E-	91.09 ± 14.61e	445.72 ± 125.30d
		E+	120.79 ± 16.51f	739.48 ± 38.62e

*The values are Mean ± Std, $n = 3$; The different letters indicate the significant difference ($p < 0.05$, one-way ANOVA, Duncan test) between the individual plant part and metal in the different treatments.

The effect of FXZ2 on Zn/Cd speciation in rhizospheric soils

Zinc and Cd chemical speciation in rhizospheric soils of E+ and E- plants were shown in **Figure 3**. It was found that under Zn stress (500, 1,000, and 1,500 mg kg^{-1} Zn), most of Zn was in F1 (exchangeable fraction). Interestingly, at 500 and 1,000 mg kg^{-1} Zn stress, the Zn content of F1 + F2 was relatively less in rhizospheric soils of E+ than E- plants, while it was rather more in E+ plants in the 1,500 mg kg^{-1} Zn treatments. This can be correlated to the Zn concentration in the shoots and roots of E+ and E- plants in 500, 1,000, and 1,500 mg kg^{-1} Zn treatments. The metal in F4 (organic matter-bound fraction) and F5 (residual fraction) was the least available to plants. Together, these fractions were found relatively more in E+ than E- plants in 500 and 1,000 mg kg^{-1} Zn treatments, while it was relatively less in E+ plants in the 1,500 mg kg^{-1} Zn treatments. Results differed from previous studies, in which arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) inoculation increased soil Zn mobility by changing Zn to high available fractions from low available fractions (Boostani et al., 2016).

Under Cd stress, no definite trend was observed in the relative percentage of the different fractions, especially at the low Cd stress (5 and 15 mg kg^{-1} Cd), while under high Cd exposure (30 and 60 mg kg^{-1} Cd), F1 + F2 were higher in E+ than E- plants. Wang et al. (2016) also reported a difference in the chemical speciation of Cd in the dark septate endophyte inoculated maize. In another study, endophyte inoculation to *Brassica juncea* increased F1 + F2 fractions of Cd in the rhizosphere compared to the control plants (Wang et al., 2020). The possible mechanism of the distinct shift in the chemical speciation of an element in rhizospheric soils is by modifying pH through the secreted root exudates (Long et al., 2013). Endophyte inoculation could affect the subcellular fractions of Cd in the host plant and its chemical forms. For example, AMF colonization increases Cd accumulation in *Medicago sativa* L. by changing Cd into inactive forms, having low toxicity (Wang et al., 2012). Similar AMF colonization affected Cd uptake and subcellular distribution by changing Cd chemical speciation in rice (Li H. et al., 2016; Luo et al., 2017). Besides, the observed results of Zn and Cd speciation might affect the anions and pH from $ZnSO_4.7H_2O$ and $CdCl_2{\cdot}2.5H_2O$ supplemented to induce Zn and Cd stress, respectively (Wang et al., 2016).

FXZ2 inoculation affected the chemical speciation in root zone soils of *D. ambrosioides* only to some extent. The effect of FXZ2 inoculation (FE) was variable for the different fractions of Zn and Cd in rhizospheric soils (**Figure 3**). The effect was not significant for all fractions of Zn in the different treatments, while in the case of Cd, there were six significant alterations out of a total 25 alterations by FXZ2

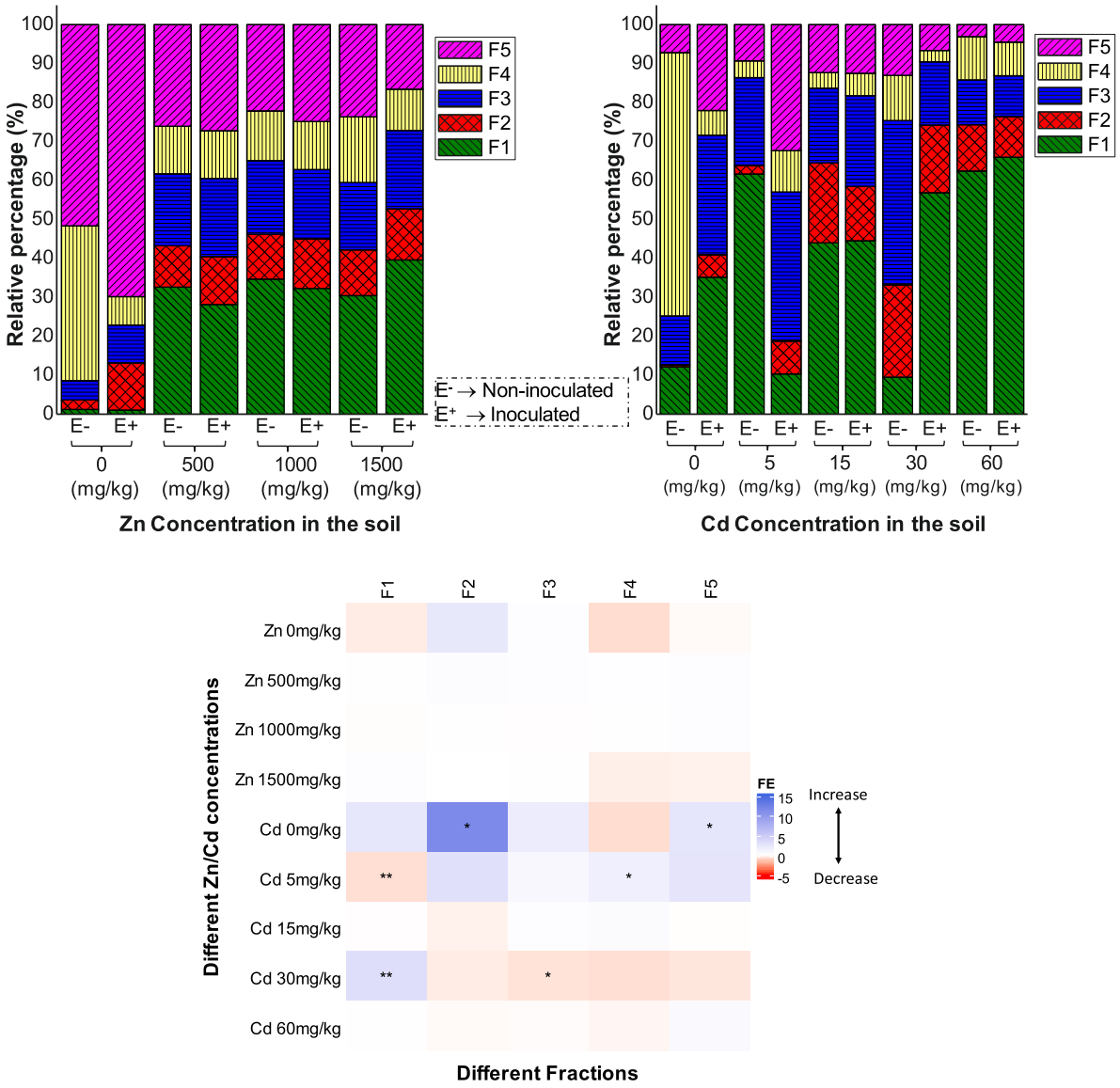

FIGURE 3
The effect of FXZ2 inoculation (FE) on the chemical forms of Zn/Cd in rhizospheric soils of *Dysphania ambrosioides* under Zn/Cd stress. F1: exchangeable fraction; F2: carbonate-bound fraction; F3: Fe-Mn oxides bound; F4: organic bound fraction; F5: residual fraction. The asterisks indicate a significant difference between F^{E+} and F^{E-} (*$p < 0.05$, **$p < 0.005$, *t*-test).

inoculation. Chemical speciation in the rhizosphere regulates toxicokinetics, i.e., the uptake and translocation of metals by the plants from the root zone (Uchimiya et al., 2020). The manipulation of the phytomicrobiome can change the rhizosphere by the secretion of root exudates, which can alter the microbial signaling compounds and chemical speciation (Bhatt et al., 2020). It has to be noted that in this study, we evaluated the chemical speciation in the rhizosphere soil only at the time of harvest (60 days). It would be interesting to evaluate how the chemical speciation of metals changes in the rhizosphere when the plant is inoculated with FXZ2 during different time intervals as the plant grows in metal stress conditions and further how it affects the rhizosphere microbial community.

The effect of FXZ2 on biochemical factors of *Dysphania ambrosioides*

FXZ2 inoculation had a positive effect on the total chlorophyll content of host plants (**Figure 4**). With the exception of 1,500 mg kg^{-1}, E+ plants had a relatively higher total chlorophyll content in Zn treatments than E- plants. The differences were significant at 0 and 1,500 mg kg^{-1} Zn

while non-significant ($p > 0.05$) at 1,000 mg kg^{-1} Zn. In Cd treatments, FXZ2 colonization significantly ($p < 0.05$) increased the total chlorophyll content of the host plants except at 30 mg kg^{-1} Cd stress ($p > 0.05$). With the increase of Zn and Cd concentration in the soil, the total chlorophyll content was decreased both for E+ and E- plants. The chlorophyll content is a significant indicator of plant growth status (Chen et al., 2010). Exceptionally high Zn in the soil can cause stress in plants, leaf chlorosis, and reduce photosynthesis (Broadley et al., 2007). Moreover, Cd-induced toxicity can adversely affect the plant chlorophyll biosynthesis by preventing δ-aminolevulinic acid dehydratase, porphobilinogen deaminase, and protochlorophyllide reductase activity and changing the photosynthetic electron transport at PS-II (Zulfiqar et al., 2021).

Our results supported the finding that the chlorophyll content decreased for the toxicity of Zn or Cd (Zhang et al., 2010; Kamran et al., 2015; Bilal et al., 2018). However, the chlorophyll of E+ plants was relatively higher than that of E- plants. The results agree with Hunt et al. (2005), who recorded that endophyte inoculation to perennial ryegrass increased chlorophyll content. Bilal et al. (2018) also reported that endophytic microbial consortia could significantly enhance the chlorophyll content of the inoculated plants under normal and Al/Zn stress. The low chlorophyll content under the influence of abiotic stress is generally due to the stress-related ROS generation and membrane lipid peroxidation, which further affects the fluidity and selectivity of the membrane (Verma and Mishra, 2005). Furthermore, in plant tissue metal stress results in the generation of ROS, which in the form of hydrogen peroxide and superoxide anion mimic and interrupt normal cellular functions by changing the oxidation/reduction cycle (Khan et al., 2015).

Tripeptide glutathione is one of the crucial plant metabolites having an essential role in the plant defense system as a ROS scavenging molecule. In plants, it occurs mainly in reduced form (GSH), and abundant production in the stress-adapted plant is related to a strongly activated defense system (Gill and Tuteja, 2010). The GSH analysis showed that FXZ2 inoculation affected the GSH content of host plants (**Figure 4**). In general, the GSH content of E+ plants was higher than E- plants under both Zn and Cd stress. The differences were significant ($p < 0.05$) at 500 and 1,000 mg kg^{-1} Zn stress, while under Cd stress, the difference was non-significant ($p > 0.05$). The thiol group of the glutathione is of high-affinity nature, linked to the complexation and detoxification of metals as a chelating compound, and takes part in the antioxidant process (Schat et al., 2002; Yadav, 2010; Cao et al., 2018). Further, it reduces phytotoxicity by forming an inactive glutathione-Cd complex and subcellular compartmentalization (Adamis et al., 2004; Zhang et al., 2019). The higher GSH content in E+ than E- plants suggests the inoculated endophyte induced counteractive mechanisms to check oxidative stress related to metal toxicity. Previous studies also indicated that inoculation of endophytic microbe can enhance the growth and tolerance of host plants to metal stress through GSH regulation, though the effect on GSH can vary with stress (Khan et al., 2015; He et al., 2017; Zhan et al., 2017).

Metal stress induces oxidative damage in plants, causing lipid peroxidation that disturbs cellular functions and membrane integrity; the injuries can be irreversible (Wan et al., 2012; Khan and Lee, 2013; Khan et al., 2015; Bilal et al., 2018). Malondialdehyde (MDA) is a byproduct of lipid peroxide breakdown. Lower MDA in plant tissue signifies lesser lipid peroxidation. The MDA content of different treatments is presented in **Figure 4**. It was found that FXZ2 inoculation lowered the MDA content of host plants. The differences were significant ($p < 0.05$) at 500 and 1,500 mg kg^{-1} Zn stress and higher Cd stress (30 and 60 mg kg^{-1}). The relatively lower MDA in E+ plants suggests that the endophyte FXZ2 had a synergistic role against the oxidative stress due to elevated Zn and Cd. Results from this study are consistent with previous research that endophyte-infected plants had lower MDA contents, for instance, *Achnatherum inebrians* inoculated with endophyte *Neotyphodium gansuense*, and *Solanum nigrum* inoculated with endophyte *Serratia nematodiphila* under Cd stress (Zhang et al., 2010; Wan et al., 2012; Khan et al., 2015), *Glycine max* L. inoculated with endophytic fungus *Paecilomyces formosus* and bacteria *Sphingomonas* sp. under Al/Zn stress (Bilal et al., 2018), and tomato inoculated with two dark septate endophytes *Phialophora mustea* under Zn/Cd stress (Zhu et al., 2018).

Phytohormone production by FXZ2

Phytohormone indole acetic acid (IAA) is responsible for apical dominance, cell elongation, evolution of vascular tissue, and improvement of plant stress tolerance (Wang et al., 2001; Eyidogan et al., 2012). And gibberellic acid (GA) is primarily responsible for seed germination, stem elongation, flower and trichome initiation, fruit development, and leaf expansion (Yamaguchi, 2008; Liu et al., 2009). Jasmonic acid (JA) has been demonstrated as a significant signaling molecule during plant defense, such as pathogens attack (Qi et al., 2016) and metals stress (Bilal et al., 2017; Per et al., 2018). JA was also reported to alter antioxidant potential, reduce H_2O_2 and MDA concentrations, and improve photosynthetic pigments concentrations under Pb and Cd stress in different plants (Piotrowska et al., 2009; Ahmad et al., 2017). Some endophytes can exogenously produce phytohormones to mitigate the effects of abiotic stress to host plants (Khan et al., 2012; Bilal et al., 2018; Chang et al., 2021). In the present study, it was found that FXZ2 exogenously secretes IAA (3.21 ± 0.59 μM L^{-1}), GA (13.76 ± 0.20 pM L^{-1}), and JA (257.70 ± 43.04 pM L^{-1}) in liquid culture. These phytohormones may play some

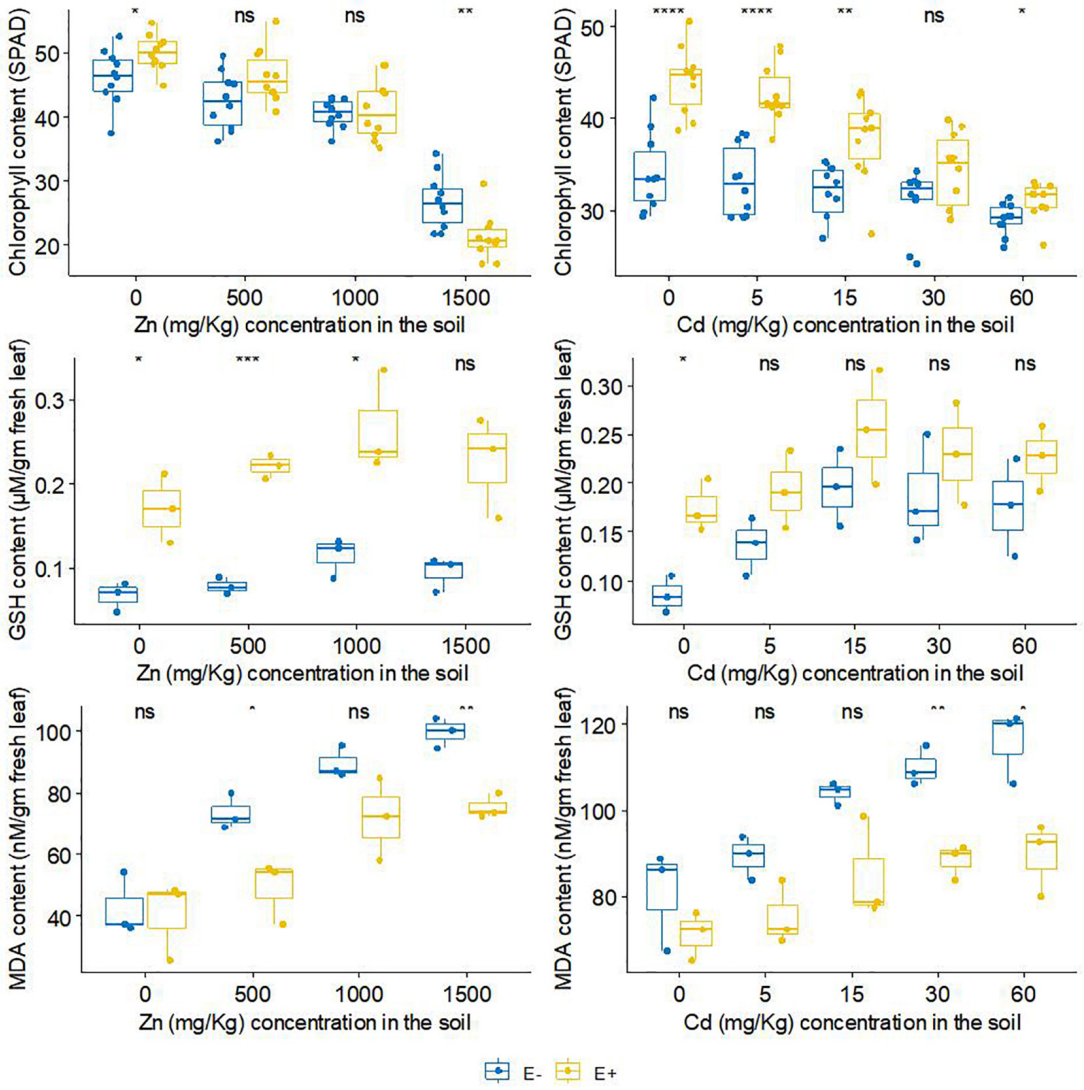

FIGURE 4
The effect of FXZ2 on the Chlorophyll, GSH and MDA content of *Dysphania ambrosioides* under Zn/Cd stress (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.00005$, ns $p > 0.05$ *t*-test).

roles in plant growth and stress tolerance under Zn/Cd stress. Similarly, some phytohormones producing fungal species, e.g., *Fusarium oxysporum*, *Piriformospora indica*, *Phoma glomerata*, *Penicillium* sp., and *Exophiala pisciphila,* have found to improve host plants' growth and crop productivity (Hasan, 2002; Yuan et al., 2010; Waqas et al., 2012; He et al., 2017). Further, the effect of FXZ2 on the endogenous production of phytohormones and host plants growth under metal stress can be tested on mutant plant cultivars not able to produce phytohormones, e.g., Waito-C (GA deficient mutant rice cultivar) (Khan et al., 2012). This can be a reliable future strategy to know how this endophyte improves the phytohormone content of the host plant and subsequently their growth under metal stress.

Conclusion

Under variable Zn/Cd stress, seed endophyte FXZ2 significantly improved *D. ambrosioides* growth and its chlorophyll and GSH content. Our results demonstrated that FXZ2 inoculation transformed the Zn/Cd speciation in the rhizosphere of host plants, subsequently affecting their uptake and accumulation. The readily available fractions, i.e., exchangeable and carbonate-bound (F1 + F2) fractions of Zn decreased in E+ as compared to E- plants at 500 and 1,000 mg kg^{-1} Zn stress, congruently, Zn in shoots of E+ plants decreased significantly ($p < 0.05$). However, under Cd stress (30 and 60 mg kg^{-1}), the effect was different, the Cd concentration in F1 + F2 increased in rhizospheric soils of E+ plants, and

subsequently, Cd accumulation in E+ plants was significantly ($p < 0.05$) increased. Therefore, FXZ2 can have different applications, for example, in agriculturally important crops it can be used to improve Zn tolerance in contaminated soils or in phytoextraction by increasing Cd bioaccumulation at high Cd stress.

Moreover, FXZ2 could exogenously secrete phytohormones IAA, GA, and JA, which could be a key mechanism for promoting host plants' growth under Zn/Cd stress. Further study is required to investigate the role of FXZ2 in the endogenous production of phytohormones in inoculated plants.

Data availability statement

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

Author contributions

VS: conceptualization, methodology, writing—original draft, investigation, formal analysis, and data curation. SP: conceptualization, methodology, writing—original draft, investigation, formal analysis, and data curation. WT: project investigation and resources and project administration. HH: writing—review and editing and validation. JW: writing—review and editing. HL: conceptualization, supervision, writing—review and editing, validation, resources, funding acquisition, and project administration. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the National Natural Science Foundation of China (41867026) and the Natural Science Foundation of Yunnan Province (2019FA019). Funding support was also provided to James White from USDA-NIFA Multistate Project W4147 and the New Jersey Agricultural Experiment Station.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 07 October 2022
DOI 10.3389/fbioe.2022.998298

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Bioprocess Engineering, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 19 July 2022
ACCEPTED 23 September 2022
PUBLISHED 07 October 2022

CITATION
Xu D, Liu B, Wang J and Zhang Z (2022), Bibliometric analysis of artificial intelligence for biotechnology and applied microbiology: Exploring research hotspots and frontiers. *Front. Bioeng. Biotechnol.* 10:998298. doi: 10.3389/fbioe.2022.998298

Bibliometric analysis of artificial intelligence for biotechnology and applied microbiology: Exploring research hotspots and frontiers

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Background: In the biotechnology and applied microbiology sectors, artificial intelligence (AI) has been extensively used in disease diagnostics, drug research and development, functional genomics, biomarker recognition, and medical imaging diagnostics. In our study, from 2000 to 2021, science publications focusing on AI in biotechnology were reviewed, and quantitative, qualitative, and modeling analyses were performed.

Methods: On 6 May 2022, the Web of Science Core Collection (WoSCC) was screened for AI applications in biotechnology and applied microbiology; 3,529 studies were identified between 2000 and 2022, and analyzed. The following information was collected: publication, country or region, references, knowledgebase, institution, keywords, journal name, and research hotspots, and examined using VOSviewer and CiteSpace V bibliometric platforms.

Results: We showed that 128 countries published articles related to AI in biotechnology and applied microbiology; the United States had the most publications. In addition, 584 global institutions contributed to publications, with the Chinese Academy of Science publishing the most. Reference clusters from studies were categorized into ten headings: deep learning, prediction, support vector machines (SVM), object detection, feature representation, synthetic biology, amyloid, human microRNA precursors, systems biology, and single cell RNA-Sequencing. Research frontier keywords were represented by microRNA (2012–2020) and protein-protein interactions (PPIs) (2012–2020).

Abbreviations: AI, Artificial Intelligence; WoSCC, Web of Science Core Collection; CPI, Compound-Protein Interactions; ACPred-FL, Anti-Cancer Peptide Predictor with Feature Representation Learning; ACPs, Anti-cancer peptides; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; AD, Alzheimer's Disease; CNN, Convolutional Neural Network; Aβ, amyloid-β; RNN, Recurrent Neural Network; RNA, Ribonucleic Acid; DNN, Deep Neural Networks; DISC, Deep learning Imputation model with semi-supervised learning for Single Cell transcriptomes; PPI, Protein-Protein Interaction; DPPI, Direct physical Protein-Protein Interactions; SVM, Support Vector Machine.

Conclusion: We systematically, objectively, and comprehensively analyzed AI-related biotechnology and applied microbiology literature, and additionally, identified current hot spots and future trends in this area. Our review provides researchers with a comprehensive overview of the dynamic evolution of AI in biotechnology and applied microbiology and identifies future key research areas.

KEYWORDS

artificial intelligence, biotechnology, bibliometric, deep learning, machine learning, applied microbiology

Introduction

Since the beginning of the 21st century, the life sciences and biotechnology and applied microbiology sectors have exemplified mankind's technological and revolutionary evolution. In these sectors, among the top 10 scientific breakthroughs published by science journals in recent decades, more than half of research outputs were revolutionarily innovative and breakthrough in nature. Emerging biological sectors include, biomedicine, bio-based chemicals, bioenergy, and genetically modified crop technology (Celikkanat Ozan and Baran, 2013). These areas are cutting-edge, and next-generation biotechnology industries are anticipated to develop rapidly in the future (Lee, 2016). As the front end of these biological industries and value chains, biotechnology and applied microbiology research has adopted a leading position in these industries. Therefore, exploring rapid developments and hot trends in basic biotechnology and applied microbiology research is pivotal in guiding biotechnology current achievements and developing new, downstream bio-industry markets.

AI represents advanced computer technology, and is a highly complex system integrating mathematics, statistics, probability, logic, ethics, and other disciplines. It primarily includes deep learning, machine learning, convolution and recurrent neural networks (CNN and RNN, respectively), full revolutionary networks (FCNs), and other specific methods. AI is extensively used in different industries, in particular biotechnology and the life sciences. In recent years, several major research developments have been achieved, including the AI-mediated prediction of protein structure, which was breakthrough of the year in 2021 (Baek et al., 2021). By exploiting complex simulation algorithms, AI has revolutionized disease diagnostics, drug research and development, functional genomics, biomarker recognition, and medical imaging diagnostics, and critically, has provided a vital reference point for disease diagnostic, prediction, and treatment strategies (Dlamini et al., 2020).

To facilitate AI research and progress in biotechnology and applied microbiology, bibliometric analyses and reviews are used to equip scientists with in-depth understandings of the application, its ongoing evolution, and future prospects. From a database search spanning 1 January 2000 to 31 December 2021, we used bibliometric methods to analyze scientific papers on AI applications in biotechnology and applied microbiology, including papers published in different jurisdictions and by institutions. We examined journals where AI biotechnological research studies were published, investigated the "top 10 cited studies", and enumerated how many times popular studies were cited. We clustered the reference network of cited studies, and investigated the subject knowledge base. Research hotspots were identified using burst keywords, which provided invaluable indicators for future research. Our research remit was to provide researchers with a macro understanding and micro analysis of the AI biotechnological field. When compared with traditional systematic reviews, we provided an intuitive, timely, and logical framework to track biotechnological developments and explore specific knowledge areas.

Methods

On 6 January 2022, we used the Web of Science Core Collection (WoSCC) to download data (2000–2021), which were independently verified by DX and ZZ. The following search terms were used: ("deep learning" OR "machine learning" OR "convolutional neural network*" OR CNN* OR RNN OR "Recurrent neural network*" OR "Fully Convolutional Network*" OR FCN*). The Web of Science category was "Biotechnology Applied Microbiology", and documents were gathered. From studies, the following basic information was gathered: authors, abstract, title, institution, journal, keywords, country/region, and references. Studies indexed in the database were included, whereas the following were excluded: 1) book chapters, data papers, meeting abstracts and proceedings papers, repeated articles, and editorials, and 2) unpublished studies with limited data for analysis. In total, 79 duplicates were excluded. A study overview (search process and analyses) is provided (Figure 1).

We described publication traits, including country, institute, journals, and keywords. The H-index is an important indicator and was used to reflect the value of scientific research (Eyre-Walker and Stoletzki, 2013). The

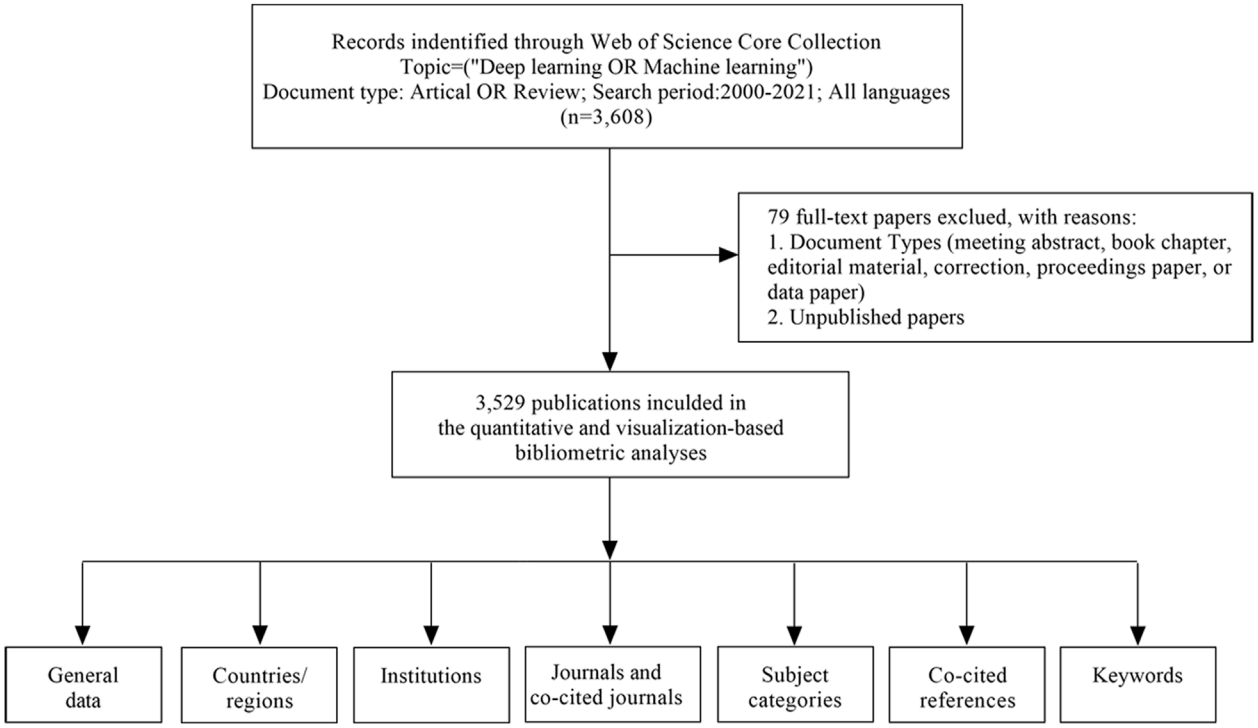

FIGURE 1
A frame flow diagram. The diagram showed details selection criteria for ABAM publications from WoSCC database and the steps of bibliometric analysis.

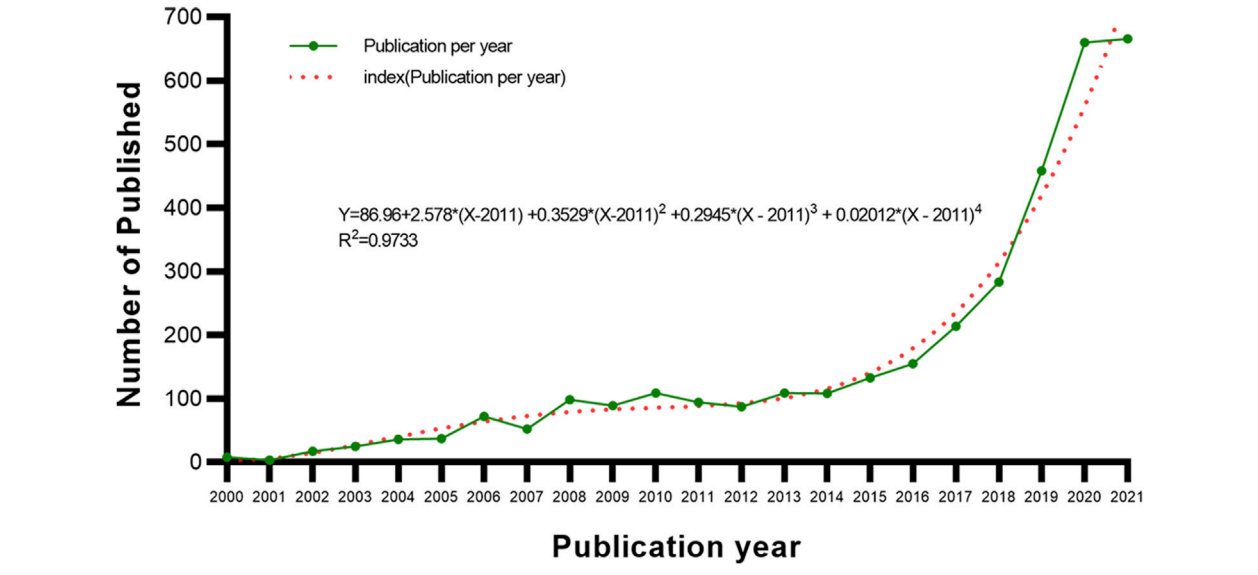

FIGURE 2
Trends in the number of publications on ABAM from 2000 to 2021.

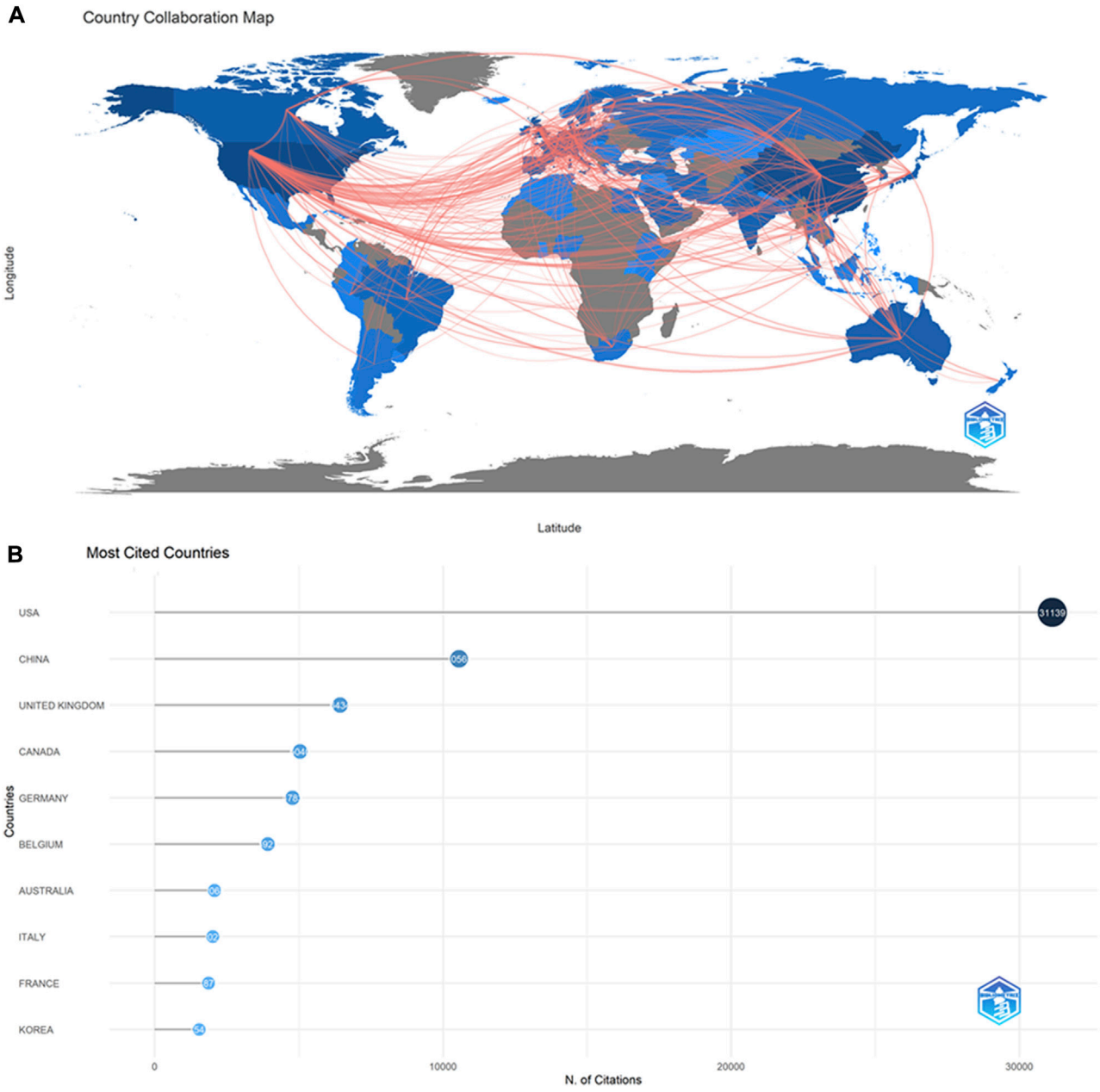

FIGURE 3
The cooperation of countries/regions contributed to publications. **(A)** Country Collaboration map. **(B)**Most Cited Countries.

Literature Metrology websites; http://bibliometric.com/, VOSviewer (Leiden University, Leiden, Netherlands), and CiteSpace V (Drexel University, Philadelphia, PA, United States) were used to visualize collaborative networks in institutes/countries/keywords/journals and co-occurrence analyses. In CiteSpace, we conducted reference co-citation analyses, constructed knowledge maps, and identified burst keywords to generate new recurrent keywords (Chen, 2006).

Results

Article distribution by publication year

The literature retrieval showed that the research on AI in this topic began in 2000. From 2000 to 2021, 3,529 papers were published, and AI with Biotechnology and Applied Microbiology (ABAM) related publication trends identified (Figure 2). Studies

TABLE 1 Top 10 countries/regions and relevant institutions.

Rank	Countries/regions	Count	Total citations	H-index	Institutions	Count	H-index
1	United States	1308	31139	91	CHINESE ACAD SCI	85	26
2	China	826	10561	61	STANFORD UNIV	52	32
3	Germany	258	4783	44	SHANGHAI JIAO TONG UNIV	46	17
4	United Kingdom	223	6434	49	UNIV CAMBRIDGE	37	16
5	Canada	158	5040	37	CARNEGIE MELLON UNIV	36	16
6	Australia	128	2068	27	TSINGHUA UNIV	32	18
7	Italy	121	2022	28	UNIV ELECT SCI & TECHNOL CHINA	31	16
8	Japan	121	1327	30	HARVARD UNIV	31	30
9	South Korea	120	1547	25	TIANJIN UNIV	30	17
10	France	99	1875	29	UNIV WASHINGTON	30	17

TABLE 2 Top 10 cited references on artificial intelligence for biotechnology and applied microbiology.

Rank	Source titles	Title of reference	Count	Interpretation of findings
1	NATURE	Deep learning.	203	This paper studied the back propagation algorithm of deep learning
2	J MACH LEARN RES	Dropout: a simple way to prevent neural networks from overfitting.	176	This study improved neural network performance in supervised learning tasks
3	NAT BIOTECHNOL	Predicting the sequence specificities of DNA-and RNA-binding proteins by deep learning.	151	This study used deep learning techniques to identify sequence specificities in DNA and RNA binding proteins
4	INT C LEARNING R	Adam: A method for stochastic optimization.	132	In this paper, a stochastic gradient descent optimization algorithm, based on the first derivative, was proposed for the first time. It was used for large data, sparse data processing, and super parameter easy adjustment.
5	J MACH LEARN RES	Scikit-learn: Machine learning, in python.	126	This article introduced scikit learning, a python module that integrated different contemporary machine learning algorithms
6	NAT METHODS	Predicting effects of noncoding variants with deep learning-based sequence model.	118	Based on deep learning, this study developed an algorithmic framework to identify functional effects from noncoding mutations
7	ACM T INTEL SYST TEC	LIBSVM: A library for support vector machines.	91	This study helped users apply support vector machine (SVM) to their applications.
8	COMMUN ACM	ImageNet classification with deep convolutional neural networks.	90	In this study, a large-scale deep CNN was used to classify 1.2 million high-resolution images
9	NATURE	An integrated encyclopedia of DNA elements in the human genome.	81	This study systematically mapped chromatin structure, transcription, transcription factor association, and histone modification regions.
10	SIGKDD EXPLORATIONS	The WEKA data mining software: an update.	79	The widely used, open source machine learning software Weka was introduced in this paper and allowed researchers access the latest technologies in machine learning.

in this area are increasing year on year, and suggest the establishment of an important research trend.

Institutes, countries, and regions

We observed that 128 countries/regions published ABAM studies: collaborations between countries (Figure 3) and the top 10 countries (Table 1) are outlined. The United States published the most studies (1308), then China (826), Germany (258), and the United Kingdom (223). Some countries, such as United States, China, Germany, and United Kingdom, showed high centrality (marked by dark blue), indicating that these countries likely played an important role in research of this topic and made great contributions.

We identified 584 institutes which contributed to ABAM publications; the top 10 are outlined (Table 1). Institutional collaborations are shown (Figure 4). The Chinese Academy of Sciences recorded the most publications (85), followed by the universities of Stanford (52), Shanghai Jiao Tong (46), and Cambridge (37).

Figure 4 emphasizes the close and complex cooperative relationship between different organizations. The VOSviewer platform can be used to analyze the centrality of organizations. The purple circle represents centrality, and the

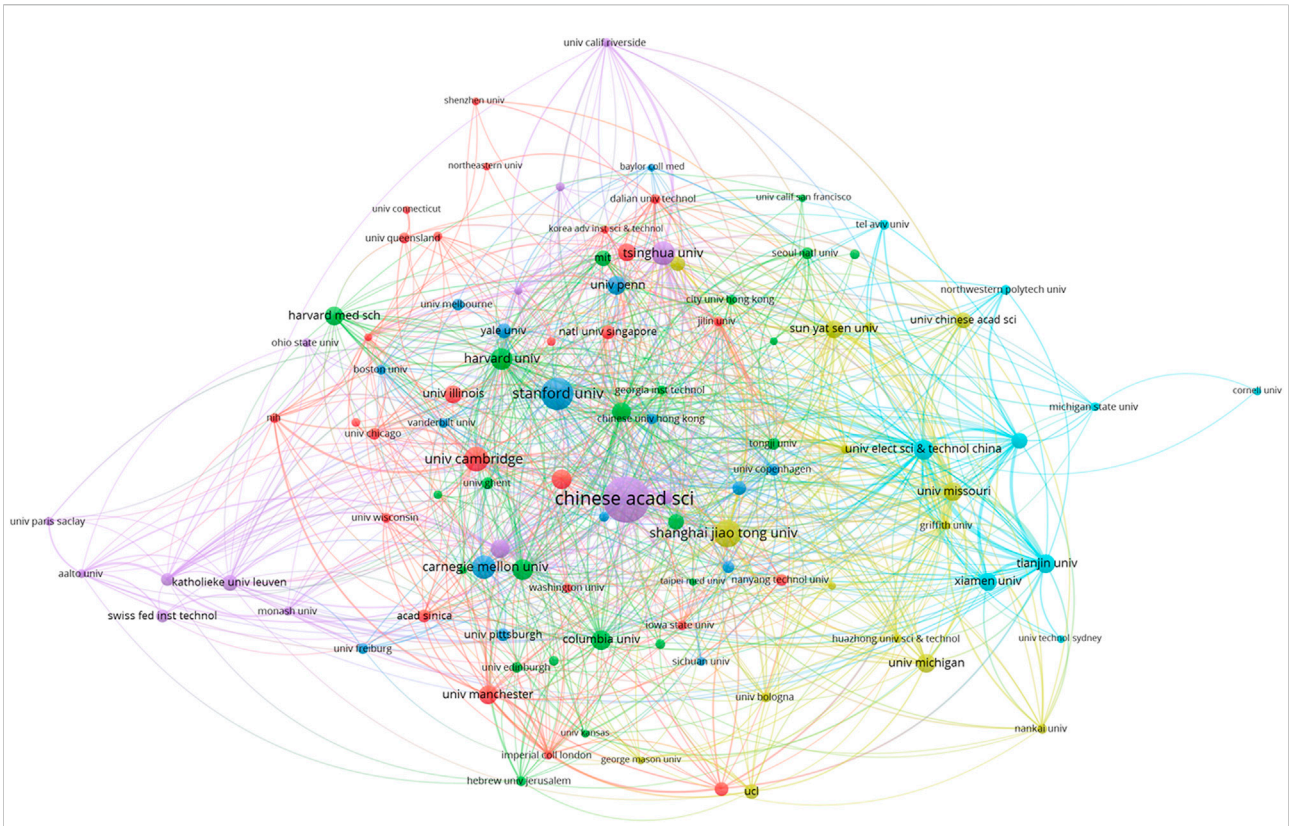

FIGURE 4
The cooperation of institutions contributed to publications.

TABLE 3 Highly link strength of the top 20 occurrence keywords.

Rank	Keyword	Occurrence	Total link strength	Rank	Keyword	Occurrence	Total link strength
1	Machine learning	782	420	11	Prediction	44	52
2	Deep learning	318	183	12	Biomarkers	39	49
3	Classification	82	95	13	Algorithms	36	40
4	Convolutional neural network	72	51	14	Bioinformatics	33	51
5	Artificial intelligence	60	61	15	Cancer	30	44
6	Random forest	57	58	16	Genomics	28	39
7	Support vector machine	56	41	17	Clustering	25	20
8	Feature selection	52	62	18	Data mining	25	29
9	Gene expression	50	61	19	Rna-seq	21	28
10	Neural networks	46	41	20	Natural language processing	19	16

area of the circle is proportional to the centrality. The Chinese Academy of Sciences and Stanford University are the most prominent organizations, showing that they conducted more research in this area.

Journals

In any research field, referential relationships between academic journals often reflect knowledge exchange, where

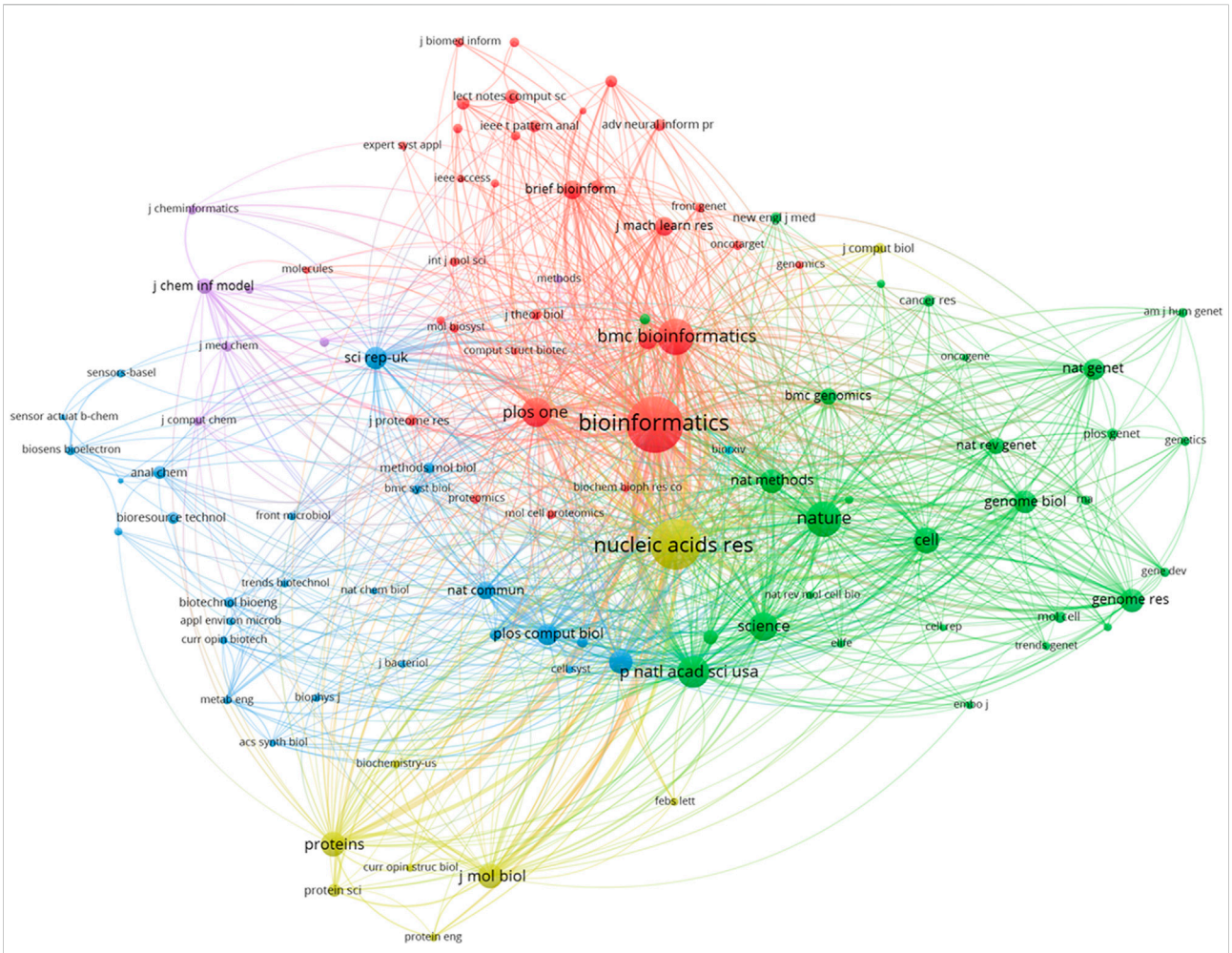

FIGURE 5
The network map of cited journals contributed to publications.

citing studies are knowledge frontiers, and referenced studies the knowledge basis. The top 10 references from studies (2000–2021) (Table 2) and collaborations between related journals (Figure 5) are outlined (Hall et al., 2009; Chang and Lin, 2011; Pedregosa et al., 2011; The ENCODE Project Consortium, 2012; Kingma and Ba, 2014; Srivastava et al., 2014; Alipanahi et al., 2015; LeCun et al., 2015; Zhou and Troyanskaya, 2015; Krizhevsky et al., 2017). Figure 5 shows that such journals as Bioinformatics, BMC Bioinformatics, Nature, Nucleic Acids Research, and PLoS One have higher centrality, and are the most popular journals for publishing research on this topic. The cooperative relationship between these journals is relatively balanced. This suggests that the research on the topic has aroused the interest of mainstream medicine and biology journals.

A dual-map overlay of journals (Figure 6) was used to show citing and cited journals on the left and right, respectively, while citation relationships were reflected by colored paths—these analyses showed that studies published in Genetics/Molecular/Biology journals were typically published in Biology/Molecular/Immunology journals.

References

Reference analysis is a vital bibliometric indicator; frequently cited studies typically and significantly influence their respective research fields. Using this approach, co-cited document-based clustering analyses generated connecting nodes and subfields for ABAM analyses.

We generated a co-citation reference network to measure the scientific relevance of related studies (Figure 7). Cluster setting parameters: top N% = 0.5 and # years per slice = 1. The Modularity Q score = 0.7135, which was > 0.5 and showed the network was reasonably separated into loosely coupled clusters. Weighted mean silhouette score = 0.9229, which was > 0.5, therefore cluster homogeneity was acceptable.

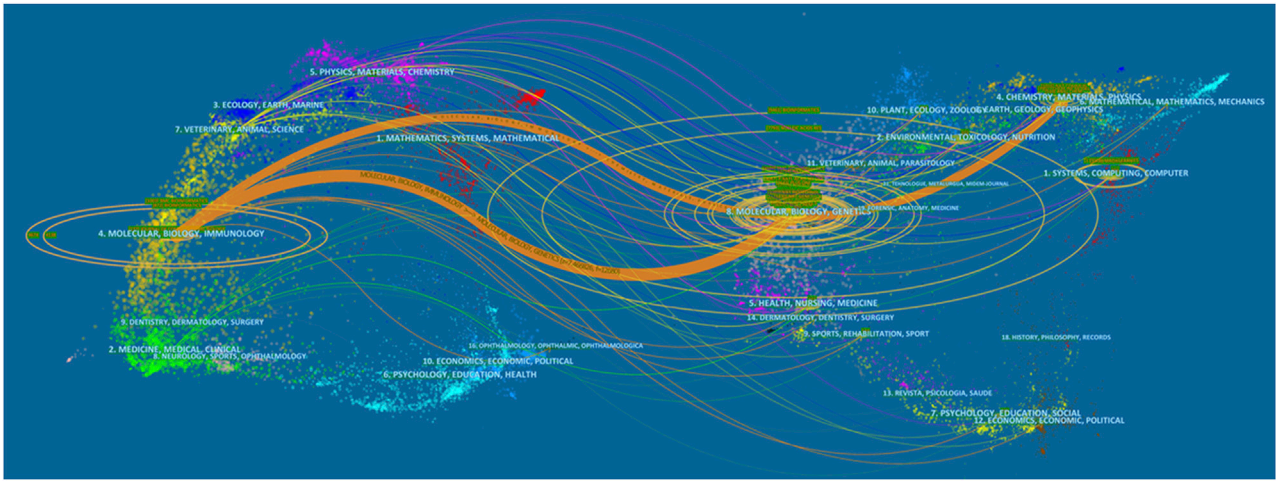

FIGURE 6
The dual-map overlay of journals contributed to publications.

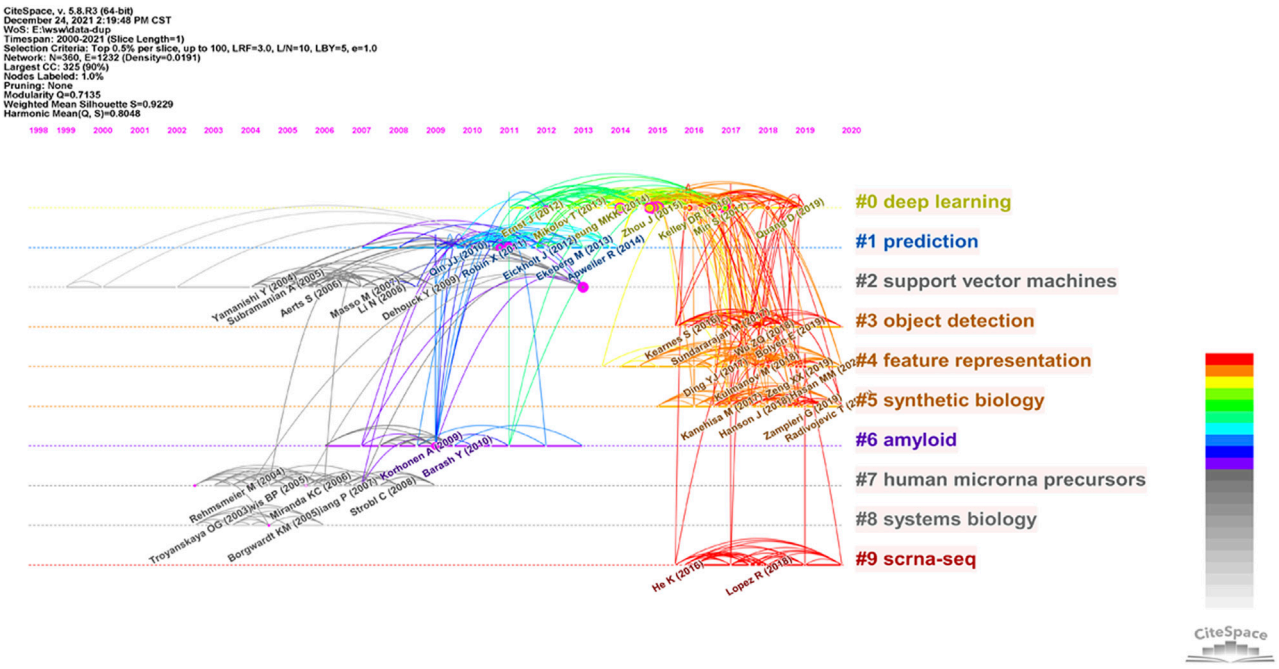

FIGURE 7
Reference co-citation map of publications on ABAM from 2000 to 2021.

Index items, as cluster markers, were extracted from studies. The largest cluster #0 was "deep learning" (Alipanahi et al., 2015), cluster #1 "prediction" (Kourou et al., 2015), cluster #2 "support vector machines (SVM)" (Furey et al., 2000), cluster #3 "object detection" (Hung et al., 2020), cluster #4 "feature representation" (Manavalan et al., 2019), cluster #5 "synthetic biology" (Wu et al., 2016), cluster #6 "amyloid" (Charoenkwan et al., 2021), cluster #7 "human microRNA precursors" (Wang et al., 2011), cluster #8 "systems biology" (Zou et al., 2015b), and cluster #9 "scRNA-Seq (single cell RNA-Sequencing)" (Arisdakessian et al., 2019).

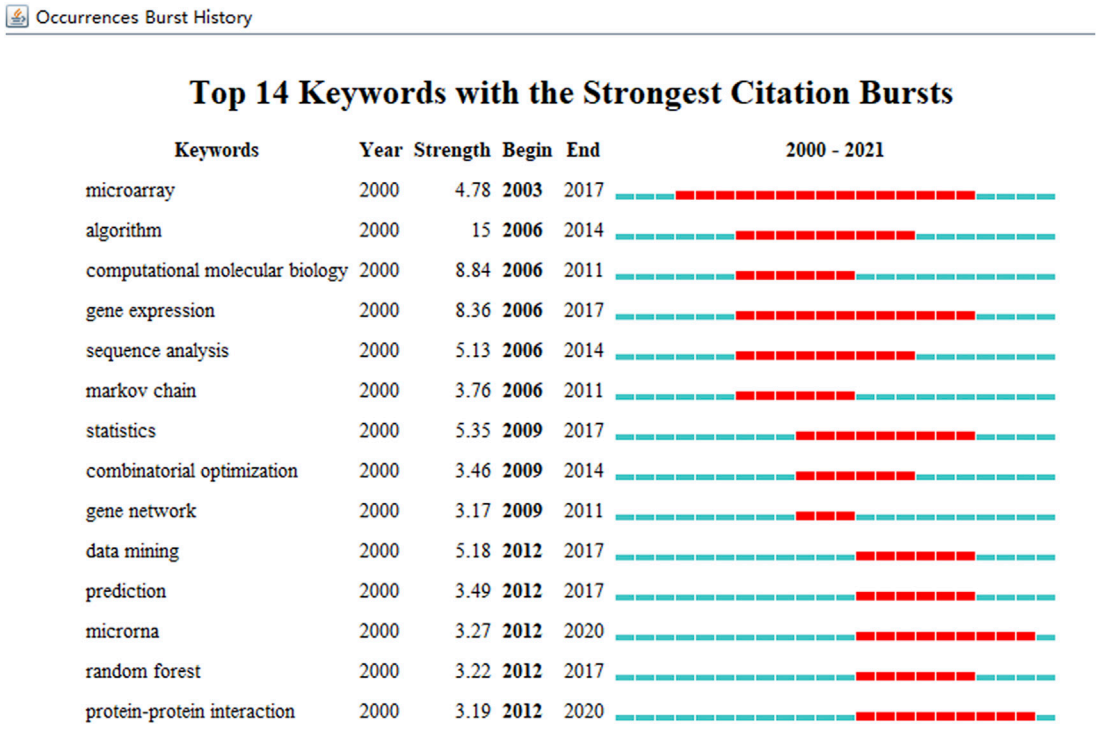

FIGURE 8
The keywords with the strongest citation bursts of publications on ABAM from 2000 to 2021.

Keywords

Analysis of keywords can provide a summary of the topics of each study and explore the hotpots and directions in this research area.

Keywords extracted from ABAM studies were processed, and the top 20 are given in Table 3. Temporal hotspot trend shifts, on the basis of the top 14 keywords with the strongest citation bursts, were analyzed and included the following. The burst keywords in 2006–2011 were computational molecular biology (2006–2011), Markov chain (2006–2011), and gene network (2009–2011). The burst keywords in 2006–2014 were algorithm (2006–2014), sequence analysis (2006–2014), and combinatorial optimization (2009–2014). The burst keywords in 2003–2017 were microarray (2003–2017), gene expression (2006–2017), statistics (2009–2017), data mining (2012–2017), prediction (2012–2017), and random forest (2012–2017). The current research hotspots are microRNA (2012–2020) and protein–protein interaction (2012–2020) (Figure 8).

Discussion

General data

In this study, 3,529 ABAM papers, confirming to search terms and inclusion/exclusion criteria, were published between 2000 and 2021. The United States published most studies (1308, 26.6%), with China second (826, 16.8%). China had five of the top 10 institutions, with four in the United States, and one in the United Kingdom. The journal in which most publications were published was Bioinformatics, which majorly contributed to ABAM research. Additionally, the top 10 cited studies were examined: the top study was cited 203 times and was published by LeCun et al. in NATURE (LeCun et al., 2015). The second rated study was cited 176 times and published by Srivastava et al. in J MACH LEARN RES (Srivastava et al., 2014).

Knowledge base

From previous studies, the application of deep learning related technologies to microbiology and biotechnology has been significant and generated many research achievements. As indicated (Figure 6), when we clustered co-cited references, key clustering nodes identified knowledge bases in this research field: #0 “deep learning”, #1 “prediction”, #2 “SVM”, #3 “object detection”, #4 “feature representation”, #5 “synthetic biology”, #6 “amyloid”, #7 “human microRNA precursors”, #8 “systems biology”, and

#9 "scRNA-Seq". Herein, we describe the knowledge bases according to different clusters.

In #0 "deep learning", a DeepBind software tool, based on deep learning, was developed by Alipanahi et al. (2015), and identified DNA and RNA binding protein sequence specificity. The tool was used to develop regulatory process models in biological systems and identify pathogenic variants. In other work, Beck et al. (2020) generated a deep learning-based, pre-trained, drug-target interaction model, Molecule Transformer-Drug Target Interaction, which identified commercially available drugs targeting SARS-CoV-2 proteins.

In #1 "prediction", Tsubaki et al. (2019) studied end-to-end representation learning of compounds and proteins, and developed a Compound-Protein Interactions (CPI) prediction strategy for virtual screening in drug discovery by combining protein convolution neural networks (CNN) and compound graph neural networks. In other work, Almagro Armenteros et al. (2017) developed a prediction algorithm based on deep neural networks which relied only on sequence information for protein subcellular localization.

In #2 "SVM", Ozer et al. (2020) showed that SVM provided solutions for high-throughput data analyses and contextualization; the approach rapidly determined timelines for invasive cancer diagnostics and treatment, and provided solutions for biomedical, bioengineering, and clinical applications. In other work, a SVM technology model constructed by Zhang et al. (2018) used joint information from multiple bone turnover markers, which improved diagnostic efficiency for osteoporosis, almost in perfect agreement with the dual-energy X-ray absorptiometry.

In #3 "object detection", the approach by Zhang et al. (2020), exploited a deep object detection technique and was used to study contacts between protein secondary structure elements, and predict tertiary structural protein topology. Einhäuser et al. (2017) developed a foveal object detector to detect eye movement, which significantly reduced metabolic costs and computational complexity, and provided insights on visual system evolution with eye movement.

In #4 "feature representation", an effective feature representation learning model ACPred-FL was developed by Wei et al. (2018), and used to rapidly and accurately identify new Anti-cancer peptides (ACPs)in many candidate proteins and peptides. The learning method developed by Peng et al. (2020) was based on feature representation learning and deep neural network (DTI-CNN), and was used to predict drug-target interactions and reduce time and experimental costs. In other research, from deep representation learning features with 107 dimensions, Lv et al. (2020) devised a sub-Golgi protein localization identification method, which exploited one feature type to accurately predict sub-Golgi protein localization.

#5 "synthetic biology" is a logical extension of recombinant technology or genetic engineering fields (Katz et al., 2018). Using integrated synthetic biology, Nguyen et al. (2021) developed a wearable face-mask, with a lyophilized CRISPR sensor, to non-invasively detect SARS-CoV-2 at room temperature within 90 min. Cubillos-Ruiz et al. (2021) proposed that synthetic biology could be used to program living cells with therapeutic functions; their cell-based therapeutic design is currently undergoing rapid development in medicine, and may provide effective treatment solutions for human diseases.

In #6 "amyloid", Charoenkwan et al. (2021) generated the first scorecard-based predictor for the accurate analysis, prediction, characterization, and identification of amyloid, on a large scale, to generate functional information for therapeutic intervention strategies. Cerebral amyloid-β (Aβ) is an Alzheimer's disease (AD) trait. Machine learning methods were used to identify cognitive performance and demographic variables for noninvasive testing of Aβ deposition, which can detect the effect of anti-amyloid drugs in the non-dementia population (Ko et al., 2019).

In #7 "human microRNA precursors", Zheng et al. (2020) used CNN and RNN approaches to automatically extract complex RNA sequence features to efficiently detect and predict human pre microRNAs. Kamenetzky et al. (2016) identified a novel pre-microRNA in the *Echinococcus multilocularis* genome using a machine learning approach, which could help control and prevent the global zoonotic infectious disease alveolar echinococcosis.

In #8 "systems biology", Reel et al. (2021) integrated different machine learning prediction algorithms to analyze different omics data to identify new biomarkers for systems biology. In their research, Weiskittel et al. (2021) outlined how systems biology algorithms layer machine learning and biological components could provide system-level analyses of single-cell omics data to clarify complex biological mechanisms. The powerful combination of systems biology, single cell omics, and machine learning could promote further, beneficial biomedical research.

In #9 "scRNA-Seq", in an unbiased manner in single cells, scRNA-Seq assesses functions in individual cells and cell-to-cell variability (Lin et al., 2020). Based on deep neural networks, Arisdakessian et al. (2019) formulated an interpolation algorithm Deepimpute based on DNN. Dropout layers and loss function were used to learn data patterns and to deal with gaps in scRNA-Seq data. He et al. (2020) developed DISC, a deep learning imputation model with semi-supervised learning for single cell transcriptomes. DISC can deduce gene expression and structures obscured by dropouts, enhanced gene and cell structures, recovered poor gene expression, and improved cell identification. Using machine learning methods (deep learning) combined with scRNA-Seq datasets, issues such as reducing dimensions, missing values, denoizing sc data, and explaining zero expansion, can be solved. Machine learning methods can be exploited to comprehensively process scRNA-Seq data, improve follow-up analyses in stem cells, identify cell subsets, and support regenerative medicine and cell therapy strategies (Yan et al., 2021).

Research frontiers and hotspots

Typically, keywords are used to concentrate on contemporary research concepts, while burst keywords represent research frontiers and emerging trends. CiteSpace was used to capture burst keywords, from which two research frontiers were identified: microRNA (2012–2020) and Protein-Protein Interaction (PPIs) (2012–2020). Importantly, we hypothesize these keywords exemplify future research frontiers.

MicroRNAs are noncoding single stranded RNAs that regulate development and gene transcription. Predicting and identifying connections between miRNAs and disease using AI-related methods is highly significant for unraveling pathogenic, preventative, prognostic, and pathological mechanisms implicated in diseases.

Zou et al. (2015a) predicted correlations between microRNAs and disease using two approaches: KATZ combined social network analysis and machine learning, while CATAPULT was a supervised machine learning method. Both were applied to 242 known associations between microRNAs and disease, and used 3-fold cross validation and leave-one-out cross-validation to evaluate method performance.

Wen et al. (2018) used the deep learning-based approach DeepMir Tar and extracted 750 features from a relatively large data set at different levels to predict human miRNA target sites. DeepMir Tar provided a new way to reveal miRNA biological function, as well as gene therapy and drug discovery for human diseases.

In large-scale RNA sequencing studies, Liu *et al.* developed a computational model called MirTarget which predicted genome-wide miRNA targets. Machine learning methods were used to train miRNA targeting feature data with miRNA binding and target down-regulation features, thus MirTarget showed better performances when compared with other algorithms (Liu and Wang, 2019).

Zheng et al. (2020) used CNN and RNN models to predict human pre-miRNAs; sequences were combined with predicted pre-miRNA secondary structures as input features to avoid feature extraction and selection processes by hand. Models were easily trained for handling training datasets; they demonstrated low generalization errors and were satisfactory for test datasets (Zheng et al., 2020).

Protein–protein interactions are very important in such cell life activities as transcriptional regulation, signal transduction, and drug signal transduction. Study of PPIs has become a research hotspot in bioinformatics. However, it is time-consuming and costly to identify PPIs using experimental methods (Chen et al., 2019).

People are more inclined to use artificial intelligence methods, like machine-learning, to automatically identify PPIs, which helps understanding of the molecular roots of disease on one hand, and provides new ideas for drug research and development on the other hand. Also, this effectively reduces experimental costs (Yu et al., 2021).

Based on a deep learning algorithm, Sun et al. (2017) designed a stacked autoencoder and investigated sequence-based PPIs predictions; the prediction accuracy of different external datasets was 87.99%–99.21%. These high-throughput methods increased our understanding of protein roles, disease etiology, and therapy design.

Hashemifar et al. (2018) developed a Direct Physical Protein-Protein Interactions (DPPI) deep learning framework, which modeled and predicted PPIs from sequence information. By adopting a deep, Siamese-like CNN which used high-quality experimental PPI data, evolutionary information from a predicted protein pair, and combined these data with random projection and data enhancement, PPIs were successfully predicted (Hashemifar et al., 2018).

Zeng et al. (2019) formulated DeepPPISP, a novel end-to-end deep learning framework. To examine local contextual features, authors used a sliding window to acquire neighbor features from target amino acids. To analyze global sequence features, a text CNN extracted features from protein sequences. To predict PPI sites, local contextual and global sequence characteristics were combined (Zeng et al., 2019).

Sequence-based deep learning technologies have been successfully used to predict PPIs. However, Yang et al. (2020) indicted these methods only focus on sequence information and ignore structural information in PPI networks. Such information, including degree, location, and adjacent nodes in graphs, are vital for PPI predictions. Theses authors generated a graph-based deep learning method for predicting PPIs, and demonstrated an accuracy of 99.15%, which improved on existing sequence-based methods (Yang et al., 2020).

In their method based on deep learning, Liu-Wei et al. (2021) developed deepviral, which predicted PPIs between humans and viruses. The method processed protein sequences and phenotypic characteristics to reveal infectious disease mechanisms and elucidate potential treatment methods (Liu-Wei et al., 2021).

Conclusion

We generated an objective, systematic, and comprehensive bibliometric analysis of scientific studies associated with deep learning, machine learning, CNN, RNN, and FCNs in ABAM. Moreover, we identified the research basis, future trends, and current hotspots in this field. Identified knowledge bases were: deep learning, prediction, SVMs, object detection, feature representation, synthetic biology, amyloid, human microRNA precursors, systems biology, and scRNA-Seq. Furthermore, microRNAs and PPIs were identified as future research frontiers and trends.

We identified some study limitations; publications over an extended period (2000–2021) were gathered, therefore, some

studies were incomplete and may have introduced publication bias into our research, potentially affecting analysis outcomes.

Author contributions

DX and ZZ conceived and designed the study; ZZ and DX wrote and integrated sections; BL and JW retrieved references. All authors read and approved the final manuscript.

Funding

Grants, supporting this study, were funded to Dongyu Xu (2021-AFCEC-529) from the Association of Fundamental Computing Education in Chinese Universities (AFCEC).

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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TYPE Original Research
PUBLISHED 17 October 2022
DOI 10.3389/fmicb.2022.1028001

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 25 August 2022
ACCEPTED 16 September 2022
PUBLISHED 17 October 2022

CITATION
Zhang Z, Wang Y, Wang S, Zhao L, Zhang B, Jia W, Zhai Z, Zhao L and Li Y (2022) Effects of antibacterial peptide-producing *Bacillus subtilis*, gallic acid, and cellulase on fermentation quality and bacterial community of whole-plant corn silage. *Front. Microbiol.* 13:1028001. doi: 10.3389/fmicb.2022.1028001

Effects of antibacterial peptide-producing *Bacillus subtilis*, gallic acid, and cellulase on fermentation quality and bacterial community of whole-plant corn silage

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In the current study, we assessed the effects of antibacterial peptide-producing *Bacillus subtilis* (BS), gallic acid (GA) and cellulase (CL) on the fermentation quality and bacterial community of various varieties of whole-plant corn silage. Three different varieties of whole-plant corn (Yuqing386, Enxiai298, and Nonghe35) were treated with 0.02% BS (fresh material basis), 0.2% GA (fresh material basis) and 0.02% CL (fresh material basis), after which 45days of anaerobic fermentation were conducted. With the exception of its low dry matter content, the results showed that Yuqing386's crude protein, water-soluble carbohydrate, and lactic acid contents were significantly higher than those of the other two corn varieties. However, its acid detergent fiber and cellulose contents were significantly lower than those of the other two corn varieties. Among the three corn variety silages, Yuqing386 had the highest relative abundance of *Lactobacillus* at the genus level and the biggest relative abundance of *Firmicutes* at the phylum level. In addition, the three additives markedly enhanced the quantity of dry matter and crude protein as compared to the control group. The application of GA considerably decreased the level of neutral detergent fiber while significantly increasing the content of lactic acid and water-soluble carbohydrates. Even though all additives enhanced the structure of the bacterial community following silage, the GA group experienced the greatest enhancement. On a phylum and genus level, the GA group contains the highest relative abundance of *Firmicutes* and *Lactobacillus*, respectively. Overall, of the three corn varieties, Yuqing386 provides the best silage qualities. GA has the biggest impact among the additions employed in this experiment to enhance the nutritional preservation and fermentation quality of whole-plant corn silage.

KEYWORDS

whole-plant corn silage, *Bacillus subtilis*, gallic acid, cellulases, fermentation quality, bacterial community

Introduction

The moist forages can be effectively preserved using silage. Silage is produced by the fermentation of lactic acid bacteria (LAB) in anaerobic conditions, which can increase feed palatability, extend storage period, and reduce nutrient loss (Ni et al., 2017). Lactic acid bacteria have the ability to transform water-soluble carbohydrates (WSC) into organic acids, primarily lactic acid, during the ensiling process, lowering the pH, and preventing the growth of harmful microorganisms (Li and Nishino, 2011). Silage has always been a significant source of roughage for ruminant diets.

The manufacture of silage has made extensive use of crop feeds, including whole-plant corn, alfalfa, natural forages and others (Dunière et al., 2013). Additionally, whole-plant corn (WPC), which has a high biological yield and a bounty of water-soluble carbohydrates, has grown to be the most extensively utilized silage material globally (McDonald et al., 1991). WPC silage quality is influenced by a range of factors, including as variety, harvest stage, additives, and so on (Nazli et al., 2018; Wang et al., 2019a). Variety is a key component among them. According to Ferraretto et al. (2015) research, feeding dairy cows various types of corn silage had an impact on their consumption of dry matter, milk production, and starch digestibility. Varying types of WPC silage have different nutritional values and economic advantages, according to research by Nazli et al. (2018). To manage the quality of WPC silage, it is crucial to choose high-quality corn varieties.

The competition between undesirable microorganisms and lactic acid bacteria during the ensiling process, which may be significantly increased by employing additives, is what determines the quality of WPC silage (Wang et al., 2019a). Numerous silage additives have been researched and used in the production of silage for many years in order to further enhance the nutritional value and fermentation quality of silage (Muck et al., 2018). Based on their biochemical characteristics, silage additives can be classified as inoculants, chemicals, or enzymes (Sun et al., 2010). These additives are crucial for enhancing the microbial community of silages, nutritional value, and fermentation properties. Previous investigations have demonstrated that some metabolites of *Bacillus subtilis* (BS) can have antibacterial properties, and it can produce lactic acid by reducing pyruvate in anaerobic environment (Gandra et al., 2017; Lanna-Filho et al., 2017). In addition, BS and its metabolites can significantly increase the amount of lactic acid bacteria and aerobic stability in corn silages when used as a silage additive (Bonaldi et al., 2021). The natural organic acid gallic acid (GA), also known as 3,4,5-trihydroxybenzoic acid, has three phenolic hydroxyl groups and one carboxyl group (Badhani et al., 2015). According to reports, GA has broad-spectrum antibacterial activities, which might means that it can disintegrate the structural integrity of bacteria or prevent the development of bacterial biofilms (Kang et al., 2008; Díaz-Gómez et al., 2013). Additionally, GA can prevent protein hydrolysis during the ensiling process, which is an advantage of its polyhydroxy structure, which enhances protein binding (Jayanegara et al., 2019). He et al. (2020) reported that adding gallic acid to high- moisture mulberry leaves and stylo silage can improved the fermentation quality and protein preservation. Wang et al. (2021) also found that adding gallic acid to whole plant soybean silage was an effective strategy to protect feed nutrition and improve silage quality. As a consequence, GA could be the potential silage additive. Cellulase (CL) are currently the most popular enzyme in silage. In silage, CL primarily breaks down plant cell walls, releases a significant quantity of soluble sugar, and supplies a enough substrate for lactic acid bacteria fermentation (Muck et al., 2018). The combined addition of CL and galactosidase to alfalfa silage has been shown to dramatically lower the amount of ammonia nitrogen while increasing the amount of lactic acid and the relative abundance of lactic acid bacteria (Hu et al., 2021).

Few studies, to our knowledge, have looked into the impact of antibacterial peptide-producing BS and GA, on WPC silage. As a result, three varieties of corn, Yuqing386 (YQ), Enxiai298 (EXA), and Nonghe35 (NH) were ensiled in the current study with three different types of silage additives (BS, GA, and CL), and their chemical compositions, fermentation traits, and bacterial communities were evaluated after 45 days of fermentation. The purpose was to evaluate the application effect of antibacterial peptide-producing BS and GA in WPC silage, at the same time, high-quality corn varieties were selected. We intend to offer a theoretical foundation and statistical backing for the actual use of WPC silage.

Materials and methods

Raw materials and silage preparation

At the experimental teaching base of the College of Animal Science and Technology, Henan University of Science and Technology (Monsoon climate of medium latitudes: 34°35′N, 112°24′E, elevation 140 m, annual mean temperature 12.2–24.6°C, and average annual precipitation 528–800 mm), three varieties of corn, YQ, EXA, and NH, were planted on June 28, 2020 and harvested on October 7, 2020. Within an hour, a forage cutter (zengguang9zp-0.4, Zengguang group Co. Ltd., Yongkang, China) chopped the collected WPC into pieces measuring 1–2 cm. Following that, the chopped WPC were immediately ensiled with: (1) No additive (CK); (2) 0.02% *Bacillus subtilis* CP7 (BS) of fresh material (FM; Zhangye Aolin Beier Biological Technology Co, Ltd., China); (3) 0.2% Gallic acid (GA) of FM (Shanghai Yuanye Biological Technology Co. Ltd., China); and (4) 0.02% Cellulase (CL) of FM (Shanghai Yuanye Biological Technology Co. Ltd., China). After fully mixed, put the sample into a unidirectional exhaust fermentation bag (23 cm × 30 cm; Chenguang Shiye Co. Ltd., Wenzhou, China) in the amount of 500 g per bag. A total of 36 bags (3 varieties of raw material × 4 treatments × 3 replicates) were made and stored at room temperature and avoid light. After

45 days of ensiling, the chemical composition, fermentation characteristics, and bacterial community diversity were assessed.

Chemical composition and fermentation characteristics analysis

After 45 days of ensiling, the silages and fresh materials were dried in a 65°C oven for 48 h, and then ground into powder with a knife mill (Zhejiang Yili Industry and Trade Co. Ltd., Zhejiang, China) through a 1 mm screen for subsequent layer analysis. The dry matter (DM) content of silages was measured by drying at 105°C in an electric blast drying oven for 4 h. The total nitrogen (TN) content was determined by Kjeldahl method (Krishnamoorthy et al., 1982; K1301, Chensheng Instrument Co. Ltd., Shanghai, China), and then the crude protein (CP) was equal to the TN × 6.25. Ether extract (EE), acid detergent lignin (ADL) and Ash shall be detected according to the procedures described by (AOAC, 2000). The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined with the ANKOM 2000i fiber analyzer (ANKOM 2000i, Anborui Science and Technology Co. Ltd., Beijing, China) according to the method of Van Soest et al. (1991). The contents of water-soluble carbohydrates (WSC) was determined by anthrone method (Murphy, 1958). The hemicellulose (HC) content was calculated by subtracting ADF from NDF, cellulose (CEL) content was the difference between ADF and ADL, and the holocellulose (HoC) content was the sum of HC and CEL (Ren et al., 2015). In addition, the biological degradation potential (BDP) was calculated by reference to Agneessens et al. (2015) using HoC divided by ADL.

Meanwhile, to determine the fermentation characteristics of silage, 20 g of each sample was weighed, added 180 ml distilled water, shake well, and then placed it in a refrigerator at 4°C for 24 h. After extraction, the filtrate was filtered through 4 layers of cheese cloth followed by 2 layers of filter paper. One aliquot of the filtrate was used to determine the pH of the filtrate immediately with a glass electrode pH meter (FE28, METTLER TOLEDO, Shanghai, China), and another aliquot was frozen at −20°C for the determination of organic acids and ammonia nitrogen (NH_3-H). The content of NH_3-N was determined by phenol hypochlorite colorimetry (Broderick and Kang, 1980). The content of lactic acid (LA) was determined by p-hydroxybiphenyl colorimetry method as the process described by Barnett (1951). Prior to examining the butyric acid (BA) and acetic acid (AA) concentrations, the filtrate was centrifuged at 10,000 rpm/min for 10 min, and the supernatant was detected by gas chromatography (GC-6800, Beifen Tianpu, Beijing, China). The analytical column was a quartz glass filled column (Φ 6 mm × 2 m), with a column temperature of 150°C and inlet temperature of 220°C; the injection volume was 1 μl; the FID detector temperature was 280°C; the carrier gas was high-purity N_2 with a flow rate of 30 ml/min and a pressure of 200 kPa; the gas was H_2 with a flow rate of 30 ml/min; the auxiliary gas was air with a flow rate of 300 ml/min. Finally, the flieg score (FS) was computed using equation given by Wang et al. (2017).

16S rDNA sequencing analysis

DNA extraction and PCR amplification

By using Power Soil DNA Isolation Kit (MO BIO Laboratories), total bacterial DNA was isolated from samples in line with the guidelines provided by the manufacturer. The ratios of 260 nm/280 nm and 260 nm/230 nm were used to evaluate the quality and amount of DNA. Following that, DNA was kept at −80°C until further processing. The common primer pair (Forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′; Reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′) together with adapter sequences and barcode sequences were used to amplify the V3-V4 region of the bacterial 16S rRNA gene. 50 μl of a total volume were used for the PCR amplification, including 10 μM of each primer, 10 μl of buffer, 10 μl of high GC enhancer, 1 μl of dNTP, 0.2 μl of Q5 High-Fidelity DNA Polymerase, and 60 ng/μl of genomic DNA. Following a preliminary denaturation at 95°C for 5 min, there were 15 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. Through the use of VAHTSTM DNA Clean Beads, the PCR products from the first step were purified. After then, a second round of PCR was carried out in a 40 μl reaction that comprised 10 μM of each primer, 20 μl of 2 × Phsion HF MM, 8 μl of ddH_2O, and 10 μl PCR products from the previous round. Following a preliminary denaturation at 98°C for 30 s, there were 10 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Finally, Quant-iTTM dsDNA HS Reagent was used to quantify each PCR products and pool them all together. The purified, pooled sample was subjected to high-throughput sequencing analysis of bacterial rRNA genes on the Illumina Miseq 2500 platform (2 × 250 paired ends) at Beijing Tsingke Biotechnology Co, LTD in Beijing, China.

Bioinformatic analysis of sequencing data

Firstly, Trimmatic (version 0.33) was used to filter the quality of the raw reads, then FLASH (version 1.2.11) was used to splice double-ended reads and remove chimeras (UCHIME, version 8.1). Finally, high-quality sequences were obtained for subsequent analysis. Operational taxonomic units (OTUs) were clustered using USEARCH (version 10.0) with a 97% sequence similarity. By default, 0.005% of the number of sequences was used as the threshold to filter OTUs. The α-diversity was calculated by using QIIME2.[1] R was also used to analyzed and display cluster heat maps, relative abundances of various microorganisms, and principal component analysis (PCA).

Statistical analysis

The fermentation characteristics and chemical composition data of silages were analyzed by two-way ANOVA in SPSS

1 https://qiime2.org/ (Accessed September 9, 2022).

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software. The significant difference was then indicated at the level of $p < 0.05$ using Duncan's multiple range tests to assess differences between treatments.

Results

Characteristics of fresh whole-plant corn of three different varieties before ensiling

The chemical compositions of the three different varieties of corn pre ensiling are shown in Table 1. The contents of several important indicators such as DM, CP, WSC, and NDF in YQ were 31.38% / FM, 8.02% / DM, 11.03% / DM, and 56.27% / DM, respectively. The contents of these components in EXA were DM (33.81% / FM), CP (8.57% / DM), WSC (9.86% / DM), and NDF (55.33% / DM). In NH, the contents of DM, CP, WSC, and NDF were 35.37% / FM, 7.85% / DM, 9.13% / DM, and 54.86% / DM, respectively.

Effects of varieties and additives on the chemical composition of whole-plant corn silage

The effects of varieties and additives on the chemical components of WPC silage such as DM, CP, EE, Ash, and WSC are presented in Table 2. When the variety was the main effect, EXA and NH had significantly larger DM contents than YQ ($p < 0.05$), the CP content in each treatment group showed that YQ and EXA were significantly higher than those of NH ($p < 0.05$). Only in CL treatment group, EE content showed that YQ was significantly less than EXA ($p < 0.05$). Ash content showed that there were significant differences among the three cultivars in GA treatment group ($p < 0.05$), and YQ > NH > EXA. WSC content in CK group YQ was significantly higher than EXA ($p < 0.05$), and there were significant differences in GA and CL groups ($p < 0.05$), and the expression was YQ > EXA > NH. When the additive was the main effect, in YQ and NH, the content of DM in each additive group was significantly higher than that in the CK group ($p < 0.05$). In YQ and EXA, the content of CP in each additive was significantly higher than those in the CK group ($p < 0.05$). In NH, the content of CP in GA and CL groups were significantly higher than BS and CK groups ($p < 0.05$); EE only in YQ, CL groups were significantly lower than BS and CK groups ($p < 0.05$); WSC in YQ, the GA and CL groups were significantly higher than the CK group ($p < 0.05$); and the BS group was significantly lower than the CK group ($p < 0.05$). In EXA and NH, the content of WSC in GA group was significantly higher than the CL group ($p < 0.05$), BS and CK groups were significantly lower than the CL group ($p < 0.05$). And the interaction effect of varieties and additives had a significant effect on CP of WPC silage ($p < 0.05$), had a highly significant effect on WSC ($p < 0.01$) but not significant effect on DM, EE and Ash ($p > 0.05$).

TABLE 1 Chemical composition of fresh whole-plant corn of three different varieties before ensiling (±SD, n=3).

Items	Corn varieties		
	YQ	EXA	NH
Dry matter / % FM	31.38 ± 3.37	33.81 ± 4.11	35.37 ± 3.94
Crude protein / % DM	8.02 ± 0.59	8.57 ± 0.30	7.85 ± 0.26
Ether extract / % DM	3.78 ± 0.66	3.82 ± 0.35	3.69 ± 0.28
Ash / % DM	4.90 ± 0.61	5.03 ± 0.76	4.60 ± 0.56
Acid detergent fiber / % DM	19.62 ± 1.46	21.39 ± 2.45	23.98 ± 2.04
Neutral detergent fiber / % DM	56.27 ± 2.08	55.33 ± 2.26	54.86 ± 3.59
Water soluble carbohydrate / % DM	11.03 ± 1.72	9.86 ± 1.32	9.13 ± 1.85

FM, fresh matter; DM, dry matter; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35.

Effects of varieties and additives on lignocellulosic composition of whole-plant corn silage

The effects of varieties and additives on the lignocellulosic components of WPC silage such as NDF, ADF, ADL, CEL, HC, and HoC are demonstrated in Table 3. When variety was the main effect, the content of NDF and HoC did not differ significantly between varieties ($p > 0.05$); the content of ADF and CL were not significantly different among the varieties in the CK group ($p > 0.05$), in the other treatment groups, YQ and EXA were significantly lower than NH ($p < 0.05$); The ADL content of YQ and EXA was significantly higher than that of NH only in the GA group ($p < 0.05$); the HC content of YQ and EXA was significantly higher than that of NH only in the BS group ($p < 0.05$). When the additive was the main effect, the NDF content in YQ only showed that the GA group was significantly lower than the other treatment groups ($p < 0.05$). The ADF content in YQ showed that the CL group was significantly lower than the BS and CK groups ($p < 0.05$), and in EXA, the CL and GA groups were significantly lower than the BS and CK groups ($p < 0.05$). ADL in YQ, the CL group was significantly lower than the BS group ($p < 0.05$), and in NH, the additives groups were significantly lower than the CK group ($p < 0.05$). Only in EXA, the CEL content of the GA and CL groups was significantly lower than that of the CK group ($p < 0.05$); the HoC content only in YQ, the GA and CL groups were significantly lower than the CK group ($p < 0.05$). HC content in different treatments groups showed no significant difference ($p > 0.05$). The interaction between varieties and additives only had a significant effect on CEL content ($p < 0.05$).

10.3389/fmicb.2022.1028001

TABLE 2 Effects of varieties and additives on the chemical compositions of whole-plant corn silage (±SD, n=3).

Items	Treatments	Varieties			Significance		
		YQ	EXA	NH	V	T	V×T
[2]Dry matter / % FM	CK	[1]27.59 ± 0.06^{Aa}	31.93 ± 0.16^{Ca}	30.84 ± 0.10^{Ba}	**	**	NS
	BS	29.82 ± 0.24^{Ab}	32.47 ± 0.44^{Ba}	32.33 ± 0.26^{Bab}			
	GA	30.31 ± 0.38^{Ab}	33.58 ± 0.17^{Ba}	33.87 ± 0.46^{Bb}			
	CL	31.09 ± 0.30^{Ab}	32.27 ± 0.29^{ABa}	33.42 ± 0.19^{Bb}			
Crude protein / %	CK	8.11 ± 0.18^{Ba}	8.39 ± 0.10^{Ba}	7.00 ± 0.14^{Aa}	**	**	*
DM	BS	9.90 ± 0.20^{Cb}	8.96 ± 0.02^{Bb}	7.07 ± 0.14^{Aa}			
	GA	9.81 ± 0.12^{Bb}	9.53 ± 0.06^{Bc}	8.77 ± 0.13^{Ab}			
	CL	9.72 ± 0.24^{Bb}	9.68 ± 0.13^{Bc}	8.16 ± 0.09^{Ab}			
	CK	3.85 ± 0.11^{Ab}	3.39 ± 0.08^{Aa}	3.72 ± 0.15^{Aa}	NS	NS	NS
Ether extract / % DM	BS	3.72 ± 0.10^{Ab}	3.29 ± 0.06^{Aa}	3.40 ± 0.11^{Aa}			
	GA	3.39 ± 0.07^{Aab}	3.31 ± 0.05^{Aa}	3.65 ± 0.17^{Aa}			
	CL	3.04 ± 0.01^{Aa}	3.55 ± 0.10^{Ba}	3.40 ± 0.07^{ABa}			
Ash / % DM	CK	5.16 ± 0.09^{Aa}	6.29 ± 1.22^{Aa}	4.52 ± 0.04^{Aa}	NS	NS	NS
	BS	4.85 ± 0.09^{Aa}	5.11 ± 0.27^{Aa}	5.08 ± 0.06^{Aa}			
	GA	5.33 ± 0.12^{Ca}	3.57 ± 0.07^{Aa}	4.52 ± 0.10^{Ba}			
	CL	4.97 ± 0.16^{Aa}	4.45 ± 0.17^{Aa}	4.85 ± 0.10^{Aa}			
Water soluble	CK	4.51 ± 0.08^{Bb}	4.11 ± 0.24^{Aa}	4.28 ± 0.16^{ABa}	**	**	**
carbohydrate / % DM	BS	4.07 ± 0.23^{Aa}	4.11 ± 0.18^{Aa}	4.21 ± 0.10^{Aa}			
	GA	6.48 ± 0.04^{Cc}	6.04 ± 0.04^{Bc}	5.18 ± 0.27^{Ac}			
	CL	6.55 ± 0.03^{Cc}	5.52 ± 0.17^{Bb}	4.85 ± 0.08^{Ab}			

[1]Capital letters indicate significant differences between varieties under the same treatment ($p<0.05$). Lowercase letters indicate significant differences among different treatments of the same variety ($p<0.05$).

[2]FM, fresh matter; DM, dry matter; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM; V, corn varieties effect; T, treatments effect; V×T, the interaction effect of treatments and corn varieties. *$p<0.05$; **$p<0.01$; NS, no significant effect.

Effects of varieties and additives on fermentation quality of whole-plant corn silage

The effects of varieties and additives on the fermentation quality of WPC silage such as pH, LA, AA, PA, BA, and NH_3-N / TN were displayed in Table 4. When variety was the main effect, the pH value only in the CL group showed that EXA and NH were significantly lower than YQ ($p<0.05$); the LA content of the three corn varieties in the CK and CL groups showed significant difference ($p<0.05$), and YQ > NH > EXA, in the BS group, YQ and NH were significantly higher than EXA ($p<0.05$). There was no significant difference in AA content among different varieties in each treatment group ($p>0.05$); BA content in CK group and GA group showed that YQ was significantly higher than the other two varieties ($p<0.05$), and in CL group showed that YQ was significantly higher than NH ($p<0.05$). NH_3-N / TN in both GA and CK groups showed that YQ was significantly higher than EXA and NH ($p<0.05$), and in BS group the performance of YQ was significantly higher than that of EXA ($p<0.05$). When the additive was the main effect, the pH value of the GA group was significantly lower than that of the BS group only in EXA ($p<0.05$). The LA content in YQ showed that the BS group was significantly higher than the GA and CL groups ($p<0.05$), and the CK group was significantly lower than the GA and CL groups ($p<0.05$), in EXA, all treatments showed significant differences ($p<0.05$), and GA group > BS group > CL group > CK group, in NH, BS. and GA groups were significantly higher than CL and CK groups ($p<0.05$). There was no significant difference in the contents of AA and BA among different treatment groups in each variety ($p>0.05$); NH_3-N / TN in YQ showed that CL group was significantly lower than BS and CK groups ($p<0.05$), and GA group was significantly lower than the CK group ($p<0.05$), in EXA, the GA group was significantly lower than the BS and CK groups ($p<0.05$), and in NH, the GA, and CL groups were significantly lower than the BS and CK group ($p<0.05$). The interaction of varieties and additives only had a very significant effect on LA content ($p<0.01$). PA was not detected in all groups in this experiment.

Effects of varieties and additives on BDP and FS of whole-plant corn silage

The effects of varieties and additives on BDP and FS of WPC silage are presented in Figure 1. When variety was the main effect, BDP showed significant differences among the three varieties in the GA group ($p<0.05$), and NH > EXA > YQ, in the CL group, showed NH and EXA were significantly

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TABLE 3 Effects of varieties and additives on lignocellulosic compositions of whole-plant corn silage (±SD, *n*=3).

Items	Treatments	Varieties			Significance		
		YQ	EXA	NH	V	T	V×T
[2]Neutral detergent fiber / % DM	CK	[1]51.93 ± 1.22^{Ab}	50.07 ± 2.46^{Aa}	49.40 ± 2.16^{Aa}	NS	NS	NS
	BS	50.31 ± 0.33^{Aab}	48.45 ± 1.33^{Aa}	49.21 ± 0.47^{Aa}			
	GA	45.47 ± 0.97^{Aa}	47.84 ± 1.55^{Aa}	49.37 ± 0.63^{Aa}			
	CL	46.83 ± 0.72^{Aab}	46.63 ± 1.47^{Aa}	48.30 ± 0.20^{Aa}			
Acid detergent fiber / % DM	CK	18.83 ± 0.52^{Ab}	20.74 ± 1.09^{Ab}	21.64 ± 0.65^{Aa}	**	**	NS
	BS	18.50 ± 0.80^{Ab}	18.01 ± 0.60^{Aab}	22.09 ± 0.17^{Ba}			
	GA	17.27 ± 0.38^{Aab}	14.08 ± 0.64^{Aa}	22.55 ± 0.84^{Ba}			
	CL	15.28 ± 0.33^{Aa}	14.42 ± 0.29^{Aa}	20.16 ± 0.57^{Ba}			
Acid detergent lignin / % DM	CK	2.99 ± 0.22^{Aab}	3.16 ± 0.22^{Aa}	3.68 ± 0.14^{Ab}	*	**	NS
	BS	3.64 ± 0.02^{Ab}	3.05 ± 0.27^{Aa}	2.68 ± 0.14^{Aa}			
	GA	3.34 ± 0.05^{Bab}	2.70 ± 0.16^{ABa}	2.31 ± 0.07^{Aa}			
	CL	2.82 ± 0.10^{Aa}	2.38 ± 0.16^{Aa}	2.08 ± 0.14^{Aa}			
Cellulose / % DM	CK	15.84 ± 0.57^{Aa}	17.58 ± 0.89^{Ab}	17.96 ± 0.56^{Aa}	**	**	*
	BS	14.87 ± 0.80^{Aa}	14.97 ± 0.44^{Aab}	19.41 ± 0.18^{Ba}			
	GA	13.93 ± 0.40^{Aa}	11.38 ± 0.60^{Aa}	20.25 ± 0.78^{Ba}			
	CL	12.45 ± 0.41^{Aa}	12.04 ± 0.41^{Aa}	18.08 ± 0.69^{Ba}			
Hemicellulose / % DM	CK	33.10 ± 1.48^{Aa}	29.32 ± 3.29^{Aa}	27.76 ± 2.29^{Aa}	NS	NS	NS
	BS	31.81 ± 0.49^{Ba}	30.43 ± 0.98^{ABa}	27.12 ± 0.63^{Aa}			
	GA	28.20 ± 0.70^{Aa}	33.76 ± 1.38^{Aa}	26.82 ± 1.46^{Aa}			
	CL	31.56 ± 0.46^{Aa}	32.21 ± 1.71^{Aa}	28.14 ± 0.59^{Aa}			
Holocellulose / % DM	CK	48.94 ± 1.02^{Ab}	46.91 ± 2.67^{Aa}	45.72 ± 2.27^{Aa}	NS	NS	NS
	BS	46.67 ± 0.34^{Aab}	45.4 ± 1.34^{Aa}	46.53 ± 0.58^{Aa}			
	GA	42.13 ± 0.96^{Aa}	45.14 ± 1.39^{Aa}	47.06 ± 0.70^{Aa}			
	CL	44.01 ± 0.76^{Aa}	44.25 ± 1.31^{Aa}	46.22 ± 0.30^{Aa}			

[1]Capital letters indicate significant differences between varieties under the same treatment ($p < 0.05$). Lowercase letters indicate significant differences among different treatments of the same variety ($p < 0.05$).

[2]FM, fresh matter; DM, dry matter; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM; V, corn varieties effect; T, treatments effect; V × T, the interaction effect of treatments and corn varieties. *$p < 0.05$; **$p < 0.01$; NS, no significant effect.

higher than YQ ($p < 0.05$). The FS of YQ in both CK and GA groups was significantly lower than that of EXA and NH ($p < 0.05$). When additive was the main effect, BDP in YQ, CL and CK groups were significantly higher than BS and GA groups ($p < 0.05$), and in NH, GA, and CL groups were significantly higher than CK groups ($p < 0.05$). FS in YQ, each additive group was significantly higher than CK group ($p < 0.05$); in EXA, GA group was significantly higher than the other groups ($p < 0.05$); in NH, GA, and CL group was significantly higher than the CK group ($p < 0.05$).

Diversity of the bacterial community in whole-plant corn silage with different treatments

The α-diversity of bacterial communities in fresh materials and WPC silage was displayed in Table 5 of this study. The reads in each group ranged from 77,928 to 48,656, with the highest reads in the YQ-CL group (77,928) and the lowest in the YQ-FM group (48,656), with an average of 67,172. Shannon and Simpson showed that it was significantly lower than that of fresh corn material after silage ($p < 0.05$); Shannon showed the highest NH-FM and the lowest YQ-GA in all groups; Simpson showed the highest EXA-FM and the lowest YQ-GA. Among the three corn varieties, OTUs, ACE, Chao1, Shannon, and Simpson of YQ were significantly lower than the other two varieties ($p < 0.05$). The interaction of varieties and additives had very significant effect on OTUs, ACE, Chao1 and Simpson ($p < 0.01$). The Coverage of each group in this experiment was above 99%, indicating that the sequencing results represented the real situation of the microorganisms in the samples. The results of β-diversity analysis of fresh raw materials and WPC silage are shown in Figure 2, it could be seen from the principal component analysis (PCA) that PCo 1 and PCo 2 were 61.90 and 11.55%, respectively. At the same time, the bacterial communities of the fresh raw materials of the three corn varieties were separated from each other, and the bacterial communities of the fresh raw materials and WPC silage were also significantly separated.

10.3389/fmicb.2022.1028001

TABLE 4 Effects of varieties and additives on fermentation quality of whole-plant corn silage (±SD, *n*=3).

Items	Treatments	Varieties			Significance		
		YQ	EXA	NH	V	T	V × T
[2]pH	CK	[1]3.88 ± 0.01^{Aa}	3.91 ± 0.02^{Aab}	3.92 ± 0.01^{Aa}	NS	NS	NS
	BS	3.90 ± 0.00^{Aa}	3.96 ± 0.01^{Ab}	3.91 ± 0.02^{Aa}			
	GA	3.87 ± 0.02^{Aa}	3.87 ± 0.01^{Aa}	3.93 ± 0.01^{Aa}			
	CL	3.90 ± 0.00^{Aa}	3.93 ± 0.00^{Bab}	3.93 ± 0.01^{Ba}			
Lactic acid / (mg.	CK	67.08 ± 0.28^{Ca}	47.82 ± 0.41^{Aa}	64.30 ± 0.46^{Bb}	**	**	**
g^{-1} DM)	BS	73.43 ± 0.63^{Bc}	60.66 ± 0.08^{Ac}	73.44 ± 0.43^{Bc}			
	GA	69.65 ± 0.26^{Ab}	71.8 ± 0.70^{Ad}	72.35 ± 0.59^{Ac}			
	CL	69.68 ± 0.41^{Cb}	58.06 ± 0.41^{Ab}	61.01 ± 0.50^{Ba}			
Acetic acid / (mg.	CK	7.49 ± 0.36^{Aa}	6.08 ± 0.26^{Aa}	7.43 ± 0.32^{Aa}	NS	NS	NS
g^{-1} DM)	BS	6.83 ± 0.57^{Aa}	6.50 ± 0.14^{Aa}	7.49 ± 0.39^{Aa}			
	GA	5.95 ± 0.14^{Aa}	6.14 ± 0.16^{Aa}	7.08 ± 0.36^{Aa}			
	CL	5.80 ± 0.33^{Aa}	6.46 ± 0.56^{Aa}	6.72 ± 0.11^{Aa}			
Propionic acid /	CK	ND	ND	ND	—	—	—
(mg.g^{-1} DM)	BS	ND	ND	ND			
	GA	ND	ND	ND			
	CL	ND	ND	ND			
Butyric acid / (mg.	CK	0.20 ± 0.01^{Ba}	0.04 ± 0.01^{Aa}	0.06 ± 0.01^{Aa}	**	NS	NS
g^{-1} DM)	BS	0.07 ± 0.02^{Aa}	ND	0.05 ± 0.01^{Aa}			
	GA	0.17 ± 0.01^{Ba}	0.03 ± 0.00^{Aa}	0.05 ± 0.01^{Aa}			
	CL	0.17 ± 0.04^{Ba}	ND	0.05 ± 0.00^{Aa}			
NH_3-N / TN / %	CK	5.03 ± 0.23^{Bc}	3.33 ± 0.17^{Abc}	3.82 ± 0.05^{Ab}	**	NS	NS
	BS	4.75 ± 0.12^{Bbc}	3.70 ± 0.15^{Ac}	4.01 ± 0.19^{ABb}			
	GA	3.66 ± 0.22^{Bab}	2.31 ± 0.08^{Aa}	2.59 ± 0.19^{Aa}			
	CL	3.21 ± 0.28^{Aa}	2.73 ± 0.20^{Aab}	2.82 ± 0.11^{Aa}			

[1]Capital letters indicate significant differences between varieties under the same treatment ($p < 0.05$). Lowercase letters indicate significant differences among different treatments of the same variety ($p < 0.05$).

[2]FM, fresh matter; DM, dry matter; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM; V, corn varieties effect; T, treatments effect; V × T, the interaction effect of treatments and corn varieties. *$p < 0.05$; **$p < 0.01$; NS, no significant effect.

Bacterial relative abundance of whole-plant corn silage with different treatments

The relative abundance of bacterial communities for fresh raw material and WPC silage with different treatments at the phylum is presented in Figure 3 (Circos map). As seen by the Circos map, *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Actinobacteria* were the top five phyla bacteria attached to the 3 fresh corn raw materials. The relative abundances of the top five phyla that attached to YQ-FM were *Proteobacteria* (76.08%), *Bacteroidetes* (18.35%), *Cyanobacteria* (2.68%), *Firmicutes* (1.73%) and *Actinobacteria* (0.66%), respectively; The relative abundances of the top five phyla that attached to EXA-FM were *Proteobacteria* (64.88%), *Bacteroidetes* (14.09%), *Cyanobacteria* (8.10%), *Actinobacteria* (5.27%), and *Firmicutes* (4.44%), respectively. The relative abundances of the top five phyla that attached to NH-FM were *Proteobacteria* (45.69%), *Cyanobacteria* (21.15%), *Bacteroidetes* (11.69%), *Actinobacteria* (8.29%), and *Firmicutes* (6.40%), respectively. After fermentation, the dominant phylum in each treatment group was *Firmicutes*. In YQ, the GA group had the highest relative abundance of *Firmicutes*, and the BS group had the lowest relative abundance of *Firmicutes*, at 91.47 and 79.10%, respectively. In EXA, the relative abundance of *Firmicutes* was the maximum in the CK group and the minimum in the GA group, 73.86 and 68.60%, respectively. In NH, the relative abundance of *Firmicutes* was the highest in the GA group and the lowest in the CL group, 66.80 and 59.02%, respectively. The relative abundance of *Firmicutes* in the three varieties WPC silage was YQ > EXA > NH. After fermentation, each treatment group's relative abundance of *Proteobacteria* declined noticeably, and the relative abundance of *Bacteroidetes* likewise trended lower. In YQ, the BS group had the largest relative abundance of *proteobacteria*, that was 18.40%, while the GA group had the lowest relative abundance, which was 6.38%; In EXA, the relative abundance of *Proteobacteria* was the highest in the CL group and the lowest in the CK group, 23.71 and 20.74%, respectively; In NH, the relative abundance of *Proteobacteria* in the CL group was the highest, and the GA group was the lowest, 31.57 and 23.62%, respectively; And the relative abundance of *Proteobacteria* in the three varieties

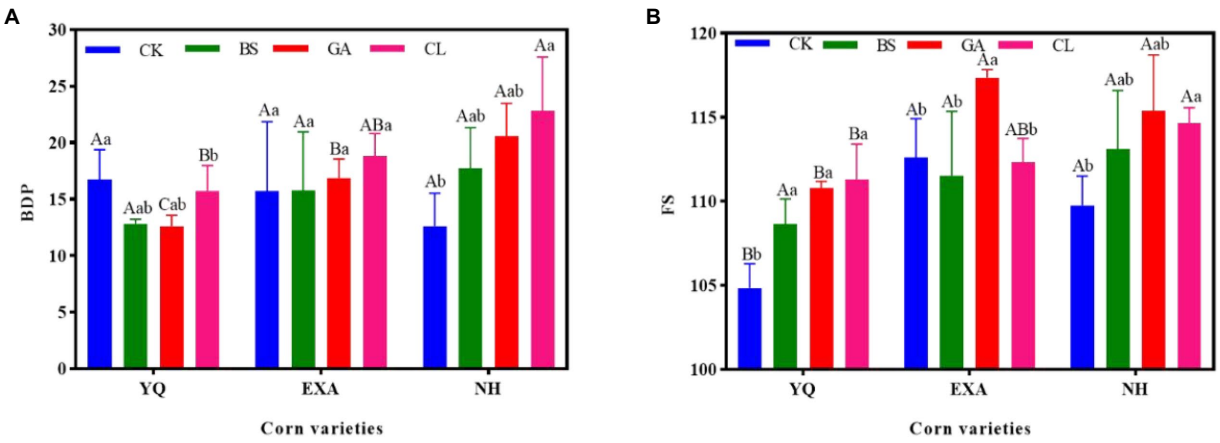

FIGURE 1
Effects of Varieties and Additives on **(A)** BDP and **(B)** FS of Whole-plant corn silage. BDP, biological degradation potential; FS, flieg score; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM; capital letters indicate significant differences between varieties under the same treatment ($p<0.05$). Lowercase letters indicate significant differences among different treatments of the same variety ($p<0.05$).

WPC silage was NH > EXA > YQ. The relative abundance of *Bacteroidetes* was reduced to less than 1% in YQ by the application of several additives; In EXA and NH, the effects of each additive were reversed, increasing the relative abundance of *Bacteroidetes* bacteria to more than 1%; while the relative abundance of *Bacteroidetes* bacteria in the three species of WPC silage was ranked NH > YQ > EXA.

The bacterial community at the genus level was assessed to further show the impact of varieties and additives on the bacterial community in WPC silage (Figures 4, 5). The accumulation columnar map (Figure 4) illustrated the diversity and abundance of bacterial community at various genus levels. *Enterobacteriaceae* (36.62%), *Sphingobacterium* (8.61%), and *Chryseobacterium* (7.16%) were the top three dominant genera attached to YQ-FM; *Enterobacteriaceae* (11.57%), *Serratia* (8.87%), and *Acinetobacter* (7.76%) were the top three dominant genera that attached to EXA-FM; and the top three dominant genera attached to NH-FM were *Chloroplast* (18.49%), *Rosenbergiella* (6.07%), and *Mitochondria* (5.63%). After fermentation, *Lactobacillus* emerged as the dominant genus in each treatment group. In YQ and NH, the relative abundance of *Lactobacillus* was both the highest in the GA group, at 87.56 and 62.18%, respectively, and the lowest both in the BS group, at 73.84 and 49.29%, respectively; in EXA, the relative abundance of *Lactobacillus* was the highest in CK group, and the GA group was the lowest, at 71.01 and 65.68%, respectively. The relative abundance of *Lactobacillus* in the silage of the three corn varieties was ranked YQ > EXA > NH. The relative abundance of *Enterobacteriaceae* decreased significantly in YQ and EXA, remained basically unchanged in NH, and only increased slightly in NH-CL group. In all groups, the relative abundance of *Enterobacteriaceae* was the lowest in YQ-GA group and the highest in NH-CL group, which were 0.92 and 6.62%, respectively. After the silage was completed, *Klebsiella* increased in all groups except the YQ-BS group. The relative abundance of *Sphingobacterium* showed a downward trend after fermentation. Except for the four groups of YQ-CK, NH-BS, NH-GA, and NH-CL, the relative abundance of *Sphingobacterium* in the other groups was at the level of 1%. In addition, corn raw materials were grouped into one category, and WPC silage of three varieties were grouped into one category, respectively. The bacteria of the first three genera of relative abundance (*Lactobacillus*, *Klebsiella*, and *Enterobacteriaceae*) were grouped into one class, and the other different genera were grouped into another class.

Discussion

Chemical compositions, lignocellulosic compositions, and fermentation quality of whole-plant corn silage with different treatments

DM content reflects the nutritional value and fermentation quality of whole-plant corn silage, and further determines the economic benefits of silage (Kennington et al., 2005). Khan et al. (2015) reported that corn silage feeding dairy cow with dry matter content in the range 30–35% had a positive effect on improving their milk production. In this experiment, after silage, the content of DM in YQ and NH used different additives were significantly higher than those in the CK group. At the same time, except for the YQ-CK and YQ-BS groups with lower DM content, the DM content of the other treatment groups were within the range 30–35%, this might be a good phenomenon for the dairy cow breading industry. Guo et al. (2013) showed that higher moisture content of silage raw material can lead to *Clostridium*-based fermentation, which produces butyric acid and results in poor silage quality and huge economic losses. The lower DM content of YQ-CK and YQ-BS in this experiment may be because the

TABLE 5 Alpha diversity of bacterial communities in whole-plant corn silage with different treatments.

Items	Treatments	Varieties			Significance		
		YQ	EXA	NH	V	T	V × T
[2]OTUs	FM	[1]268.67 ± 4.04^{Aab}	454.00 ± 4.64^{Bb}	496.33 ± 1.37^{Cc}	**	**	**
	CK	236.67 ± 20.27^{Aa}	394.33 ± 2.31^{Ba}	480.00 ± 1.70^{Bbc}			
	BS	327.33 ± 5.72^{Ab}	440.33 ± 14.03^{Bab}	469.00 ± 8.18^{Bb}			
	GA	322.33 ± 1.81^{Ab}	468.00 ± 3.68^{Cb}	416.33 ± 1.29^{Ba}			
	CL	322.00 ± 6.69^{Ab}	445.67 ± 2.78^{Cb}	395.00 ± 1.70^{Ba}			
ACE	FM	294.41 ± 4.68^{Aa}	464.35 ± 3.77^{Bb}	500.27 ± 1.30^{Cb}	**	*	**
	CK	300.44 ± 21.05^{Aa}	430.19 ± 2.48^{Ba}	490.51 ± 2.09^{Bb}			
	BS	383.49 ± 2.51^{Ab}	467.46 ± 9.16^{Bb}	480.98 ± 5.46^{Bb}			
	GA	384.60 ± 9.41^{Ab}	481.46 ± 1.39^{Bb}	445.00 ± 1.78^{Ba}			
	CL	380.45 ± 2.55^{Ab}	459.76 ± 2.55^{Cab}	428.99 ± 2.77^{Ba}			
Chao1	FM	306.19 ± 7.82^{Aab}	468.49 ± 2.89^{Bab}	502.18 ± 1.22^{Bb}	**	NS	**
	CK	300.08 ± 24.22^{Aa}	438.87 ± 4.38^{Ba}	496.65 ± 3.28^{Bb}			
	BS	382.80 ± 3.05^{Aab}	477.53 ± 6.78^{Bb}	487.42 ± 5.55^{Bb}			
	GA	392.30 ± 9.73^{Ab}	484.96 ± 1.07^{Bb}	447.86 ± 1.73^{Ba}			
	CL	377.00 ± 3.23^{Aab}	467.66 ± 3.92^{Cab}	434.18 ± 4.22^{Ba}			
Shannon	FM	4.33 ± 0.21^{Ab}	6.15 ± 0.12^{Bc}	6.30 ± 0.28^{Bb}	**	**	NS
	CK	1.57 ± 0.09^{Aa}	2.27 ± 0.06^{Ba}	3.57 ± 0.08^{Ca}			
	BS	2.06 ± 0.17^{Aa}	2.51 ± 0.07^{Bab}	4.05 ± 0.06^{Ba}			
	GA	1.23 ± 0.08^{Aa}	2.85 ± 0.07^{Bb}	3.24 ± 0.05^{Ba}			
	CL	1.62 ± 0.12^{Aa}	2.46 ± 0.05^{Bab}	3.49 ± 0.02^{Ca}			
Simpson	FM	0.87 ± 0.02^{Ac}	0.96 ± 0.00^{Ac}	0.93 ± 0.02^{Ac}	**	**	**
	CK	0.35 ± 0.03^{Aab}	0.49 ± 0.01^{Ba}	0.71 ± 0.01^{Cab}			
	BS	0.48 ± 0.04^{Ab}	0.53 ± 0.01^{Aab}	0.75 ± 0.01^{Bb}			
	GA	0.24 ± 0.02^{Aa}	0.56 ± 0.01^{Bb}	0.65 ± 0.00^{Ca}			
	CL	0.34 ± 0.03^{Aab}	0.53 ± 0.01^{Bab}	0.74 ± 0.00^{Cb}			
Coverage	FM	0.9990	0.9996	0.9998	—	—	—
	CK	0.9989	0.9992	0.9995			
	BS	0.9987	0.9993	0.9996			
	GA	0.9986	0.9995	0.9993			
	CL	0.9991	0.9994	0.9993			
Reads	FM	48,656	65,471	51,324	—	—	—
	CK	68,503	76,053	67,463			
	BS	62,553	73,266	67,362			
	GA	65,463	65,044	74,515			
	CL	77,928	69,011	74,961			

[1]Capital letters indicate significant differences between varieties under the same treatment ($p < 0.05$). Lowercase letters indicate significant differences among different treatments of the same variety ($p < 0.05$).

[2]FM, fresh matter; DM, dry matter; YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM; V, corn varieties effect; T, treatments effect; V × T, the interaction effect of treatments and corn varieties. *$p < 0.05$; **$p < 0.01$; NS, no significant effect.

moisture content of YQ raw material is higher, which leads to the growth of undesired microorganisms and consumes more DM. CP is an important indicator reflecting the nutritional value of silage. WSC is an important microbial fermentation substrate in the silage process. It is generally believed that the content of WSC > 5% DM can meet the needs of microorganisms in the fermentation process, thereby ensuring the fermentation quality (Ni et al., 2017). The WSC content of each type of corn raw materials used in this investigation complied with the aforementioned standards. After silage, in YQ and EXA, the content of CP in BS group was significantly higher than that in CK group, and in YQ, the content of WSC in BS group was significantly lower than that in CK group. Bai et al. (2020) found that the CP content of alfalfa silage increased significantly compared with the CK group after adding *Bacillus subtilis* producing antimicrobial peptide, while the WSC was lower than the CK group, which agreed with the findings of this investigation. This may be the antibacterial peptide produced by the additive can efficiently thwart undesirable bacteria'

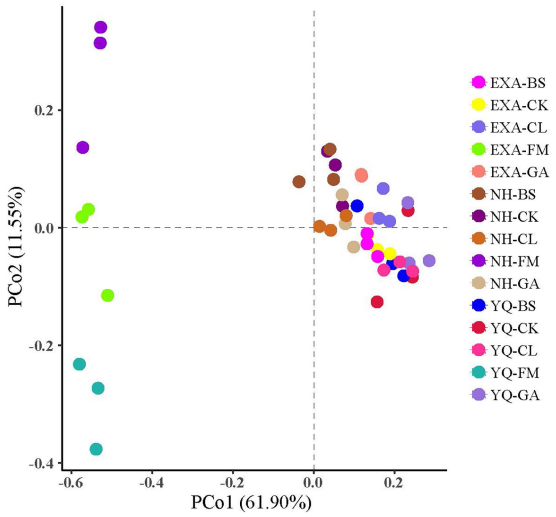

FIGURE 2
Principal component analysis (PCA) of bacterial communities for whole-plant corn silage with different treatmentsn. YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; FM, fresh raw materials; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM.

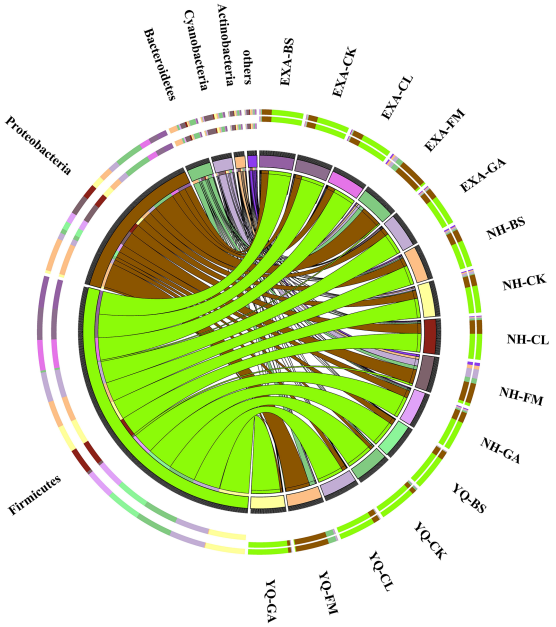

FIGURE 3
Circos map of bacterial communities at the phylum for whole-plant corn silage with different treatments. YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; FM, fresh raw materials; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM.

physiological activities and promote the activities of LAB, thus consuming a great number of WSC and better protecting CP from degradation, so that the content of CP is relatively increased and the content of WSC is significantly reduced. In this experiment, the content of CP in GA and CL group was significantly higher than that in CK group, and the content of WSC was significantly higher than that in BS group and CK group. Previous studies have shown that gallic acid has anti-fungal, anti-viral, anti-inflammatory and anti-oxidant effects (Choubey et al., 2018; Kahkeshani et al., 2019). This might be the cause of the greater CP and WSC contents in the GA group compared to the CK group. Cellulase can improve the degradation rate of fiber in plant cell wall and produce more WSC as fermentation substrate, this can accelerated the fermentation process by promoting the physiological activities of lactic acid bacteria, protect proteins from degradation by undesired microorganisms, which further improves the content of CP and WSC (Cai et al., 2019). In addition, there were some differences in the contents of DM, CP, EE, ash and WSC of WPC silage of different varieties. The DM contents of EXA and NH were higher, and the CP and WSC contents of YQ and EXA were higher. The research of Cherney et al. showed that there will be differences in the content of chemical components of whole-plant corn silage of different varieties, which is consistent with the results of this study (Cherney et al., 2004). This might be because there were some differences in the growth period of different corn varieties, so the accumulation law of chemical components was also different, resulting in different content of chemical components after silage.

Lignocellulosic components mainly include NDF, ADF, ADL, Cl, HC, and HoC, which are related to the palatability and biodegradability of silage. In this study, the NDF and ADF contents of all varieties of WPC decreased after silage, this was consistent with the research results in whole-plant corn silage with lactic acid bacteria and organic acid by Jiang et al. (2020). Such results indicate that silage process can be used as biochemical pretreatment to promote the degradation of lignocellulose components, so as to realize further utilization (Aichinger et al., 2015; Zhao et al., 2018). The NDF content in YQ, ADF content in EXA and ADL content in NH all showed the same rule that was compared with CK group, the content of this index was significantly reduced after adding gallic acid. In addition, the CEL and HoC contents of GA group were significantly lower than those of CK group in all corn varieties in this experiment. This might be because the use of gallic acid played its acidic role, which could rapidly reduce the pH of silage, thus promoting the acid hydrolysis reaction of NDF, ADF, and ADL. The ADF and ADL contents of CL group were significantly lower than those of CK group in corn variety YQ, CEL, and HoC contents of CL group were significantly lower than those of CK group in all corn varieties. It is explained that the used of cellulase causes an enzymatic reaction in the silage process, which decomposes the cellulose structural carbohydrates in the plant cell wall, resulting in a decrease in the contents of NDF, ADF, HoC, and so on (Desta et al., 2016). BDP is an index to measure the degradability of silage (Agneessens et al., 2015), which is derived from HoC and ADL. In this experiment, in corn varieties EXA and NH, the BDP of each additive group was higher than that of CK group, indicated that several additives used in this

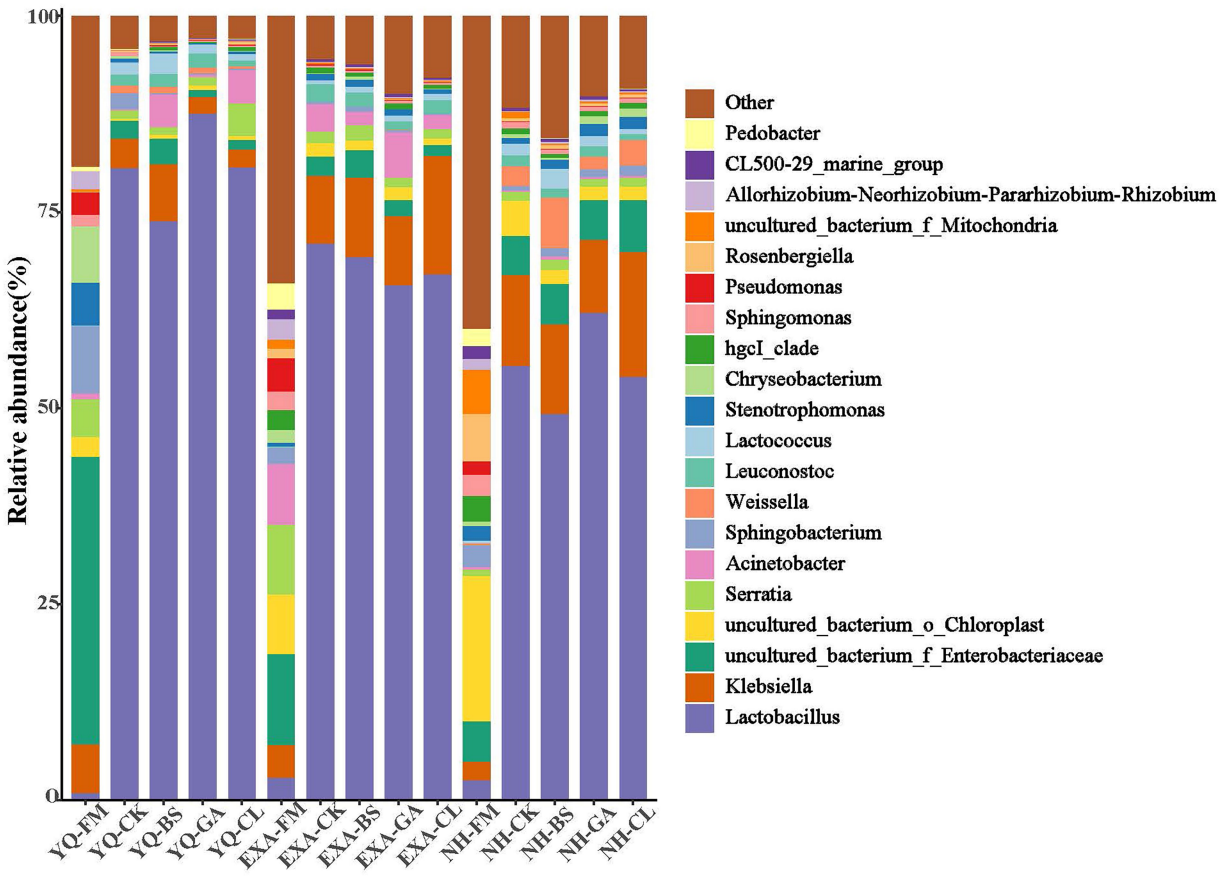

FIGURE 4
Relative abundance of bacterial communities at the genus levels for whole-plant corn silage with different treatments. YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; FM, fresh raw materials; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM.

experiment were helpful to improve the biodegradability of silage. In addition, in this experiment, ADF, ADL, HC, and CL were different among different varieties WPC silage. Benefield et al. (2006) and Borreani et al. (2018) found that the composition of CP, EE, and other chemical components varied significantly and NDF, ADF, and other lignocellulosic components between different varieties of whole-plant corn silage. This is similar to the results of this experiment. It may be that different corn varieties have different genetic backgrounds, which leads to this difference.

pH, lactic acid, and NH_3-N / TN are the main indexes to measure the fermentation quality of silage. It is generally believed that high-quality silage has pH <4.2, lactic acid content of 4–6%, NH_3-N / TN <10% (Wang et al., 1999; Shao et al., 2005; Yuan et al., 2012). In this research, the pH values of all treatment were between 3.8 ~ 4.0, indicated that the WPC is easy to be prepared into high-quality silage. Lara et al. (2016) found that the addition of *Bacillus subtilis* will reduce the LA content in corn silage. In this experiment, the LA content of BS group in YQ and NH was considerably larger than that of CK group and CL group. *Bacillus subtilis* can create LA by pyruvate reduction under anaerobic circumstances, according to Cruz Ramos Hugo et al. (2000), which might account for the elevated LA level in the BS group in our test. It also might be that the addition of antimicrobial peptide-producing BS promoted the proliferation of homofermentative lactic acid bacteria in WPC silage, resulting in more LA (Bai et al., 2020). Among all corn varieties, LA content in GA group was significantly increased than that in CK group, aside from the acidity of GA, it should be owed to its anti-bacterial property benefiting the dominance establishment of lactic acid bacteria and reduced negative nutrient competition (He et al., 2020). In YQ and EXA, compared with CK group, LA content in CL group was significantly increased. Zhao et al. (2021) found that cellulase can increase LA content in silage, in the study of the interaction between cellulase and lactic acid bacteria on the mixed silage of soybean residue and corn straw. Which is consistent with the results of our experiment, it is explained that the structural carbohydrates in the WPC were degraded to a great extent, so that the soluble sugars are released, providing additional fermentation substrates for the fermentation of lactic acid bacteria, thus producing more LA (Stokes, 1992). Furthermore, the presence of PA was not detected at all in this test, and the content of BA was extremely low, which is similar to

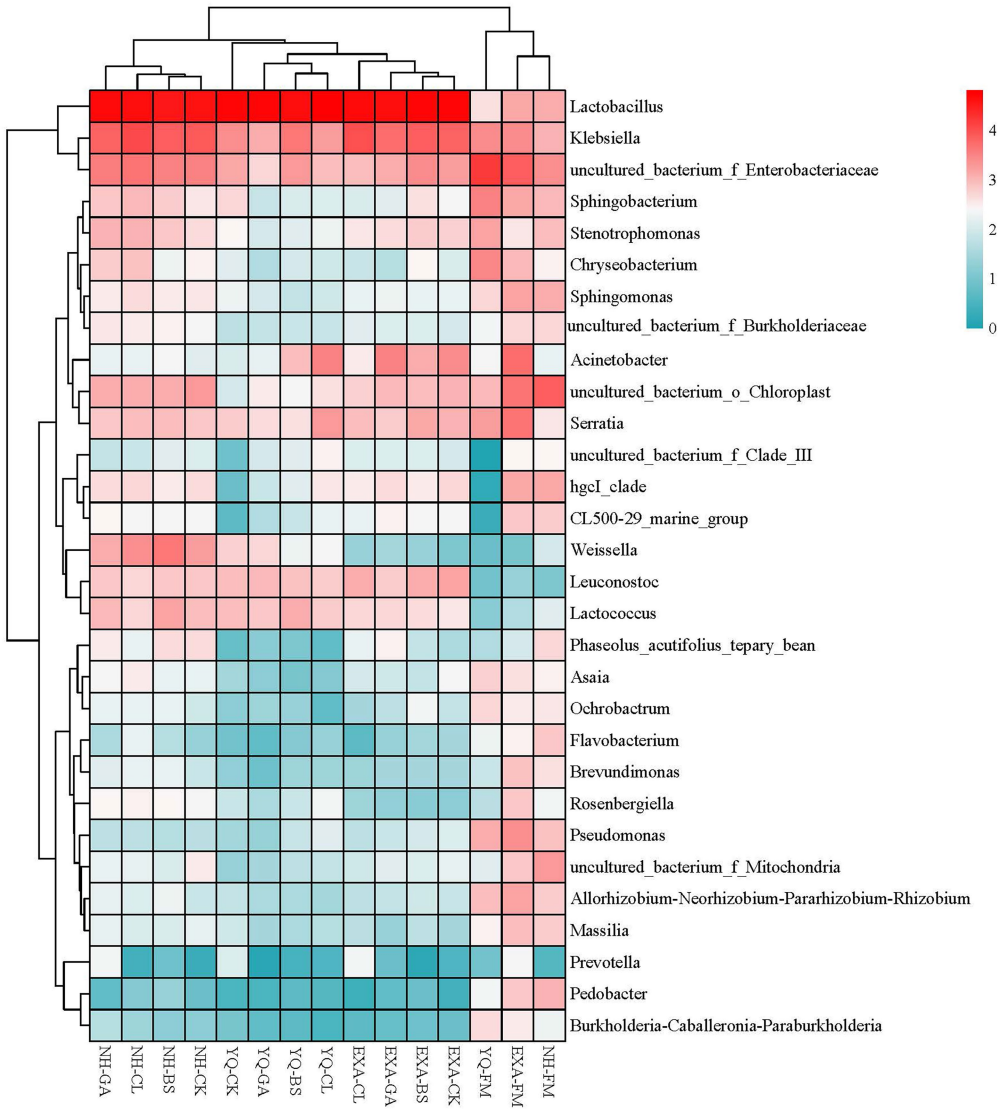

FIGURE 5
Community heat map of bacterial genera for whole-plant corn silage with different treatments. YQ, corn variety Yuqing 386; EXA, corn variety Enxiai 298; NH, corn variety Nonghe35; FM, fresh raw materials; CK, no additives; BS, 0.02% *Bacillus subtilis* CP7 of FM; GA, 0.2% gallic acid of FM; CL, 0.02% cellulase of FM.

the study of Wang et al. (2019b) in alfalfa and stylo silage mixed with Moringa oleifera leaves. This indicates that the WPC silage had better quality in this experiment. It is reported that heterofermentative lactic acid bacteria can produce acetic acid and propionic acid during silage (Xu et al., 2021). We speculated that the fact that propionic acid was not detected in this experiment might be due to the absence of heterofermentative lactic acid bacteria activity during silage in this experiment. In principle, protein degradation will not be avoided during silage. This converts total nitrogen (TN) to non-protein nitrogen (NPN) including small peptides, amino acid free nitrogen, and NH_3-N (He et al., 2020). In addition, ammonia nitrogen is a more accurate indicator of protein hydrolysis, reflecting the deamination of amino acids or peptides (Li et al., 2018). In present study, NH_3-N / TN of GA group and CL group were significantly lower than that of BS group and CK group among all varieties. Wang et al. (2021) showed that gallic acid can reduce the content of NH_3-N in whole plant soybean silage, which is consistent with the results of our test. The deamination of peptides or amino acids in silage may be restricted by the GA addition, resulting in less NH_3-N being produced and greater nutrient protection in WPC silage. FS is calculated by DM and pH, and it is a comprehensive reflection of DM content and pH value (Wang et al., 2017). In this test, FS of each group is greater than 100, which belongs to excellent level. This also shows that WPC is a high-quality raw material for silage production. In all groups, the FS of EXA-GA group was the highest, which also benefited from its higher DM and lower pH. In addition, some

fermentation indexes, such as pH, LA, and NH_3-N / TN, were significantly different among corn varieties. Darby and Lauer (2002) showed that the fermentation quality of whole-plant corn silage would not be different due to different corn varieties. This was different from the results of this experiment, which may be because the corn varieties he selected have the same growth period and similar genetic background, resulting in no difference in the fermentation quality of WPC silage.

Bacterial community diversity of whole-plant corn silage with different treatments

Silage process is a complex microbial symbiosis system, in which many microorganisms participate. Therefore, its structural composition and diversity affect the fermentation quality and nutrients (Zhou et al., 2016). Bacterial alpha diversity is mainly used to reflect species richness, evenness and sequencing depth (Caporaso et al., 2012), which is represented in diversity (Shannon and Simpson indexes), richness (Chao1 index), and OTUs. In this study, in YQ and EXA, the ACE, and Chao1 index of each additive group were higher than those of CK group, and GA group was the highest. In YQ and NH, Shannon and Simpson index of GA group and CL group were lower than CK group, and both of them were the lowest in GA group. This indicates that GA group has high species richness and low biodiversity. According to records, the greater the abundance of dominant bacteria, the lower the diversity of microbial community, and vice versa (Ogunade et al., 2018). This may be due to the fact that the antibacterial and acidic properties of gallic acid inhibit the activities of non acid tolerant microorganisms, thereby increasing the relative abundance of acid tolerant microorganisms (Xu et al., 2019). In addition, among the three varieties, the ACE, Chao1, Shannon and Simpson index are the lowest in YQ, which indicates that YQ silage has lower species richness and diversity. This may be caused by the difference of microbial composition carried by different varieties of corn silage materials, or the difference of microbial community structure caused by different nutritional composition of different corn silage materials.

The results of principal component analysis can be used to distinguish the bacterial communities of different treatment groups. Fresh WPC materials of three varieties (YQ-FM, EXA-FM, and NH-FM) gathered in the second and third quadrants, and all WPC silage gathered in the first and fourth quadrants. The raw materials of the three corn varieties were clearly separated. Therefore, it can be explained that there are differences in bacterial communities among the three different varieties of WPC raw materials. There were also differences in bacterial communities between WPC silage and corn raw materials. This is due to the anaerobic conditions of silage. Under anaerobic conditions, a large number of microbial life activities attached to fresh corn raw materials were inhibited and gradually replaced by facultative anaerobic and acid resistant lactic acid bacteria (Ni et al., 2017).

Bacterial relative abundance of whole-plant corn silage with different treatments

Silage is formed by microorganisms community under extremely complex environment, and bacteria play an important role in the whole fermentation process (Li et al., 2019; Yang et al., 2019). In this study, before silage, *Proteobacteria* was the dominant phylum of fresh raw materials of three varieties of corn, followed by *Bacteroidetes* and *cyanobacteria*. After silage, *Firmicutes* evolved into a new dominant phylum in all treatment groups. However, the relative abundance of *Proteobacteria* decreased significantly and became the second dominant phylum, such results are also reflected in the research of Keshri et al. (2018). Previous studies have shown that *Firmicutes* can produce acid and secrete a variety of enzymes in anaerobic environment, and anaerobic and low pH environment help to promote the growth and reproduction of *Firmicutes* (Wang et al., 2018; Ali et al., 2020). In this experiment, after silage, among corn varieties YQ and NH, the relative abundance of *Firmicutes* was the highest in GA group, and the relative abundance of *Proteobacteria* was the lowest in GA group. This may be because the addition of GA rapidly reduces the pH of WPC silage, which provides strong conditions for the growth and reproduction of *Firmicutes*, and promotes the relative abundance of *Firmicutes* in GA group to increase significantly and the relative abundance of *Proteobacteria* to decrease significantly. *Firmicutes* can decompose macromolecules like cellulose and starch (Romero et al., 2017). Therefore, it is inferred that the changes of lignocellulosic composition in this study may also be related to *Firmicutes*. Furthermore, among the three corn varieties, the relative abundance of *Firmicutes* is the highest in YQ, which might be related to the higher WSC content of YQ, which also indicates that corn variety YQ might have higher potential to make silage.

Further, we studied the changes at genus level of bacterial community in WPC silage. Before silage, the dominant bacteria of different varieties of WPC silage raw materials were different. But mainly *Enterbacteriaceae*, *Chloroplast*, *Sphingobacterium*, *Serratia*, *Chryseobacterium*, *Acinetobacter*, *Rosenbergiella*, and *Mitochondria*. However, the relative abundance of *Lactobacillus*, which plays a major role in the silage process, was very low. After silage, the dominant genus in all groups was *Lactobacillus*, followed by *Klebsiella*, *Enterobacteriaceae*, *Acinetobacter*, and *Serratia*, and different groups have different relative abundances. As is known to all, *Lactobacillus* plays a key role in increasing LA and reducing pH, and high-level *Lactobacillus* has also become a symbol of high-quality silage (Mu et al., 2020). In this study, we observed that the addition of GA to corn varieties YQ and NH increased the relative abundance of *Lactobacillus*. This may be because the acidity of GA rapidly lowered the pH of silage or because its antibacterial properties prevented the growth of undesirable microorganisms and created an ideal environment for the *Lactobacillus* to flourish. However, this phenomenon is not shown in the variety EXA, so it is inferred that this may be caused by the amplification of the differences in the genetic

background of different corn varieties. *Klebsiella* is a facultative anaerobic bacterium, which is a genus of *Enterobacteriaceae* under *Proteobacteria*. It can produce carbon dioxide and cause a variety of diseases (Podschun and Ullmann, 1998; Wang et al., 2022). In this experiment, *Klebsiella* is the second dominant genus in silage, and previous studies have had similar results (Ogunade et al., 2018). Compared with that before fermentation, the relative abundance of *Klebsiella* only decreased in YQ silage, and the use of BS and GA seemed to promote the growth of *Klebsiella*, which was obviously undesirable. However, it is worth noting that in YQ silage and NH silage, the addition of GA inhibited the growth of *Klebsiella* compared with CK group. Hu et al. (2018) reported that adding mixed organic acid salts to WPC silage can reduce the relative abundance of *Klebsiella*, which might be an effective means to improve this phenomenon. Because *Enterobacteria* can compete with LAB to produce acetic acid during fermentation, resulting in the loss of nutrients, it is undesired in silage (Muck, 2010). Although there was a noticeable decline in relative abundance of *Enterobacteria* in the current research compared to that before silage, the relative abundance of *Enterobacteraceae* was still rather high in silage. Fortunately, in YQ silage and EXA silage, the addition of GA and CL has a certain effect on reducing the relative abundance of *Enterobacteria*. Although *Acinetobacter* requires oxygen, it can rely on acetate to survive in an anaerobic environment (Fuhs and Chen, 1975). Previous studies have reported that the increase of the relative abundance of *Acinetobacter* was associated with the increase of acetic acid content (Ogunade et al., 2017). However, this correlation is not reflected in this study, and the specific reasons need to be further studied. *Serratia* usually produces 2,3-butanediol (McDonald et al., 1991), but no 2,3-butanediol was detected in this study.This is irrelevant, because even if *Serratia* exists, it becomes next to nothing after the fermentation process. Remarkably, the addition of GA seems to have a certain inhibitory effect on the survival of *Serratia*, although this effect is very weak. This was might decided by the antibacterial properties of GA. There was another interesting phenomenon, Parvin et al. (2010) found that *Serratia* can be detected in ryegrass silage, but not in raw materials before silage, which is quite different from the results of this test. It may be attributed to the difference of silage materials or geographical location. After all, forage species, geographical location, maturity, the type of fertilizer used and the competition of epiphytic microbial communities are all factors that affect the spatial structure of microbial communities in silage (McGarvey et al., 2013). It can be seen from the community heat map of bacterial genera (Figure 5) that the grouped clusters were mainly grouped into four categories. The fresh corn raw materials were grouped into one class, and the WPC silage of each variety after silage were grouped into three other classes. This was not only consistent with the previous results of this experiment, but also confirmed that corn varieties had an important impact on WPC silage. Additionally, we noticed that among the three fresh corn raw materials, NH and EXA were classified as one class, YQ was classified as another one class and carried less *Lactobacillus*. However, the relative abundance of *Lactobacillus* in YQ after silage was the highest among the three varieties, suggesting that YQ might had high potential for producing high-quality WPC silage. Overall, we screened out the best corn variety among the three test varieties. Moreover, several additives were helpful to improve the quality of WPC silage, but the most significant effect was GA. This indicated the potential of GA as a new silage additive.

Conclusion

It is concluded that, after silage, YQ has higher CP, WSC, LA contents and lower NDF, ADF contents; its *Firmicutes* and *Lactobacillus* has the highest relative abundance on the phylum and genus levels, respectively. The three additives increased the contents of DM, CP and LA to a certain extent, reduced the value of NDF, ADF and NH_3-N/TN, and the value of BDP was relatively higher than that of the CK group; In addition, the used of additives also improved the bacterial community structure, in which the relative abundance of *Firmicutes* on the phylum level and *Lactobacillus* on the genus level were the highest in GA group. The above results showed that YQ quality was the best among the three corn varieties, and GA has the most significant effect on the quality improvement of WPC silage among the three silage additives, which can be recommended to be used in actual operation.

Data availability statement

The data presented in the study are deposited in the NCBI repository (https://www.ncbi.nlm.nih.gov/), accession number PRJNA882789; 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

ZZhan, YW, LiZ, and YL designed the experiment. ZZhan wrote the original draft. YW contributed to the review of the paper and the acquisition of the funding. ZZhan, BZ, and ZZhai conducted experiments. SW, LuZ, and WJ contributed to the experiment methods and material consult. ZZhan and ZZhai performed data analysis and result visualization. All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by the China Agriculture Research System (CARS-38) and the National "13th five-years plan" Key Research and Development Project sub topic of China (2018YFD0502001).

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.2022.1028001/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 17 October 2022
DOI 10.3389/fmicb.2022.933017

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 30 April 2022
ACCEPTED 12 September 2022
PUBLISHED 17 October 2022

CITATION
Chaudhary P, Agri U, Chaudhary A, Kumar A and Kumar G (2022) Endophytes and their potential in biotic stress management and crop production.
Front. Microbiol. 13:933017.
doi: 10.3389/fmicb.2022.933017

Endophytes and their potential in biotic stress management and crop production

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Biotic stress is caused by harmful microbes that prevent plants from growing normally and also having numerous negative effects on agriculture crops globally. Many biotic factors such as bacteria, fungi, virus, weeds, insects, and nematodes are the major constrains of stress that tends to increase the reactive oxygen species that affect the physiological and molecular functioning of plants and also led to the decrease in crop productivity. Bacterial and fungal endophytes are the solution to overcome the tasks faced with conventional farming, and these are environment friendly microbial commodities that colonize in plant tissues without causing any damage. Endophytes play an important role in host fitness, uptake of nutrients, synthesis of phytohormone and diminish the injury triggered by pathogens *via* antibiosis, production of lytic enzymes, secondary metabolites, and hormone activation. They are also reported to help plants in coping with biotic stress, improving crops and soil health, respectively. Therefore, usage of endophytes as biofertilizers and biocontrol agent have developed an eco-friendly substitute to destructive chemicals for plant development and also in mitigation of biotic stress. Thus, this review highlighted the potential role of endophytes as biofertilizers, biocontrol agent, and in mitigation of biotic stress for maintenance of plant development and soil health for sustainable agriculture.

KEYWORDS

biotic stress, endophytes, plant growth, sustainable agriculture, biocontrol

Introduction

Agricultural strengthening is an important factor to the food safety for the rising world population. The recovery of soil fertility and crop heath by the usage of chemical fertilizers not only affects the soil health by decreasing the water holding capacity, depleting soil fertility, and diminishing soil nutrient and microflora but also poses a

threat to human health and ecosystem. By considering all these problems, researchers are attentive for the substitution of chemical fertilizers with microbial-based fertilizers (Granada et al., 2018). Application of endophytes as biofertilizers can be a better approach to improve soil microbial status that stimulates the natural soil microbiota, therefore influencing nutrient accessibility and decomposition of organic matter (Fasusi et al., 2021). Endophytes are microbes that live within the host plant and have the capability to colonize plant roots without causing harm to the plants. They increase plant growth, act as biocontrol agent and protect the host from pest naturally, and endure tolerance against numerous biotic/abiotic stresses. Endophytes capable of producing several growth hormones such as IAA, ACC deaminase, increased in uptake of K ions in plant tissues, and decreased ethylene level are an alternate mechanism to alleviate stress conditions in various plants (Fan et al., 2020; Agri et al., 2022). They are also able to improve the uptake of nutrients such as nitrogen, magnesium, zinc, sulfur, and phosphorus from soil and provide to the host plant for better growth and survival (Agri et al., 2021).

Both bacterial and fungal endophytes hold tremendous potential for being used as biocontrol agent. Endophytes show antagonistic activity against disease-causing phytopathogens and diminish the damage attributed to phytopathogens. They produce several bioactive antimicrobial and antiviral metabolites along with producing various antioxidants to suppress pathogens (Gouda et al., 2016). Moreover, diverse range of fungal species especially entomopathogenic fungi have been known to exert long-term preventive measure for insect population (Litwin et al., 2020). Different bacteria such as *Bacillus*, *Pseudomonas*, *Pedobacter*, and *Acidobacterium* involved in mineral solubilization, metabolite production, and N_2 fixation. Several fungal strains including *Beauveria bassiana*, *B. metarhizium*, *M. robertsii*, *Chaetomium globosum*, and *Acremonium* spp. are successful in plant protection (Grabka et al., 2022). With a wide host range, endophytic fungus becomes advantageous as compared to other biocontrol agents. Notably, *Trichoderma viride* isolated from *Spilanthes paniculata* showed broad range activity against *Colletotrichum capsici*, *Fusarium solani*, and *Pythium aphanidermatum* (Qi et al., 2019).

Crop plants undergo various environmental stresses during their growth period that ultimately results in reduced crop productivity. Genetic and physical growth alteration due to several environmental cues restricts the full plant development in their growth period. One such biotic stress occurs by the recurrent attack on plants by phytopathogens such as bacteria, virus, fungi, and herbivores, which ultimately reduce plant vigor and death of host plant in extreme conditions (Pandey et al., 2017). In agricultural field, biotic stress especially caused by bacteria and fungal phytopathogens is the major cause of pre- and post-harvest losses. Plant being sessile in nature responds to stress conditions accordingly through various stimulatory mechanisms. They have evolved unique physiological, biological, and molecular adaptation strategies to adjust the adverse conditions and promote plant growth. However, the extent of stress and climatic extremity makes them unable to cope up with the challenges raised by the environment (Chitnis et al., 2020). The generalized defense system in plants is unable to fully relieve the pressure and meet the demands of multistress tolerance to thrive and survive. So far, genetic engineering and other chemical and physical methods have been used to get stress tolerant cultivars. But they do not provide stress tolerance capacity for a very long time, and also, they are not ecofriendly. Thus, harnessing the potential of beneficial endophytes present in the nature for disease management could be an alternative strategy for improving plant resistance and resilience in crop varieties (Zheng et al., 2021). This will not only reduce chemical inputs but mitigate environmental stress without causing adverse effects. Useful endophytic microbes residing in the plant tissues are promising measure to remediate stressful conditions in a natural way.

Endophytes

Plants are associated with a wide range of microbial community having positive, negative, or neutral kind of response in their host plant. Majority of the research is focused on the known epiphytic beneficial microbes colonizing the rhizosphere zones. However, plant growth-promoting endophytes are the subset of rhizosphere microbiome that is important determinants of plant microecosystems (Khati et al., 2018; Chaudhary et al., 2021a). The potential of endophytes as a bioinoculant is thus far to be sightseen to the completest potential due to few shortcomings. Such endosymbiont groups of microbes are diverse and harbored in almost every other plant species found in nature (Nair and Padmavathy, 2014). They mutually reside and proliferate within the plant tissues such as stems, roots, seeds, fruit, buds, and leaves deprived of producing any damage to the host plants (Specian et al., 2012). A small change in the diversity of plant endophytic communities can have significant impact over plant growth regulation and environmental adaptation (Vandenkoornhuyse et al., 2015). Gradual co-evolution in plant endophytic associations has eventually led to a positive response toward each other existence and influence vital activities in their host plant (Wang and Dai, 2011).

Endophytes are potent microbial resource needed to be explored for their application in agriculture sector. Most of the beneficial growth-promoting species belongs to the facultative group of endophytes that live in soil freely but colonizes crop plants under suitable conditions (Gaiero et al., 2013). Almost every other plant species hosts various bacterial, fungal, or actinomycete endophytes that may regulate plant and soil health. Various plant growth parameters are regulated by the colonization of endophytes and based on the microenvironment

and the host's metabolic capacity; they biosynthesize various compounds emanating growth-promoting activities similar to rhizospheric microbes (Chaudhary et al., 2021b, 2022). They maintain stable symbiosis through secreting various bioactive compounds contributing to colonization and plant growth (Gouda et al., 2016). The attributes associated with endophytes include the production of extracellular enzymes (Khan et al., 2014), bioremediation, synthesis of secondary metabolites against phytopathogens (Mousa and Raizada, 2013), and induced systemic resistance (Constantin et al., 2019). But mainly, endophytic bacterial and fungal strains confer propound impacts on the overall health and maintenance of crop plants under different environmental conditions *via* nitrogen fixation, phosphate solubilization, siderophore, and phytohormones production and by conferring tolerance to various stresses. Additionally, N-fixing endophytes *Novosphingobium sediminicola*, *Ochrobactrum intermedium* (from sugarcane) and *Bradyrhizobium*, *Kosakonia*, and *Paraburkholderia* (from rice) carry nitrogen fixation genes (Muangthong et al., 2015; Okamoto et al., 2021).

Both climatic and edaphic factors equally contribute to the nature and action of endophytes toward plants (Kandel et al., 2017a). Under different condition, they also enhance the levels of plant growth-promoting hormones (cytokines, gibberellins, and auxin) and facilitate nutrient cycling whenever required (Egamberdieva et al., 2017; Chaudhary and Sharma, 2019). Few are known to produce polyamines, including putrescine, cadaverine, spermidine, and spermine, which involved in lateral root development and stress adaptations (Couee et al., 2004). Numerous growth-promoting bacterial and fungal endophytes have been reported till date. Microbial symbionts are suitable to maximize crop productivity, but more research is required to understand the significance in plant growth (Chaudhary et al., 2021c). However, complete understanding of the mechanisms and the genetic regulation utilized by endophytes in plant growth regulation is an important aspect to be studied for their application under field conditions.

Diversity and distribution of endophytic microbes for maintenance of soil health and plant productivity

Microbial root endophytes

Roots are the main habitat and colonization route for the bacterial and fungal endophytes. The main entry points for bacterial colonization are root hairs, root cracks, or wounds formed by microbial or nematode activities. The other major sites for root colonization include intercellular spaces in cortex and epidermis (Compant et al., 2005). Endophytes such as *Pseudomonas putida* and *P. fluorescens* colonized the olive through root hairs (Mercado-Blanco and Prieto, 2012). An axenically phytopromotional fungal root endophyte *Piriformospora indica* begins root colonization in the cortex region by a biotropic growth phase and continues with cell death-dependent phase. Inoculation of *P. indica* promotes plant growth, early flowering, higher seed yield, and adaptation to stresses in various host plants such as *Phaseolus vulgaris*, *Triticum aestivum*, and *Cicer arientum* (Varma et al., 2012; Ansari et al., 2014).

Rhizospheric microorganisms are enriched with nutrients and influence plant growth through soil nutrient recycling and nutrient uptake (Kukreti et al., 2020; Kumari et al., 2020). Overall root endosphere is metagenomically diverse and most often dominated by beneficial *Proteobacteria* (50%), *Actinobacteria*, *Firmicutes*, and *Bacteriodetes* (10%) (Liu et al., 2017). In association with roots, such microbe produces several compounds that influence plant development. Plant hormones such as gibberellins, cytokinins, and indole acetic acid (IAA) highly facilitate plant growth. In addition, few are known to promote plant mycorrhization. For instance, ACC deaminase (1-amonocyclopropane-1-carboxylic acid) containing *Arthrobacter protophorniae* enhanced nodulation in *Pisum sativum* (Barnawal et al., 2014). The other best-known fungal root colonizers are known as dark septate endophytes (DSE). The *Phialocephala fortinii* s.l- Acephala applanata species complex (PAC) species of *Ascomycetes* are the DSE fungi in forestry systems. In the study, dual inoculation of PAC positively increases plant biomass in spruce (Reininger and Sieber, 2013).

Endophytes living under extreme conditions such as Antarctica are also known to boost crop productivity. Under stressed condition, where mycorrhizae are generally low in abundance, different fungal endophytes potentially act as the prime root mutualistic symbionts (Mandyam et al., 2010). In terms of increasing nutrient acquisition of nutrients such as phosphorus from the roots and increasing the host fitness, both root-associated endophytes and mycorrhizal fungi provide benefits in a very similar manner. However, furthermost fungal endophytes do not endure an obligate biotrophic life phase and live at smallest part of their life cycle separated from the plant (Park and Eom, 2007). The only two known vascular plant, i.e., *Colobanthus quitensis* and *Deschampsia Antarctica* from such extreme condition harbors *Penicillium* species. *Penicillium* (root endophyte) helps in growth of vascular plants in Antarctic region *via* enhancing nitrogen acquisition and nutrient uptake by significantly increasing yield. The mechanism involved in nitrogen acquisition is attributed to the litter protein breakdown and amino acid mineralization (Oses-Pedraza et al., 2020). In total, two fungal strains isolated from Antarctic plants rhizosphere, i.e., *Penicillium brevicompactum* and *P. chrysogenum* isolated from plants rhizosphere, i.e., *Colobanthus quitensis* and *Deschampsia Antarctica* increased

the final yield by 42% in lettuce and 68% in tomato plants in comparison with control (Molina-Montenegro et al., 2020). Several genera of beneficial root endophytes have been reported from medicinal plants such as *Pseudomonas*, *Xanthomonas*, *Bacillus*, *Inquilinus*, and *Pedobacter*. They have been associated with stimulation of growth activities such as production of secondary metabolites, solubilizing phosphate, and upregulating the expression of certain stress regulating genes under stress conditions (Rat et al., 2021).

Horizontal transmission colonization of the root endosphere *via* the rhizosphere. Types of endophytes: Passive endophytes – They penetrate through cracks present at root emergence area, root tips, or those created by pathogens; facultative endophytes – They live exterior to the host in certain phase of their life cycle and are frequently allied with plants from its adjoining soil; obligate endophytes – they depend plant metabolism for their survival; endofungal bacteria – Bacterial symbionts of fungi occur inside fungal spores and hyphae.

Endophytic community in aerial tissues (phyllosphere)

Not all endophytes enter *via* root zones and move through the xylem vessels, they harbor diverse communities that enter the aerial tissues *via* above-ground surfaces too. Different entry routes chosen by many plant-promoting endophytes are stem (laimosphere), fruits (carposphere), leaves (Phyllosphere), seeds (spermasphere), and flowers (anthosphere) (Lindow and Brandl, 2003). Endophytes that live within the leaf tissues and stems are well documented. Phyllosphere microbes are an important component of microbial communities that live asymptomatically within leaves and also known for plant health maintenance (Ritpitakphong et al., 2016). Besides being the largest microbial habitat on Earth, the functional roles of phyllosphere residents are still less understood over the rhizosphere microbiome. It is estimated that their abundance in nature may exceed 10^{62} cells globally. *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most abundant phyla associated with *A. thaliana*, *Populas*, and *Salix* (Redford et al., 2010; Firrincieli et al., 2020). The most abundant genus of phyllosphere region is *Pseudomonas* in tomato plants (Dong et al., 2019). Leaf endophytes including bacteria and fungi are the subset of phyllosphere endophytes. Leaf endophytes most of the times comprise five phyla, *Proteobacteria* (90%), *Actinobacteria* (2.5%), *Plancomycetes* (1.4%), *Verrucomicrobia*, and *Acidobacteria* (1.1 and 0.5%) (Romero et al., 2014). They live inside the leaf and maintain symbiotic relationship with the host plants.

It is evident to suggest that endophytes enter leaves and stems through openings such as stomata and hydathodes through dispersion with the help of rain, soil, or pollinators (Frank et al., 2017). For instance, *Gluconobacter diazotrophicus* enters through stomata in sugarcane plants (James et al., 2001). After reaching this site, endophyte strains multiply and form a thin layer of biofilm. Apart from this, some may enter to the inner tissues and start residing as endophytes where further microbes could colonize themselves into xylem. They further colonize and multiply in different organs including anthrosphere, phylloplane, carposphere, and caulosphere (Meyer and Leveau, 2012). Numerous growth-promoting foliar endophytes have been identified through high-throughput screening procedures. Despite this, the gaps still hinder their field application and practical exploitation in agriculture. Not only bacterial species but also fungal strains equally promote plant growth through nutrient recycling, i.e., carbon and nitrogen, provide resistance to pathogens and assist in leaf litter decomposition (Arnold et al., 2007). Various fungal species such as *Penicillium aurantiogriseum*, *Fusarium incarnatum*, *Trichoderma harzianum*, and *Fusarium proliferatum* have been reported from wheat plant (Ripa et al., 2019). Seed-borne endophytic microbes are not fully explored and are of great interest. They potentially produce phytohormones, enzymes, and antimicrobial compounds and improve plant development. The main property of seed endophytes is their vertical transmission. Such microbes are naturally useful in that they signify not only a termination for the community assemblage in the seed, but also an early idea for community gathering in the new seedling (Shahzad et al., 2018). Seed-borne endophytes (bacterial and fungal) benefit seeds by facilitating the germination of seeds in soil.

They are of great interest because they pass their characters to next generation through vertical transmission. This provides important traits in plant growth which are determined by both microbe and plant genomes. Also, seed consists of a limited range of microbial species and has progressed *via* co-selection with the host plant species (Vujanovic et al., 2019). Additionally, this could probably result in reducing the phytopathogenic asset in demand to the sustenance of plant development (Cope-Selby et al., 2017). In addition, they have the ability form endospores and maintain plant growth by phytase activity, regulating cell motility, modulating endogenous phytohormones such as cytokinins that break seed dormancy, enhancing soil structure, and degrading xenobiotics. For instance, fungal endophytes *Epichloe* are stated to support their host plants in growth promotion. Similarly, fungi *Penicillium chrysogenum*, *Trichoderma*, and *Phoma* sp. isolated from *Opuntia* spp. are known to be involved in seed germination (Delgado-Sánchez et al., 2013). In a study, *Paraburkholderia phytofirmans* PsJN actively colonized different seeds of maize, soy, and pepper. Also, wheat seeds colonized with *Paraburkholderia phytofirmans* PsJN showed significant alteration in spike onset compared with non-treated plants under pot and field experiments (Mitter et al., 2017). There are different pathways adapted by seed-borne endophytes. Few enter *via* xylem tissues, through stigma and

exogenous pathway where seeds are dirtied from the exterior source. The floral parts of the plant tissue have not been studied extensively for the growth-promoting endophytes. An endophytic fungus, *Lasiodiplodia* sp. ME4-2 isolated from floral parts of *Viscum coloratum* which involved in production of important metabolites regulating plants growth such as indole-3 carboxylic acid and secondary metabolites such as 2-phenylethanol (Qian et al., 2014).

Endophytic plant growth-promoting mechanisms

Endophytes being potential agent impart beneficial effects on their host plant are well-acknowledged inoculants to encourage the plant growth directly/indirectly. Plant growth occurs directly (endophyte–pathogen interaction) through regulating the attainment of vital nutrients such as phosphorous and nitrogen, modulating level of hormones. Indirectly through enhanced plant defense, endophytes could help in biocontrol of phytopathogens by production of antibiotics, regulating defense mechanism by induced systemic resistance, declining the quantity of iron accessible to pathogen, and pathogen inhibition through volatile compounds (**Figure 1**). Here are the few direct mechanisms involved in plant development.

Production of phytohormones

Numerous endophytes are identified to produce plant growth hormones (**Supplementary Table 1**). Hormones stimulate plant growth through regulating structural and morphological changes in response to gravity or light stimuli. They secrete gibberellic acid, cytokinin, auxins such as indole acetic acid, and ethylene. They do not only increase the overall root biomass through enhancing root surface area and root length but are known to act as signal molecules between endomicrobes and plants (Spaepen et al., 2007). In addition, they have been well known to enhance root length and root surface area, control the rate of vegetative growth, and increase the rate at which root and xylem develop. Other indole-related compounds such as indole-3-lactic acid (ILA) and indole acetamide (IAM) also found in different endophytic strains such as *Azospirillum brasilense* which is formed as an intermediate during the auxin biosynthetic pathways. For instance, the root endophyte *Piriformospora indica* produced auxin through utilizing IAA biosynthetic pathway (Xu et al., 2018). The IAA production by endophytes is considered an important factor in plant growth regulation. Khan et al. (2014) reported that *Sphingomonas* sp. (endophyte) isolated from the foliar region of *Tephrosia apollinea* improved growth activity in tomato plants through indole acetic acid (11.23 μm mL^{-1}). In another study, *Micrococcus yunnanensis* RWL-2, *Pantoea dispersa* RWL-3, *Micrococcus luteus* RWL-3, and *Staphylococcus epidermidis RWL-7* were analyzed using GC-MS and found to produce IAA (11.50–38.80 μg ml^{-1}). When inoculated in rice plants, they significantly increased main growth-promoting attributes in rice plants, i.e., dry biomass, shoot and root length, chlorophyll, and protein contents (Shahzad et al., 2017). Endophytic fungi (*Falciphora oryzae*) helped in lateral root growth while reduced the primary root height (Sun et al., 2020). Also, IAA activity in endophytes also reported to increase nitrogenase activity in rice through showing transcriptional changes in nitrogen-fixing root nodules (Defez et al., 2016). Fungi are also able to produce gibberellins, auxins, and cytokinins important as chemical signaling. Endophytic fungus (*Porostereum spadiceum*) produces gibberellins and rescue growth of soybean under normal and salt affected by promoting seed germination and increasing chlorophyll content (Hamayun et al., 2017). Several endophytic fungi including *A. flavus*, *Paecilomyces formosus*, *P. glomerata*, *Penicillium corylophilum*, *Rhizopus stolonifer*, and *Pochonia chlamydosporia* (Khan et al., 2012). Almost all the gibberellic acid producing fungal endophytes belong to *Ascomycetes* group; however, *P. spadiceum* belonging to the Basidiomycota is the first endophyte to produce gibberellic acid and involved in phytostimulation (Waqas et al., 2012). Cytokinins are important group of plant hormones that are involved in apical dominance, chloroplast maturation, cell proliferation and differentiation, seed germination, prevention of senescence, and plant–pathogen signaling mechanisms. Bacterial endophytes *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, and *Arthrobacter* sp. isolated from humic-treated cucumber plants produced several cytokinins (cis-zeatin cytokinin, riboside type zeatin, isopentyladenine, and isopentenyladenosine) greater than 30 pmol/ml (De Hita et al., 2020).

Endophytic diazotrophic bacteria as biofertilizer

Endophytes being successful colonizers of different plants act potentially as biological nitrogen fixers and act as an alternative nitrogen source for crop production. They face less competition over other rhizospheric microbes and directly fix atmospheric N_2 make it accessible to plants. Moreover, the partial pressure of oxygen inside the plant tissue is suitable in comparison with the outer surface for efficient nitrogen fixation as low partial pressure supports the proper functioning of O_2-sensitive nitrogenase enzyme (Cocking, 2003). Nitrogen is a vital macronutrient that the plants require because it promotes

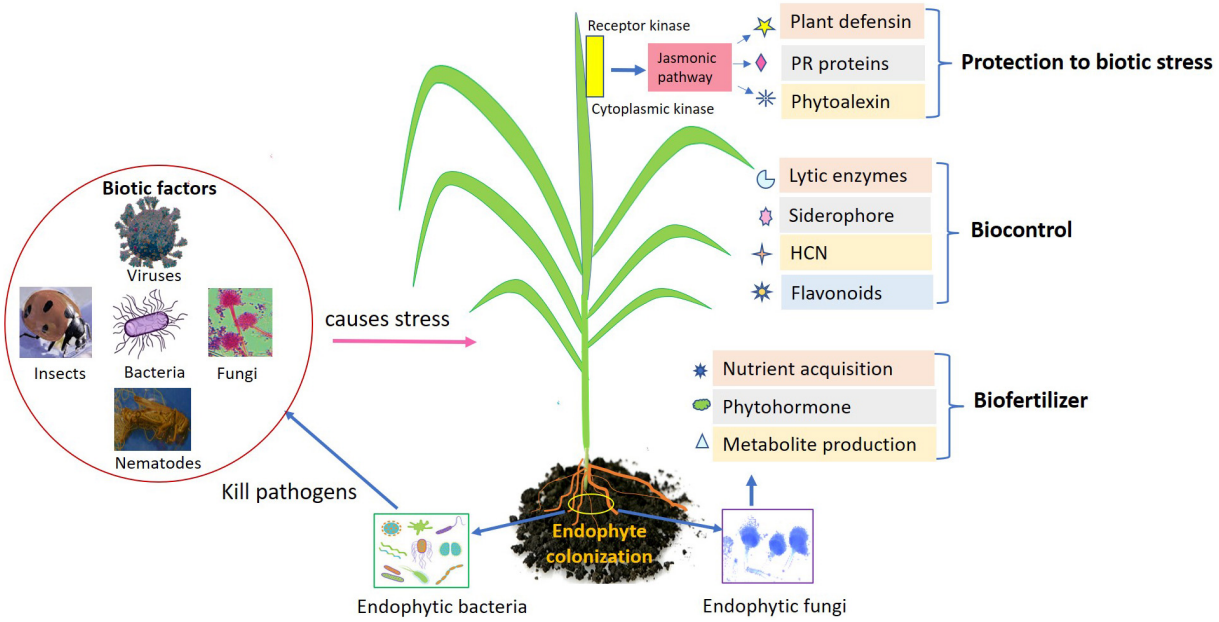

FIGURE 1
Role of endophytes as biofertilizers, biocontrol, and biotic stress management in agricultural crops.

shoot growth and aid in reproduction and main constituent of chlorophyll. Dinitrogen is an inaccessible form of nitrogen present in air and converted by diazotrophs into soluble, non-toxic form ammonia *via* biological process of nitrogen fixation. The ammonia-oxidizing bacteria and the nitrifying bacteria then transform this ammonia into nitrite and nitrate, respectively. Denitrifying occurs in the deeper soil horizons, converting the unused nitrate to atmospheric nitrogen, which ultimately escapes to the atmosphere as dinitrogen gas. This is the usual nitrogen cycle pathway (Mahanty et al., 2017). Several nitrogen-fixing bacteria have been reported such as *Azospirillum brasilense*, *Acetobacter diazotrophicus*, *Klebsiella oxytoca*, *Rhizobium* sp., and *Burkholderia cepacia* (Kong and Hong, 2020). In addition, various non-leguminous plants such as wheat, sorghum, maize, and rice harbor free-living nitrogen-fixing bacteria. For instance, *Gluconacetobacter diazotrophicus*, *Herbasprillum rubrisubalbicans*, and *Burkholderia silvantantica* can fix nitrogen in the intercellular spaces of sugarcane stems (Lery et al., 2011). Endophytes isolated from rice such as *Bradyrhizobium sp. and Paraburkholderia* sp., showed acetylene reduction properties and high sugar content contributing to high nitrogen-fixing ability. High content of sugar in different crops such as sweet potato, pineapple, and sugar has known to assist endophytic N-fixing activity among non-leguminous plants (Okamoto et al., 2021). *Acetobacter diazotrophicus* and *Azoarcus* isolated from sugarcane and kallar grass potentially fixed atmospheric nitrogen up to 150 kg N ha^{-1} $year^{-1}$ (Gupta et al., 2012).

Phosphate solubilization

Phosphate solubilization is an important mechanism involved in solubilizing the insoluble phosphate into soluble form like orthophosphate. Plant requires a major amount of phosphorus for enhanced productivity in the range of 30 μmol l^{-1}, but limited amount is available to plants which make this nutrient a limiting factor in soil. Endophytes have the capability to solubilize unsolvable phosphates or have the ability to liberate organic phosphates though production of acids such as malic, gluconic, and citric acids. Endophytic bacteria that have been reported to mobilize phosphorus through mineralization and solubilization include *Pseudomonas* spp., *Bacillus megaterium*, *Azotobacter*, *Paenibacillus*, *Thiobacillus*, and *Serratia* (Jahan et al., 2013; Kang et al., 2014).

Pseudomonas fluorescens strains isolated from *Miscanthus giganteus* showed great variation in phosphate solubilization capacity with highest solubilization recorded about 1,312 mg L^{-1}. Furthermore, when inoculated with the potential strains, high weight of shoot and root was observed in pea plants as compared to control (Otieno et al., 2015). The major endophytic fungi belong to genera *Curvularia*, *Piriformospora*, *Penicillium*, and *Aspergillus* and *Trichoderma.* Symbiotic association of mycorrhizal fungi with plants has been recognized to surge the passage of phosphorus in plants. It is evident from a study that apart from mycorrhizal associations, endophytic bacteria equally contribute to the P solubilization. Poplar samples when inoculated with P solubilizing *Rahnella* and *Burkholderia* sp.

strains showed a root architecture with greater root volume under tomography-based root imaging (Varga et al., 2020). Endophytic fungi *Penicillium* and *Aspergillus* isolated form roots of *Taxus wallichiana* solubilized P and produced phosphatase and phytase enzymes (Adhikari and Pandey, 2019). Kang et al. (2014) observed that *Bacillus megaterium* regulates the content of amino acids and carbohydrates to promote the growth of mustard plant.

Siderophore biosynthesis

Siderophores are low molecular weight composites produced by several microorganisms including endophytes to scavenge iron and make it available to plants. Endophytes are known to synthesize hydroxamate, carboxylate, and phenolate type of siderophore to converse plant protection against phytopathogens. It also assists plant growth and yield by providing iron to plants under iron deficient conditions (Rajkumar et al., 2010). It also facilitates better nutrient mobilization in comparison with rhizospheric counterparts. They are better adapted to the activities of internal tissues of the plants, in terms of originating from the internal microbiome (Verma et al., 2021). Large numbers of bacterial endophytes are there to contain property of iron chelation such as *Azotobacte*r, *Bacillus*, *Enterobacter*, *Arthrobacter*, *Nocardia*, and *Streptomyces* (Bokhari et al., 2019).

Biofortification of *Enterococcus hirae* and *Arthrobacter sulfonivorans* in wheat grains not only efficiently makes bioavailability of iron and zinc micronutrients but it also significantly increases plant growth up to 20% in comparison with control (Singh et al., 2018). Bacterial siderophore (catechol and hydroxamate type) isolated from *Arabidopsis thaliana*, *F. rubra* and *Agrostis capillaris*, growing on the heavy metals contaminated area significantly improved growth rate in *Festuca rubra* and *Brassica napus* (Grobelak and Hiller, 2017).

Role of endophytes as biocontrol agents

Many researchers have previously reported the use of bacterial and fungal endophytes for disease management in plants. *Serendipita indica* conferred resistance against *Fusarium* and *Rhizoctonia solani* and demonstrated antioxidant capacity *in vitro* (del Barrio-Duque et al., 2019). In another study, production of Bacillomycin D protein by *Bacillus amyloliquefaciens* helped in showing antagonistic activity against fungus *Fusarium graminearum* (Gu et al., 2017). Seed application of *B. bassiana* 11-98 efficiently colonized tomato and cotton seedling and protect plants against *Rhizoctonia solani* and *Pythium myriotylum*. Possible mechanisms were coiling of hyphae, induction of resistance, and production of lytic enzymes, thus protecting the older plants from root rot. However, biocontrol practices through endophytes may be achieved through direct inhibition of pathogens or indirectly by establishing the plant's systemic resistance (Santoyo et al., 2016). The other involved mechanisms include competition for niche and resources, production of cell wall degrading enzymes, initiation of induced systemic resistance (ISR), and quenching the quorum sensing of pathogens (Rajesh and Rai, 2014). Apart from this, several antibiotic compounds and lytic enzymes produced by endophytes reduce disease severity in many plants. For instance, many fungal genera *Fusarium*, *Trichoderma*, and *Botryosphaeria* secrete enzymes such as cellulose, 1,3- glucanases, amylase, and glutaminase which can aid in reducing phytopathogens through inhibiting the cell wall (Ait-Lahsen et al., 2001). Biological control also depends upon many factors such as host specificity, physical structure of soil, inoculum used, and the prevalent environmental conditions. The ability to colonize the plant tissue makes them a better biological control agent than others in having better biological compatibility when applied to plants (Rabiey et al., 2019). Under genomic studies, endophytes were also found to contain several notable genes pertaining to pathogenesis regulation which were previously not found in rhizospheric bioinoculants (Brewer et al., 2016). Also, endophytes are more protected from external factors such as radiations, temperature, and pressure when compared to epiphytes (Andreote et al., 2014). However, a deeper understanding on their mechanism and mode of action is still required to better exploit endophytes as biocontrol agents. Here are the few mechanisms employed by endophytes in controlling diseases in plants.

Production of secondary metabolites with antifungal and antibacterial properties

Most of the endophytes are known to produce secondary metabolites exhibiting good antibacterial and antifungal activities preventing the growth of harmful microorganisms. Various metabolites such as alkaloids, phenols, flavonoids, peptides, steroids, and terpenoids are isolated from both bacterial and fungal endophytic strains (**Supplementary Table 2**). Alkaloids possess firm potential in inhibiting the proliferation of microbes. Fungal endophytes such as *Clavicipitaceae* sp. isolated from grass family showed production of alkaloids, which are harmful for aphids (Panaccione et al., 2014). Alkaloids are identified as to contaminate precise hosts and causes slight damage to non-target organisms. Altersetin alkaloid isolated from *Alternaria* spp. displayed a strong antibacterial effect on pathogenic bacteria (Hellwig et al., 2002; Akutse et al., 2013). GS-MS analysis showed production of thermostable metabolites such as d-norandrostane and

longifolenaldehyde by *A. alternata* AE1 isolated from neem leaves. Both the compounds have bactericidal and antioxidant properties and showed zone of inhibition against numerous gram-positive and gram-negative bacteria (Chatterjee et al., 2019). Gond et al. (2015) evaluated the effect of antifungal proteins such as iturin A, bacillomycin, and fengycin isolated from *Bacillus* spp. in controlling fungal pathogen *Fusarium moniliforme*. Antifungal protein designated as Efe-AfpA isolated from *Epichloe festucae* showed disease resistance against pathogen *Sclerotinia homoeocarpa* causing dollar spot disease (Tian et al., 2017). Apart from this, many endophytes are widely reported being associated with antibiotic activity. Lipopeptides produced by several endophytes may show antimicrobial and surfactant activities and well known for their antibiotic activity. *Bacillus amyloliquefaciens* strain produces lipopeptides having biocontrol activity toward *Erysiphe cichoracearum* (fungal pathogen). The fengycin, iturin, and surfactin produced by *Bacillus* sp. helped in inhibiting the growth of fungal pathogen. Also, pellicle biofilm formation affected the colonization ability of pathogens (Jiao et al., 2021).

Bio control strategies through quorum quenching

Quorum sensing (QS) is a signaling mechanism that controls growth and metabolism in single-cell microorganisms such as bacteria. Density-dependent cell-to-cell communication controls most of the traits which are helpful in endophytes as well a key controller of virulence in pathogens (Frederix and Downie, 2011). The factors responsible for virulence such as biofilm formation, toxin production, antibiotic resistance, exopolysaccharides (EPS), and degradative exoenzymes secretions are highly regulated by quorum sensing signaling. This mechanism takes place *via* small diffusible signaling molecules called autoinducers (Seitz and Blokesch, 2013). For instance, many pathogenic bacteria such as *Pseudomonas* and *Ralstonia* primarily use acylated homoserine lactones (AHLs) to communicate while producing virulence (Mansfield et al., 2012). They cause great damage to crops. Therefore, antiquorum sensing approach could be harnessed to trigger the phenotype of pathogen to block infection (Chen et al., 2013). Quenching process is regulated by interfering with virulence-associated activities such as modification of signals, catalysis of degrading enzymes such as AHL-lactonase, and inhibition of signal synthesis (Dong et al., 2002). Lactonase enzyme works through removing the lactone ring from the acyl moiety of AHLs and ultimately inactivates AHLs (Murugayah and Gerth, 2019). Endophytic bacteria and fungi provide plethora of bioactive molecules, which can act as an inhibiting agents including QS quenching enzymes such as lactonase, acyclase, and QS inhibitor molecules (LaSarre and Federle, 2013). Such agents can provide promising approach to control phytopathogens and suppress virulence expression in them. They assist in degrading quorum-sensing signals from pathogenic microbes and disrupt intercellular communication (Rutherford and Bassler, 2012). Endophytes with quorum quenching activity attenuate virulence factors rather than killing the microbes or limit the cell growth. This property effectively reduces the selective pressure associated with bactericidal agents (Chen et al., 2013). QS and *in- silico* analysis showed antiquorum sensing and antibiofilm potential of *Alternaria alternata* isolated from *Carica papaya* against pathogen *Pseudomonas aeruginosa*. Significant decrease in cyanin, alginate, and rhamnolipid production was observed. Protease activity such as LasA protease activity and Las B protease activity responsible for virulence was correlated with decrease in biofilm formation (Mishra et al., 2020).

Endophytes such as *B. firmus* and *Enterobacter asburiae* PT39 showed effective degrading capability of AHL by preventing violacein production (80%) in biosensor strain. Still, cell-free lysate when applied to *P. aeruginosa* PAO1 and PAO1-JP2 biofilm caused decrease in biofilm formation (Rajesh and Rai, 2014). In a study, AHL-degrading bacteria *Pseudomonas nitroreducens* potentially degraded diverse variety of AHL including *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) in *D. Zeac* EC1. It fully degraded OdDHL (0.2 mmol/L) in 48 h. Furthermore, the application of this strain as a biocontrol agent might considerably reduce soft rot disease produced by *D. zeae* EC1 to suppress tissue maceration in numerous host plants (Zhang et al., 2021). These observations demonstrate that QQ strains have huge potential to reduce the disease harshness due to QS-modified pathogenic bacteria. Antivirulence activity can also be achieved by an engineered endophytic bacterium through introducing quorum-quenching gene. For instance, to control *Burkholderia glumae* which causes grain rots of rice, an *N*-acyl-homoserine lactonase (*aiiA*) gene from *Bacillus thuringiensis* was inoculated into *Burkholderia sp.* KJ006 to repress *N*-acyl-homoserine lactone (Cho et al., 2007). Thus, quorum-quenching microbes provide great potential as biocontrol agents. There are several advantages of introducing quorum-quenching microbes into plants. Being compatible in nature endophytes occupies most of the cellular space without leaving space for later-invading phytopathogens (Kung and Almeida, 2014).

General plant defense responses against biotic stress

Plants are attacked by various pathogens, parasites, and herbivores, all of which cause biotic stress. Various pests belonging to Lepidoptera, Hemiptera, Orthoptera, and Diptera are well known for damage crop plants. Pests destroy more than 40% of the world's crops every year (FAO, 2021). Also,

the fungal parasites are hidden robbers that inhibit the plants growth either by killing the host cell through secretion of toxin or biotropic fungi that feed on living host cell. Host plants become a source of nutrients for such harmful parasites. In some biotropic fungi, haustoria plays a major role in absorbing nutrients from host tissues (Szabo and Bushnell, 2001). Plant viruses also cause leaf chlorosis, spotted wilt, stunted growth in several important plants such as tomato, cucumber, potato, and sugarcane (Roossinck et al., 2015). In addition, nematodes feed on different plant parts (seeds, roots, flowers, leaves, and stems) and cause wounds on the plants. Quick reproduction ability in mites and insects also makes them vectors of other pathogens such as virus and bacteria (Maafi et al., 2013; Adam et al., 2014).

Plants have evolved a plethora of defense mechanisms to combat broad-spectrum pests and pathogens (Rejeb et al., 2014). The defense mechanism could be performed, with toxic metabolites deposited, and it could be inducible. Upon pathogen attack, the innate immune system gets activated that prevents the pathogen entry and terminate their growth. It is a primary defense that contains physical barriers such as waxy cuticles, rigid cell wall, and trichomes to avoid phytopathogens. Cuticle not only restricts the entry of liquid and gas fluxes but also protects plants against pathogens, xenobiotics, and irradiation (Serrano et al., 2014). Trichomes can also have negative or positive effects depending on the target pests through their impact on the behavior of herbivore natural enemies. For instance, the presence of leaf trichomes positively inhabited predatory mite *Typhlodromus pyri* on grapes. On the other hand, European ride mite favored grape varieties with low trichomes (Loughner et al., 2008). Plants can also produce a variety of secondary metabolites to protect themselves from herbivores and harmful microorganisms. Numerous metabolites, such as amines, peptides, alkaloids, cyanogenic glucosides, phenolics, polyacetylenes, non-protein amino acids, and quinines, contribute significantly to disease reduction in plants. Different concentrations and compositions of such compounds work synergistically for defense mechanism (Wink, 2018).

Few defense mechanisms are consecutive (production of phytoanticipins) that are preformed and induced (phytoalexin production) that are activated after pathogen attack. Phytoalexins are low molecular weight compounds that possess antimicrobial. There are wide varieties of phenolic compounds, which assist in phenotypic plasticity and act as inhibitors, pesticides and contain anti herbivory roles (Kant et al., 2015). As rapidly the host plant is infested by pathogen, it displays accretion of phenolics and causes increase in host metabolism. Mainly, hydroquinones, caffeic acid, gallic acids, hydroxycinnamates, and 5-hydroxynapthoquinones are effective allelochemicals (Cheng and Cheng, 2015). Caffeic acid (200 μg/ml) in tobacco root exudates defends tobacco plants from infection by *Ralstonia solanacearum*. It resulted in

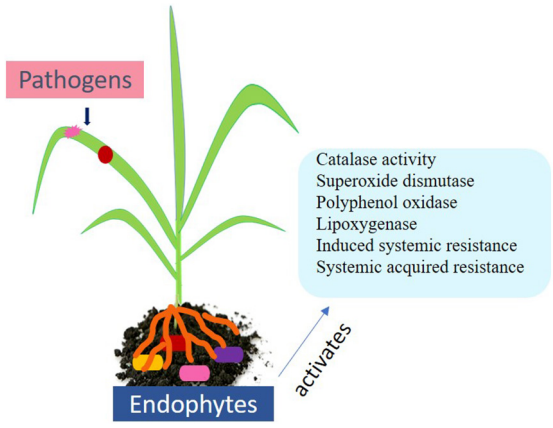

FIGURE 2
Endophytes and their role in biotic stress management.

thinning of cell membrane and created irregular cavities in cells. Moreover, expression of IecM and *epsE* genes associated with inhibition of biofilm formation was also observed and exhibited important prospect in plant defense (Li et al., 2021). In plants, complex network of antioxidative defense system to counter harmful reactive oxygen species (ROS) comprised free radicals such as OH•, $O^{\cdot -}$, and non-radicals such as H_2O_2 and $^1O_2$ which are formed under unfavorable circumstances (Huang et al., 2019). ROS scavenging mechanism includes enzymatic components such as catalase, guiacol peroxidase, superoxide dismutase, dehydroascorbate reductase, and glutathione reductase. Non- enzymatic antioxidants such as reduced glutathione, ascorbic acid, carotenoids, and flavonoids help in scavenging oxidative stress (Das and Roy Choudhury, 2014).

Additionally, plant hormones such as salicylic acid, ethylene, and jasmonic acid play central role in biotic stress signaling. Plants also possess an innate immunity system to recognize microbe-associated patterns (PAMP) such as lipopolysaccharides, peptidoglycan, and bacterial flagellin. Such immunity is called PAMP triggered immunity. Herbivores are recognized through herbivore-associated molecular patterns (HAMPs) (Zhang and Zhou, 2010). Other immune response includes transcription methods in the host nucleus and recognizing Avr proteins that are avirulent in nature. Effector triggered immunity arouses hypersensitive responses (HRs) and causes programmed cell death (PCD) in diseased and nearby cells (Howden and Huitema, 2012). A long-lasting and broad-spectrum pathogen resistance against secondary infection known as systemic acquired resistance (SAR) is conserved among diverse plants (**Figure 2**). Diverse group of molecules including salicylic acid is increased in tissues that occur systematically after localized exposure to a pathogen or after treatment with synthetic or natural compounds (War et al., 2011).

Endophytes as parasites: Hyperparasitism

It is a biocontrol strategy in which the parasitic host is plant pathogen. In fungi, hyperparasitism is frequently observed, but it is rarely seen in bacteria. Instead of using chemicals, it is frequently used to protect plants against pathogens. *Trichoderma* species, a well-known necrotrophic mycoparasite that targets host mycelium, is the most prevalent hyperparasite (Steyaert et al., 2003; Qualhato et al., 2013). Fungal parasite *Trichoderma harzianum* has a potential ability to parasitize *Epichloe typhina*, an agent that causes choke disease in grasses (Węgrzyn and Górzyńska, 2019). It showed the capability of parasitizing the already-grown mycelium of *E. typhina*. Predatory bacterium such as *Bdellovibrio bacteriovorus* has the uncommon property to use the bacterial cytoplasm as nutrients (Harini et al., 2013). Several pathogenic microbes are predated by *Xanthomonas vesicatoria* including *Erwinia carotovora*, *Pseudomonas syringae*, and *E. herbicola* (McNeely et al., 2017). *Trichoderma* spp. has been found to parasitize *Rhizoctonia solani* hyphae, thus inhibiting the disease production (Harman et al., 2004). This property can be used to treat plant diseases such as damping off in soybean seedlings and root rot in sugar beet.

Competition for space, infection, and nutrients

Pathogen adapts to nutrient-rich niches such as the rhizosphere, phyllosphere, phloem, and xylem. Pathogens choose different routes into the plant based on their survival needs. Few enter through stomata such as *Pseudomonas syringae*, while others use nectarthodes such as *Erwinia amylovora*, which causes potato fire blight disease (Melotto et al., 2008; Gudesblat et al., 2009). Furthermore, some pathogens have a distinct acquisition strategy and rely entirely on the host plant for nutrition (Fatima and Senthil-Kumar, 2015). Biotrophic pathogens consume nutrients from host tissues. Such pathogens invading plant tissues are competitively prevented by non- pathogenic endophytes already residing in the tissue. Endophytes being ubiquitously present can act through colonization and can resist the pathogen attack through competing for resources which could be available to pathogens through niche overlap. This could be understood from the study by Blumenstein et al. (2015) showing elm (*Ulmus* spp.) endophytes exhibiting extensive niche overlap against Dutch elm disease pathogen. Carbon utilizing profiles of asymptomatic endophytes showed high competition with respect to the utilization of sugar alcohols, monosaccharides, and tri- and tetra-saccharide. In another study, *Lecanicillium* reduced the available nutrients on the leaves while also inducing plant responses during root colonization (Litwin et al., 2020).

Lytic enzymes as plant disease antagonist

Extracellular enzymes that exhibit biocontrol activity are being increasingly explored as potential antimicrobials to target pathogenic microbes. Numerous endophytes have been reported to produce different lytic enzymes such as chitinase, cellulose, proteases, hemicelluloses, and amylase, which aid the hydrolysis of polymers (Dutta et al., 2014; Bodhankar et al., 2017). Lytic enzymes play vital role in the colonization of endophytes in the host cells through formation of polysaccharide and protein biofilms (Limoli et al., 2015). However, it also helps in controlling plant pathogens through cell wall degradation process (Cao et al., 2009). Specifically, fungal cell wall mostly comprises of polysaccharides that provide structural stiffness to the cell wall in phytopathogens. Therefore, the interference in the glycosidic bonds through enzymatic lysis can deteriorate the cell wall and thereby cause cell death. For instance, extracellular enzyme chitinase isolated from *P. aeruginosa* suppressed phytopathogen *Xanthomonas campestris*, which causes black rot disease in cruciferous vegetables (Mishra and Arora, 2012).

Lytic enzymes chitinases, β 1-3 glucanases, and proteases secreted from *Trichoderma harzianum*, and *Trichoderma viride* significantly reduced the incidence of collar rot disease by *Aspergillus niger* (Gajera and Vakharia, 2012). It assists in the breakdown of glycosidic bond. Similarly, β-1, 3-glucanases synthesized from *Trichoderma harzianum* showed antagonistic activity through hydrolyzing *O*-glycosidic linkage of β- glucan chains in cell wall of parasitic fungi *Sclerotinia sclerotiorum.* It is a serious disease that causes white mold in *Phaseolus vulgaris* (Vázquez-Garcidueñas et al., 1998). However, individual applications of lytic enzymes producers are ineffective, whereas application with another mechanism works well.

Induced resistance in plants

It is an indirect mechanism through which endophytes inhibit pathogens. Endophytes behold the property to decrease disease susceptibility upon pathogen attack by triggering induced resistance in their host plant (Card et al., 2016). Resistance patterns primarily ISR mediated by phytohormones such as ethylene or jasmonic acid and systemic acquired resistance (SAR) linked with the salicylic acid regulation is the known signaling pathways (**Figure 2**). Root colonization by endophytes and expression of pathogenesis-related genes is often correlated with the elicitation of induced systemic

resistance against infection. For instance, root endophyte *Fusarium solani* has been shown to reduce infection in tomato through activating pathogenesis-related genes such as PR5 and PR7 (Kavroulakis et al., 2007). The endophyte *Bacillus pumilus* along with synthetic benzothiadiazole triggered ISR in contrast to bacterial spot disease in pepper occurred due to *Xanthomonas axonopodis* (Yi et al., 2013). *Fusarium oxysporum* strain Fo47 *via* endophytic-mediated resistance (EMR) was found to suppress various wilt diseases in tomato, flax, watermelon, and pepper (Larkin and Fravel, 1999; Trouvelot et al., 2002). *Epichloe* spp. showed the ability to potentiate expression of salicylic acid defense mechanism against *Blumeria graminis* (Kou et al., 2021). Expression of pathogenesis-related PR1 protein and callose deposition by *Bacillus cereus* induced ISR against *Botrytis cinera* and simultaneously activated the SA- and JA/ET (Nie et al., 2017).

Modulation of biotic stress controlling mechanisms by endophytes

Microbial endophytes are well identified for their potential role in plant growth-promoting activities. However, their multidimensional interaction with broad range of host plants makes them potential candidate in stress tolerance mechanism (Tamosiune et al., 2017). Endophytic microbes are reported to have numerous beneficial effects in comparison with other PGPRs in colonizing the internal tissues and remain protected from the harsh environment and less nutritional requirement (Pandey et al., 2019). Endophytes commonly reside in plant tissues and benefit their host plant by eliciting defense response toward pathogen outbreak and protect them from different environmental stress (Nanda et al., 2019). Microbial endophytes being inhabitants of plant tissues are known to exhibit unique host's gene expression, physiological and metabolic response essential in conferring resistance against pests, herbivores, and phytopathogens. Pathogens cause various harmful diseases in plants and interfere with growth mechanisms of plants. It reduces photosynthetic rate, results in stunted growth, and damages plant tissues (Pérez-Bueno et al., 2019). Endophytes produce numerous compounds that help plants to interfere with pathogen by recognizing pathogen related structures. Several metabolites such as volatiles and antibiotics and hormones effectively control the expression genes related to stress response and improve plant growth through induced resistance (Lu et al., 2021).

Some studies reported the similarity of bioactive compounds by endophytic microbes to those formed by host plants (Puri et al., 2006). Different antioxidant enzymes such as peroxidase (POD), polyphenol oxidase, phenylalanine ammonia lyase (PAL), lipoxygenase, and chitinase alleviate biotic stress. Peroxidase enzymes are involved in the wide range of progressions with hypersensitive response, cross-linking of phenolics, lignifications, phytoalexin production, and suberization (Prasannath, 2017). Lipoxygenase belongs to non-heme iron containing deoxygenase that participates in stress response through lipid oxidation and acts as signal molecule to communicate with plants, pathogens, and allied endophytes as reported by Veronico et al. (2006). Different endophytes are known to produce peroxidase enzyme, which play important part in the conversion of H_2O_2 into H_2O as reported by Caverzan et al. (2012). Endophytes boost plant immunity by ISR, SAR, pathogenesis-related proteins and *via* production of numerous phytohormones to overcome the pathogen stress (Romera et al., 2019; Oukala et al., 2021). Several microbes produce surfactin, mycosubtilin, and lipopeptides, which activated the plant innate immune response. It was observed that surfactin production suppresses the *Fusarium* invasion during seed germination (Eid et al., 2021). Suppression of virulence genes such as vir A and vir G and expression of defense-related genes such as PR1, STS, and ANTS induced resistance toward *N. parvum* and *B. cinerea* as reported by Haidar et al. (2016).

Remodeling and reinforcement of cell wall to cause physical barriers against pathogens

Bacterial and fungal endophytes change chemical and physical characteristic to confer resistance against phytopathogens and herbivory (**Supplementary Table 3**). High deposition levels of callose in guard cells protect plants from herbivory that cause extensive tissue damage. Callose is β-(1,3)-D-glucan which protects plant tissues from pathogen attack. It is usually deposition among the cell wall and plasma membrane at site of pathogen invasion, at the plasmodesmata and on other plant tissue to slow down pathogen attack (Wang et al., 2021). For instance, endophytic bacteria *B. amyloliquefaciens* and *P. fluorescens* increase callose deposition in guard cells and immunize the *W. somnifera* plant leaves against *A. alternata* (Mishra et al., 2018). Callose deposition and increased lamina density provides resistance to the host plants. It protects plants from different herbivores precisely from leaf wounding ants and aphids (Khare et al., 2018). Upregulation of genes related to cellulose and lignin deposition and hardening of host cell wall were enhanced through inoculation of foliar endophytic fungus *Colletotrichum tropicale* isolated from *T. cocoa.* High cellulose and lignin deposition protects cocoa tree from black pod disease caused due to *Phytophthora* spp. (Mejía et al., 2014). In most cases, fungal endophytes limit insect growth rate, reducing insect survival and oviposition. Consortium of chitinase producing endophytes *Chitiniphilus* sp. MTN22 and *Streptomyces* sp. MTN14 showed uniform lignifications and callose deposition in *B. moneri* protecting against *Meloidogyne*

incognita nematode. Callose deposition in leaves was found preferentially in the interveinal region of host leaves (Gupta et al., 2017). Succession of structural variations is observed in *Arabidopsis thaliana* seedlings through callose deposition when inoculated with *Gluconacetobacter diazotrophicus* and protected the plant from *Ralstonia solanacearum* infection (Rodriguez et al., 2019).

Protection efficacy of *B. phytofirmans* PsJN against *Botrytis cinera* was correlated with the callose deposition and H_2O_2 production. Further primer expression of PR genes (PR1, PR2, PR5, and JAZ) and modulation in leaf carbohydrate metabolites and sugar levels after pathogen attack were reported from the study (Miotto-Vilanova et al., 2016). Rapid creation of papillae upon pathogen attack especially against fungal pathogens acts as physical fence to limit pathogen entry into the host tissues. Resistance to fungal pathogen is often correlated with the rapid formation of cell wall appositions called papillae, which forms specifically upon interaction between plant and endophytes in response to pathogen attack (Collins et al., 2003). Furthermore, to papillae, phenolic conjugates associated with papillae contribute directly in antifungal activity that forms cross-linking to form a toughened wall that cannot be simply degraded by pathogens and their associated enzymes (Zeyen et al., 2002). These are some successful cell wall-associated defense response mediated through endophytes that can stop invasive pathogens at an initial phase, before the creation of disease in plants.

Stimulation of bioactive metabolites

Secondary metabolites involved in defense response toward pests, herbivores, and pathogens. Different plant microbes specially endosymbionts secrete various metabolites and regulate defense mechanisms and having antimicrobial properties. Plant secondary metabolites such as steroids, alkaloids, phenolics, flavonoids, and terpenoids function in innate immunity and defense response signaling (Isah, 2019). *Phomopsis* sp. (fungal endophyte) produce VOCs comprised of butanol, acetone, sabinene, 1-butanol, and phenethyl alcohol, which inhibit the *Ascomycetes* and *Deuteromycetes* growth (Singh et al., 2011). VOCs such as caryophyllene, 2-methoxy-4-vinylphenol, and 3,4-dimethoxystyrol having antifungal actions released from *Sarocladium brachiariae* endophytic fungi found to be effective against *Fusarium oxysporum* (Yang et al., 2021). Colonization of asexual *Epichloe festucae* in agricultural forage grasses provided protection against herbivorous insects (Hennessy et al., 2016).

Alkaloid production from Clavicipitaceae and Ascomycota decreases herbivore feeding and virus transmission. Oxidative burst and phytoalexin production improved resistance against *Botrytis cinera* by grapevine cells and leaf-associated bacteria *Pseudomonas fluorescens* (Verhagen et al., 2011). Phytoalexins are low molecular compound containing antimicrobial, antifungal, and antiviral activities, which involved in electron transport and phosphorylation, causes rapid and complete termination of respiration in *B. cinerea* conidia (Pezet and Pont, 1990). Endophytic bacteria (*P. migulae* 8R6) showed ACC deminase activity, which limits the phytoplasma-induced damages in periwinkle through regulating the stress-related hormone such as ethylene. It improved resistance toward infection of phytoplasma and reduced the quantity of symptomatic plants up to 93% (Gamalero et al., 2017). Analysis of free amino acid in diseased leaves showed significant impact of *P. citrinum* and *A. terreus* to disease resistance and promotion of sunflower growth (Waqas et al., 2015). Change in the amino acids delays and changes the progression of pathogenic microbes. Surfactin especially surfactin A and other lipopeptides purified from *Bacillus subtilis*, *Fusarium oxysporum*, *F. moniliforme*, and *F. solani* were known to play major role in antifungal activity (Sarwar et al., 2018).

Priming of the plant defense system

Endophytes can protect plants against pathogen attack *via* the host by triggering induced resistance *via* several molecular events. Upon pathogen attack, the interaction between plant endophytic associations leads to an alteration in second messenger such as Ca^{2+} in the cytosol (Vadassery and Oelmüller, 2009). It acts as signaling molecule in sensing microbe-associated molecular patterns (MAMPs) and initiates induction of complex immune response. After activation of certain signals, bacterial and fungal endophytes that are attached to cell surface receptors activate kinases (cell surface receptor). When kinases are stimulated, they phosphorylated and send signals to ethylene/jasmonic acid or salicylic acid against phytopathogens which triggers ET/JA transduction pathways (Conn et al., 2008; Ryan et al., 2008). Endophytic colonization with the host plants downregulates the expression of genes associated with biotic stress defense response.

Usually, different phytohormones such as jasmonic acid, ethylene, and salicylic acid triggers induced resistance. JA and ET pathways are known to encourage resistance toward necrotrophic pathogens, but the SA pathway activates resistance toward the biotrophic and hemibiotrophic pathogen (Ding et al., 2011). ISR is normally triggered upon endophytic colonization of roots and immunes the plant body for future attack from pathogens. Several compounds such as flavonoids, polyphenols, phytoalexins, and signal transduction pathways were activated by jasmonate/SA or ethylene (Leon-Reyes et al., 2009; Lebeis et al., 2015). The first report indicating the induced systemic resistance by *Pseudomonas fluorescens* 89B-61 elicited resistance against cucumber anthracnose (Wei et al., 1991).

Increased synthesis of phenolic metabolites is often correlated with induced systemic resistance. Contact among *B. distachyon* and *Microdochium bolleyi* (endophytic fungus) isolated from wheat roots induced ISR against pathogen attack of *Fusarium culmorum*. Endophytic fungi upregulated expression of certain genes such as *chitinase 1*, *BdLOX3*, and *TaBH1* induced ISR in wheat (Matušinsky et al., 2022).

Some endophytes can also regulate stress management through SAR mediated by salicylic acid (Pieterse et al., 2014). SA is often associated with building up of pathogenesis-related (PR) proteins and chitinase. *Paenibacillus* strain (PB2) used to control *Mycosphaerella graminicola* induced pathogenesis-related proteins (PR1), which is considered as a marker of SAR (Samain et al., 2019). *Bacillus subtilis* activated a durable defense response in *Arabidopsis thaliana* against *P. syringae* pv. tomato DC3000 facilitated through salicylic acid/ethylene and NPR1 protein (Rudrappa et al., 2010). *Bacillus subtilis* and *Pseudomonas fluorescens*-mediated systemic alleviated the biotic stress in *Solanum lycopersicum* against *Sclerotium rolfsii* (Cappellari et al., 2019). *B. aryabhattai* showed induction of defense-related genes protein (PR1) and phytoalexin-deficient 3 in *A. thaliana*. PR1 gene expression was higher in treated plants (Portieles et al., 2021). Endophytes shows the upregulation of different genes and unique signaling pathway according to dissimilar colonization tactics as reported by Morelli et al. (2020).

There are reports indicating the distinction of endophytic mediated resistance from ISR and SAR as jasmonate, salicylic acid, and ethylene are not involved (Pieterse et al., 2014). Root endophytes *Fusarium oxysporum* strains *Fo 47* and CS-20 have the ability to induce endophytic mediated resistance in tomato and cucumber and protect them against vascular and root pathogens such as *Verticillium dahliae* and *Pythium ultimatum* (Benhamou et al., 2002). Endophytic mediated resistance in case of Fusarium species differs from ISR and SAR in terms of no association of resistance with jasmonic acid and ethylene. Also, tomato plant established a tri-partite interaction with endophytic *Fusarium oxysporum* and other organisms residing in the host plants. Grasses often establish tripartite association among endophytic fungi, arbuscular Mycorrhizal fungi, and *Leymus chinensis* (Liu et al., 2020).

Defense-related enzymes

Defense mechanisms through endophytes are mediated through the activation of multiple defense compounds and enzymes at the site of pathogen attack. Various enzymes such as PAL, POD, and superoxide dismutase (SOD) are important antioxidant enzymes, which help in defense oxidative stress and lipid peroxidation during pathogen invasion (Birben et al., 2012). Other defense enzymes such as ammonia lyase, chitinase, and β-1-3 glucanase are associated with resistance induction in plants. Several endophytic strains confirmed the production of chitinase enzyme. Some of them are *Colletotrichum sublineolum*, *Streptomyces hygroscopicus*, and *Bacillus cereus*, which are known to inhibit the growth of phytopathogenic fungi such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Aspergillus niger*, and *B. cinerea* (Wang et al., 2001; Brzezinska and Jankiewicz, 2012). ROS that are harmful for plants are neutralized enzymes such as superoxide dismutases, catalases, peroxidase, glutathione-*S*-transferases, and alkyl hydroperoxide reductases. Consortium of *Polyporus vinctus*, *Trichoderma reesei*, and *Sphingobacterium tabacisoli* accumulated defense enzymes such as PAL, POD, and polyphenol oxidase. It triggered systemic resistance contrary to *Fusarium* wilt of banana and showed first line of defense (Savani et al., 2020). Various enzymes are known to mitigate oxidative stress. *Bacillus subtilis* (EPC5) isolated from coconut root samples showed biocontrol activity against *Ganoderma lucidum*, which is the causal agent of basal stem rot on coconut palm through higher induction of phenols, peroxidase, polyphenol oxidase, and phenylalanine lyase (Rajendran et al., 2015).

Evaluation of potential *Streptomyces* spp. viz. *S. diastaticus*, *S. olivochromogenes*, *S. collinus*, and *S. griseus* triggered systemic resistance and significantly increased total phenolics, flavonoids, superoxide dismutase, ascorbate peroxidase, and guiacol peroxidase which ultimately induced resistance against *Sclertium rolsfii* in chickpea (Singh and Gaur, 2017). Endophytic fungi (*Fusarium sambucinum)* isolated from mangrove forest efficiently produced defense enzymes such as laccase (41.5 U L^{-1}), manganese peroxidase (23.6 U L^{-1}), endo-xylanase, and biosurfactant (Martinho et al., 2019). These enzymes promote the hydrolysis of lignin and decrease the degree of polymerization exposing the microfibrils to other enzymatic attack. Lipoxygenase genes detected in fungal endophyte *Paraconiothyrium variabile* isolated from conifer *Cephalotaxus harringtonia* showed inhibitory effect on *Fusarium oxysporum*, which causes vascular wilt in conifers. Lipoxygenase genes pvlox1 and pvlox2 unregulated the stress response and acted as stress marker and signaling compound when exposed to invading phytopathogens (Bärenstrauch et al., 2020). It is observed that stress factors affect growth of plants and productivity. In the present situation, thorough and efficient research on the response of endophytes on different essential crops is comparatively inadequate under field conditions. Indeed, understanding the association between crop and beneficial microbes can lead to better agricultural performs that augment plant fitness and improved the yield.

Molecular mechanism of host–endophyte interaction

It is less understood how the endophyte and host interact. To effectively manipulate the mutualistic link between the two, it is crucial to identify, isolate, and characterize the genes involved in such beneficial interactions. A novel approach for

closely examining endophytism and revealing the characteristics required to harbor plants as a habitat has been made available through endophyte genome analysis (Kaul et al., 2016). It has revealed genes important for endophytic lifestyle that are found frequently in endophyte genome such as those involved in N fixation, mineral acquisition, and stress tolerance related (Martinez-Garcia et al., 2015). Exudates such as organic acids, proteins, and amino acids are released by plants from their roots, acting as communications signals between host plant and bacterial endophytes (Kawasaki et al., 2016). Endophytic bacterial colonization is a multistage process that includes chemotactic movement toward roots, attachment to root surfaces, entry inside the root, and movement (Kandel et al., 2017b). There are various genes such as *fliC3*, *MgIB*, *pilX*, *FliI*, *Aer*, and *CheZ*, which involved in chemotaxis and motility (Samanta et al., 2016; Liu et al., 2018). *Gilmaniella* sp. inoculation in *Atractylodes lancea* upregulated the genes and proteins such as terpene skeleton biosynthesis as well as farnesene synthase related to primary metabolism (carbohydrate metabolism, carbon fixation) which improve plant growth (Yuan et al., 2019). Additionally, they noticed an increase in genes related to signaling such as those related to ethylene response factors, heat stress, trielix, and basic loop helices. Sequiterpenoid, phytoalexins such as gossypol and heliocides can protect cotton plants from herbivores infections (Yang et al., 2013). The overexpression of oryzalexin's genes (*OsTPS19*) and monoterpene S-limonene serve protective metabolite against *Magnaporthe oryzae* and provide resistance to plants toward infection (Chen et al., 2018). Wheat plants have improved resistance to *Fusarium* head blight due to the presence of *Fhb7* gene in endophytic *Epichloe* fungus, which encodes glutathione-S-transferase involved in trichothecenes detoxification (Wang et al., 2020). Dinkins et al. (2017) observed that *Epichloe coenophiala* altered the expression of several WRKY transcription factors linked to the increased resistance in *Lolium arundinaceum*. Endophytic fungus increased the expression of iron transporters and genes involved in fatty acid production to encourage the *Noccaea* plant development (Ważny et al., 2021).

Omics approach to study endophytes and host plants interaction

Multiomics, which includes genomes, transcriptomics, proteomics, and metabolomics, are becoming increasingly important in plant–microbe interaction (Kaul et al., 2016). The potential value of endophytes can be investigated using modern high-throughput genomic technology. An in-depth examination of endophytes in terms of sequencing and biological evolution has greatly increased interest in endophyte research (Selosse et al., 2022). Endophyte genome-wide analysis directly reflects endophyte colonization preferences and genetic characteristics on various hosts. This makes it much easier to find the related genes involved in host growth, development, insertion elements, metabolism, and surface attachment (Subudhi et al., 2018). *Pantoea ananatis*, an endophytic bacterium with enormous biological potential, contains genes for hydrolase and fusylic acid resistance protein (Wu et al., 2020).

Proteomic analysis using mass spectrometry identified differentially expressed proteins (DEPs) related to the endophytic *Gluconacetobacter* and sugarcane interaction which involved in signaling and cellular recognition (Lery et al., 2011). Using multiomics analysis, researchers discovered that liposaccharide and adhesins are potential molecular determinants underlying the divergent phenotypic behavior of closely related species during plant–host colonization (Monteiro et al., 2012). RNA sequencing and microarray enables the identification of differentially expressed genes, which involved in upregulation of nutrient acquisition and chemotaxis (*nifH*, *sbpA*, and *trpB*) in wheat roots colonized by *Azospirillum brasilense* (Camilios-Neto et al., 2014). Proteomics and transcriptomics were used to decode the effect of endophytes on the host *Atractylodes lancea* as reported by Yuan et al. (2019). Metabolomic analysis is a popular technique for quantifying metabolites. It can be used to complement transcriptomic and proteomic data, allowing for a well understanding of host phenotypical structures and elucidating plant–microbe interaction and mechanism (Chen et al., 2022). During various stages of plant development, endophytes synthesize a variety of secondary metabolites and mediate an increase in metabolites biosynthesis in particular species and organs (Zhai et al., 2017). The DEGs and metabolites of anthracnose-resistant cultivars of *Camellia oleifera* indicate the critical function of flavonoid biosynthesis in the defense toward anthracnose using transcriptome and metabolomics (Yang et al., 2022). Barley metabolo-transcriptome profiling revealed the activation of the HvCERK1 gene, which confers resistance to *Fusarium graminearum* as reported by Karre et al. (2017).

Microarray-based gene expression analysis revealed single inoculation of endophytic *Bacillus megaterium* isolated from black pepper root encouraged growth elevation in *A. thaliana* Col O seeds by upregulation of biotic stress related genes such as *MYB4*, *MYB7*, *WRR4*, *ATOSM34*, and *ATHCHIB*. Also, the bacterial colonization inside the host tissues triggered ethylene-responsive genes such as *ERF71* and *RAP2*. Other genes *such as BAP1*, *BTK4*, *MKK9*, and *AIBI* were found associated with jasmonic acid and salicylic acid transduction pathways (Vibhuti et al., 2017). In another study, rice seed primed with *Pseudomonas putida* BP25 endogenously colonized rice and altered root growth and defensive response against *Megnaphorthe oryzae*. Defense-related phenols, peroxidase, and both volatile and nonvolatile metabolites were found in

primed plants. Also, pathogenesis-related genes associated with systemic acquired resistance, i.e., *OsPR1-1* and *OsPR3* were downregulated by endophytic colonization. Growth-related genes playing important role in intermodal elongation such as *OsAcO4* and *OsACS6* were observed regulating plant growth and protecting it against blast disease (Ashajyothi et al., 2020).

Although endophytic microorganisms possess great potential in the agricultural field still, there are certain challenges involved with the field application of endophytes that are restricting their wide use. When introduced into a crop plant, many factors prevail which must be evaluated for their wide application from lab to field. First, many fungal endophytes produce toxic secondary metabolites such as mycotoxins which cause infection in their host plants upon colonization and reach up to fruits and seeds. There is still a need to study upon their colonization and viability of the desired inoculants (Chitnis et al., 2020). It is important to focus on their unpredictable behavior and inadequate colonization of the target site in field trial. Instead of proper establishment of the biological strain, single-strain endophyte inoculants under application do not show desired plant growth activity. Well-formulated consortia could be more promising and help in plant growth promotion through circumventing some of the critical limitations such as crop specificity of microbes. In addition, it is necessary to raise awareness among the farmers about the product's efficacy of endophytes in comparison with harmful chemical fertilizers. Main attention for the introduction of endophytes is the better understanding of plant–microbe interactions under different sets of conditions that will help in reducing bulk production of inoculum doses (Fadiji and Babalola, 2020). Modifying the root exudation chemistry of plants to choose a more beneficial microbiome is one of the most effective strategies. The use of advance biotechnological tools to investigate both the community and functionalities of endophytic microorganisms could be helpful (White et al., 2019). Understanding the genetics and engineering of their complex interactions through next generation sequencing could be helpful in revealing their taxonomic and functional diversity. However, multiple field trails, sampling at different times and locations under different environmental factors, are an important factor to improve their performance under field conditions. Also, future studies can focus on the development of endophytic nanoparticle which could provide a new aspect of metabolism regulation under extreme condition.

Conclusion

At present, increasing the productivity of crops is important without any disturbance to the soil fertility, to fulfil food needs and provide a healthy environment for our future generations. But due to the incidence of diverse kind of pest and pathogen in crops, it leads to the decrease in yield of crop plants resulting substantial crop losses every year. To diminish the loss of crop yield and to control the diseases, different effective methods should be used. Endophytes are eco-friendly, non-toxic, easily applicable, and cost-effective in nature, so farmers use them as a substitute to fertilizers for sustainable agriculture. More research needs consideration on the biochemical, molecular, and genetic mechanisms of endophytes decisive for stress resistance in different crops. Omics approach can help unravel the functions of complex plant microbiome, providing information about competent microbes in terms of stress tolerance and plant productivity. Endophytes and their metabolites must be explored to the multiomics level as potentially fruitful research in the biological control of plant diseases.

Author contributions

PC and AC performed conceptualization and wrote the manuscript. UA wrote the manuscript. AK and GK helped in editing the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors acknowledge the Microbiology Department, Govind Ballabh Pant University of Agriculture and Technology.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.933017/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 20 October 2022
DOI 10.3389/fmicb.2022.1005458

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 28 July 2022
ACCEPTED 06 October 2022
PUBLISHED 20 October 2022

CITATION
Flores-Duarte NJ, Caballero-Delgado S, Pajuelo E, Mateos-Naranjo E, Redondo-Gómez S, Navarro-Torre S and Rodríguez-Llorente ID (2022) Enhanced legume growth and adaptation to degraded estuarine soils using *Pseudomonas* sp. nodule endophytes. *Front. Microbiol.* 13:1005458. doi: 10.3389/fmicb.2022.1005458

Enhanced legume growth and adaptation to degraded estuarine soils using *Pseudomonas* sp. nodule endophytes

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The joint estuary of Tinto and Odiel rivers (SW Spain) is one of the most degraded and polluted areas in the world and its recovery is mandatory. Legumes and their associated bacteria are recommended sustainable tools to fight against soils degradation and loss of fertility due to their known positive impacts on soils. The aim of this work was to isolate and characterize plant growth promoting nodule endophytes (PGPNE) from inside nodules of *Medicago* spp. naturally growing in the estuary of the Tinto and Odiel Rivers and evaluate their ability to promote legume adaptation in degraded soils. The best rhizobia and non-rhizobia among 33 endophytes were selected based on their plant growth promoting properties and bacterial enzymatic activities. These strains, identified as *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12, were used for *in vitro* studies using *Medicago sativa* plants. The effects of individual or combined inoculation on seed germination, plant growth and nodulation were studied, both on plates and pots containing nutrient-poor soils and moderately contaminated with metals/loids from the estuary. In general, inoculation with combinations of rhizobia and *Pseudomonas* increased plant biomass (up to 1.5-fold) and nodules number (up to 2-fold) compared to single inoculation with rhizobia, ameliorating the physiological state of the plants and helping to regulate plant stress mechanisms. The greatest benefits were observed in plants inoculated with the consortium containing the four strains. In addition, combined inoculation with *Ensifer* and *Pseudomonas* increased As and metals accumulation in plant roots, without significant differences in shoot metal accumulation. These results suggest that PGPNE are useful biotools to promote legume growth and phytostabilization potential in nutrient-poor and/or metals contaminated estuarine soils.

KEYWORDS

contaminated estuarine soils, enhanced nodulation, nodule endophytes, plant growth-promoting bacteria, phytostabilization

Introduction

Industrial and mining activity releases toxic metal/loids into the environment, such as As, Cd, Cu, and Pb, all of them very harmful to human health and most living beings (FAO and ITPS, 2015). In turn this has caused the degradation of soils, either by the modification of pH or by originating a decrease in the number of arable lands causing a problem at social, economic, and environmental levels (Singh et al., 2010; Chen et al., 2013). Heavy metals are dangerous because they are not degradable either chemically or biologically, being able to remain in the environment for hundreds or thousands of years (Herrera Marcano, 2011). These metals have also been repressing the enzymatic activity of the soils, causing a decrease in the growth and respiration of the populations of microorganisms, thus altering the diversity present in the rhizosphere (Abdu et al., 2017; Aponte et al., 2020). Marshes of river estuaries are particularly sensitive to heavy metals contamination by metals deposition. A good example is the combined estuary of Tinto and Odiel rivers (Huelva, SW Spain), known as one of the most contaminated regions in the world (Hudson-Edwards et al., 1999). Levels of toxic metal/loids as high as 125 ppm of As, 890 ppm of Cu, 275 ppm of Pb or 1,500 ppm of Zn in the Odiel river marshes, and 2 to 3-fold these levels in the Tinto River marshes, have been reported in the last decade (Mesa et al., 2015a; Navarro-Torre et al., 2017). These levels exceed those allowed by regional and national legislation in natural parks, agricultural and industrial soils (Junta de Andalucía and Consejeria de Medio Ambiente, 1999).

One solution to this great environmental problem is the implantation of pioneering crops able to regenerate the soil and improve its yield, respecting the environment. This kind of strategies should include the use of leguminous plants (belonging to the *Fabaceae* family), due to their great benefits. First, legumes are an important product in the food industry both for human and animal consumption worldwide, since they are very rich in protein, fiber, vitamins, and minerals and also has a low cost, being highly consumed in underdeveloped countries (Ferreira et al., 2021). Second, legumes play a very important role in agriculture as cover crops, since they improve soil fertility increasing crops yield, help the constant movement of soil nutrients and release matter into the soil (Stagnari et al., 2017; Ferreira et al., 2021). Third, legumes are good candidates to adapt to degraded soils affected by biotic and abiotic factors, particularly these plants play an important role in the regeneration of soils degraded by heavy metals due to their ability to accumulate metals in the roots without affecting plant growth (Mesa et al., 2015b; Flores-Duarte et al., 2022a).

Legumes are capable of fixing atmospheric nitrogen through symbiosis with many genera of soil nitrogen-fixing bacteria, called rhizobia, which penetrate the roots through the root hairs, forming nodules in which nitrogen fixation takes place (Poole et al., 2018). But nodules are not only occupied by rhizobia, within the legume nodules there are another great variety of bacteria called non-rhizobial nodule-associated bacteria (NAB; Rajendran et al., 2012), non-rhizobial endophytes (NRE; De Meyer et al., 2015), or just nodule endophytes (Velázquez et al., 2013). These bacteria do not induce nodule formation but can colonize nodules accompanying rhizobia forming a beneficial association and enhancing nodulation and plant growth. Within this group, bacteria of the genera, *Acinetobacter*, *Agrobacterium*, *Bacillus, Burkholderia, Pseudomonas* and *Variovorax*, among others, have been found (Shiraishi et al., 2010; Martínez-Hidalgo and Hirsch, 2017; Bessadok et al., 2020). These nodule endophytes usually behave as plant growth promoting bacteria (PGPB).

Soil health and fertility are directly influenced by beneficial plant-microbe relationships that determine soil biodiversity (Vishwakarma et al., 2020). Plant-PGPB interactions have shown to provide benefits in plant growth and development by facilitating the acquisition of nutrients (Fasusi et al., 2021) and producing phytohormones related with plant and root lenth growth, and the formation of root hairs and lateral roots, such as cytokinin, gibberellins, abscisic acid, and IAA (Raza et al., 2019). Through atmospheric nitrogen fixation (Roy et al., 2020), phosphate mobilization/solubilization (Etesami et al., 2021) and siderophores production (Lurthy et al., 2020; Kang et al., 2021) bacteria provide N, P, Fe, and Zn to plants. PGPB also play a determinant role in plant adaptation and tolerance to biotic and abiotic stress (Chaudhary et al., 2022). For example, bacteria with ACC-deaminase activity are able to break the ethylene precursor ACC, altering plant stress perception (Penrose and Glick, 2003; Chandwani and Amaresan, 2022).

Microorganisms have an important role in pollutant detoxification and heavy metal plant stress resistance (Caracciolo and Terenzi, 2021). Soil microorganisms have developed different resistance mechanisms, such as metal biosorption, bioaccumulation, modification of metal chemical state (methylation) or production of chelating compounds, particularly siderophores and biosurfactans, that cause a lowering in metal availability for plants (Verma and Kuila, 2019), then diminishing plant metal content (Caracciolo and Terenzi, 2021). PGPB are directly involved in metal detoxification through the production of secondary metabolites, such as siderophores, ACC or IAA (Caracciolo and Terenzi, 2021). Particularly in legumes, nitrogen-fixing bacteria and endophytes inside nodules have probed to reduce metal translocation to aerial parts and increase plant nitrogen content and growth in metal contaminated soils (Navarro-Torre et al., 2020; Flores-Duarte et al., 2022b).

In a recent work, a consortium of PGPB, including *Pseudomonas*, *Chryseobacterium* and *Priestia* genera, showed their ability to promote *Medicago sativa* growth and adaptation in poor-nutrient soils (Flores-Duarte et al., 2022b). These bacteria were isolated from the rhizosphere of different legumes growing in estuarine soils with low levels of minerals and organic matter. It is well accepted that endophytes maintain a much closer relationship with the plant and have lower competition with soil microorganisms than rizospheric bacteria (Adeleke and Babalola, 2021; Dwibedi et al., 2022). The present work is aimed to determine whether a consortium of endophytes isolated from

TABLE 1 Physicochemical properties and micronutrients concentrations of soil from high marshes of the Odiel River.

Physicochemical properties								
Location	**Texture (%) ***		**Organic material (%)**		**Conductivity (μS·cm^{-1})**		**pH**	
High marsh	72/13/15		0.9 ± 0.01		13.1 ± 0.3		6.8 ± 0.2	
Metal/loid concentration (mg·kg^{-1})								
Location	**As**	**Cd**	**Cu**	**Zn**	**Mg**	**Na**	**Fe**	**P**
High marsh	27.9 ± 2.2	0.38 ± 0.01	316.7 ± 3.20	345.0 ± 7.10	0.789 ± 0.01	0.369 ± 0.01	9345.56 ± 9.35	0.056 ± 0.01

Values are mean ± S.D. ($n = 3$). *Texture (sand/slit/clay percentage).

nodules could enhance legume growth in degraded soils more efficiently than rhizosphere bacteria, introducing soil metal contamination as additional stress.

The objectives that arise in this work are the following: (i) isolation and characterization of rhizobia and endophytes from nodules of *Medicago* spp. naturally growing in the Odiel river estuary (Huelva, Spain); (ii) selection of rhizobia and nodule endophytes based on their properties, enzymatic activities and ability to tolerate metal/loids; (iii) determine the ability of the selected bacteria to promote *M. sativa* growth, nodulation and metal accumulation in Odiel marshes soils under greenhouse conditions.

Materials and methods

Collection of samples and soil characterization

Nodulated wild plants of *Medicago* spp. were collected from high marshes of the Odiel river estuary (Huelva, Spain; 37°150 N, 6°580 W; SW Spain) in February 2020. Soil samples (15–20 cm deep) were collected using a shovel, gloves and plastic bags. The samples were immediately transported to the laboratory and stored at 4°C. Three homogeneous soil samples were deposited in sterile bottles for chemical soil analysis, as described by Mateos-Naranjo et al. (2015). The soil texture (percentage of sand, silt, and clay) was determined using the Bouyoucos hydrometer method (Bouyoucos, 1936). Electrical conductivity was measured with a Crison-522 conductivity meter (Spain) and pH and redox potential with a Crison pH/mVp-506 portable meter (Spain). The concentration of nutrients in the soil was measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES; ARLFisons3410, Thermo Scientific, Walthman, MA, United States). The amount of organic matter was determined by the method of Walkley and Black (1934). Results are presented in Table 1.

Isolation of endophytic bacteria from nodules of *Medicago* spp.

Nodules were exscinded from roots with a scalpel, washed with water and placed on tubes. Samples were immersed in 70% (v/v) ethanol for 1 min with manual agitation to disinfect the surface. Then, they were washed again with sterile water and immersed in 3% (v/v) sodium hypochlorite for 15 min under continuous agitation and finally washed four times with sterile distilled water. Disinfected nodules were deposited in a mortar with 1 ml of sterile 0.9% (w/v) saline solution and grinded. Portions of 100 μl of the resulting mix were to spread and extended with a spatula in Petri dishes with TY medium (tryptone-yeast extract agar) and TSA (tryptone soya agar, Intron Biotechnology, Korea). Plates were incubated for 72 h at 28°C. Controls of nodule surface disinfection and solution sterility were performed. Bacteria were separated based on the different colony morphology and cell morphology was observed by Gram stain using an Olympus CX41 microscope with the 100x objective.

Determination of plant growth promoting properties and enzymatic activities

The ACC deaminase activity was assessed as described by Penrose and Glick (2003) and adapted by Mesa et al. (2015a). Activity was measured by monitoring α-ketobutyric acid at 540 nm in a spectrophotometer (Lambda25; PerkinElmer, Walthmam, MA, United States), and the amount of α-ketobutyric acid was determined using a standard curve with known concentrations. ACC deaminase activity was expressed in μmoles of α-ketobutyrate per mg of protein per hour. To verify nitrogen fixation, strains were plated in nitrogen free broth (NFB; Döebereiner, 1995) for 5 days at 28°C. Indole-3-acetic acid (IAA) production was determined using a colorimetric technique by incubating 3 ml of a liquid culture of TY or TSB supplemented with L-tryptophan (0.1 mg/ml) and incubated at 28°C for 72 h at 200 rpm. Cultures were then centrifuged for 5 min at 13,000 rpm and supernatants transferred to glass tubes. The appearance of a pink color after adding 4 ml of Salkowski's reagent (Gordon and Weber, 1951) indicated that the test was positive. The amount of IAA was calculated by measuring the absorbance at 535 nm in a spectrophotometer (Lambda 25; Perkin Elmer, Waltham, MA, USA). Phosphate solubilization was carried out on plates with NBRIP medium (phosphate growth medium from the National Institute of Botanical Research) as

described by Nautiyal (1999). The appearance of a transparent halo after 7 days of incubation at 28°C indicated that the bacteria had the capacity to solubilize phosphates. 100 µl of bacterial culture medium was added to each well. The formation of biofilms will be prolonged by checking the adhesion capacity of the bacteria in microplates with 96 wells in TY or liquid TSB at 28°C, incubating for 4 days, after incubation each well was stained with 200 µl of 0.01% crystal violet as described by del Castillo et al. (2012). Siderophores production was determined by the appearance of an orange halo around the well containing 100 µl of bacterial culture in CAS medium (Chromeazurol S), after incubation for 7 days at 28°C (Schwyn and Neilands, 1987). Enzymatic activities were determined on plates incubated at 28°C for 7 days. Pectinase and cellulase activities were examined as described by Elbeltagy et al. (2000). For pectinase activity, strains were plated on ammonium mineral agar (AMA). Plates were revealed with 2% CTAB and positive bacteria showed a halo around. For cellulase activity, strains were plated on solid M9 minimal medium supplemented with yeast extract (0.2%) and carboxymethylcellulose (1%). Plates were developed by covering the plate with 1 mg/ml Congo Red solution for 15 min and decolorizing with 1 M NaCl for 15 min. The appearance of a clear halo indicated positive result. Chitinase activity was performed as described by Mesa et al. (2015a). Amylase activity was performed on starch agar plates (Scharlab, Barcelona, Spain) and revealed with 10 ml lugol. The formation of a transparent halo indicated positive result. Lipase and protease activities were observed by the presence of halos around the bacteria after incubation in casein agar and Tween 80 mediums, respectively, as described by Prescott (2002). And finally, the DNAse activity was determined by cultures on DNA agar plates revealed with 1 M HCl.

For PGP properties and enzyme activities, positive and negative controls from BIO-181 group collection were used (Supplementary Table 1).

Tolerance to metals/loids

The isolated bacteria were plated in TY and TSA medium with increasing concentrations of As, Cd, Cu and Zn, from 1 M $NaAsO_2$, 1 M $CdCl_2$, 1 M $CuSO_4$, 1 M $ZnSO_4$ stock solutions. Plates were incubated for 24–48 h at 28°C and tolerance expressed as the maximum tolerable concentration (MTC), that is the maximum concentration that allows visible bacterial growth.

Analysis of diversity and identification of isolates

The diversity of the isolated endophytes was analyzed by performing a Box-PCR. Bacterial genomic DNA was isolated using a G-spin™ Genomic DNA Extraction Kit for Bacteria (Intron Biotechnology, Gyeonggi-do, Korea) with the instructions determined by the manufacturer. Box-PCR was performed using 1 µl of DNA and Box A1R primer (5′-CTACGGCAAG GCGACGCTGACG-3′) using the Maxime™ PCR PreMix kit (i-Taq™; Intron Biotechnology, Gyeonggi-do, Korea) and following the PCR conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 20 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Electrophoresis was performed in a 1.5% (w/v) agarose gel and a voltage of 70 V for 2 h. Representative bacteria of each different Box-PCR profile were identified by 16S rRNA gene amplification using 16F27 and 16R1488 primers (Navarro-Torre et al., 2016) and the Maxime™ PCR PreMix kit (i-Taq™; Intron Biotechnology, Gyeonggi-do, Korea) following the next PCR conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 10 s, extension at 72°C for 50 s, and final extension at 72°C for 5 min. Electrophoresis was performed in a 1% (w/v) agarose gel and a voltage of 120 V for 30 min. PCR products were purified with the enzyme ExoSAP IT (Affymetrix, Santa Clara, CA, USA), following the manufacturer instructions, and sequenced by the StabVida company (Caparica, Portugal). Then, 16S rRNA gene sequences were compared with those deposited in the EzBioCloud database (Yoon et al., 2017) using the Ez-Taxon e service (www.ezbiocloud.net/eztaxon; accessed July 2022). Finally, 16S rRNA gene sequences were deposited in the NCBI GenBank.

Inoculant design

Selected bacteria were grown together on TY plates to test growth compatibility as described in Navarro-Torre et al. (2016). The inoculants were prepared by cultivating selected strains separately in TSB or TY at 28°C for 24 h and 200 rpm. Afterwards, cultures containing 10^8 cells mL^{-1} were transferred to sterile Falcon tubes to be centrifuged at 8000 rpm for 10 min, washing twice with sterile 0.9% (w/v) saline solution, to remove traces of the culture medium (Navarro-Torre et al., 2016). At the end of the washes the cultures were resuspended in a sterile liquid nitrogen deficient buffered nodulation medium (BNM; Ehrhardt et al., 1992), or sterile water for experiments under greenhouse conditions. For co-inoculation and consortium inoculation designs, bacteria were mixed after resuspension. Inoculation conditions were defined as follow: C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4 + N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8 + N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer sp.* N10; N4 + N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8 + N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer sp.* N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp., N8 *Ensifer* sp. N10 and *Ensifer* sp. N12.

Labelling of *Pseudomonas* with fluorescence and microscopy

Pseudomonas strains (*Pseudomonas* sp. N4 and *Pseudomonas* sp. N8) were marked with the fluorescent protein mCherry by triparental matting, mixing the donor *Escherichia coli* DH5α containing the plasmid pMP7604 (Lagendijk et al., 2010), the helper strain *E. coli* containing pRK600 (Finan et al., 1986) and *Pseudomonas* strains spontaneously resistant to rifampicin (100 μg/ml) as recipients. 100 μl aliquots of TSB overnight cultures were plated on TSA containing rifampicin (100 μg/ml) and tetracycline (10 μg/ml). Fluorescent *Pseudomonas* resistant to both antibiotics thus containing the plasmid were selected. *M. sativa* plants were co-inoculated with the rhizobia (*Ensifer* sp. N10 or *Ensifer* sp. N12) and labelled *Pseudomonas* in square plates (12 × 12 cm), 20 seedlings per condition, as described below. After 28 days, roots and nodules were cut with a sterile scalpel and 0.5 mm cuts observed (Mesa et al., 2015b). Fluorescent bacteria in plant tissues were visualized using a laser scanning spectral confocal optical microscope (Zeiss LSM 7 DUO, Zeiss, Jena, Germany) with an objective Plan-Apochromat 20X/0.8 M27, filters of 572–727, and a laser of 561 nm (5.3%). Images were processed with ZEN2011 software (Zeiss, Jena, Germany).

Medicago sativa seeds germination and nodulation on plates

Alfalfa (*M. sativa*) seeds were surface disinfected following the protocol described by Navarro-Torre et al. (2019) by immersing them for 10 min in 70% (v/v) ethanol, followed by 30 min in sodium hypochlorite at 3% (v/v) under gentle agitation, washing 6 times with sterile distilled water. The next step was to immerse 50 seeds in each inoculant for 1 h and for the non-inoculated control (C-) seeds were immersed for 1 h in 0.9% (w/v) NaCl. Seeds were transferred to plates containing 0.9% (w/v) water-agar (5 repetitions per condition and 10 seeds per plate) or water-agar plates containing a mixture of 7.5 μM As, Cd, Cu, and Zn, prepared from the stock solutions described above (paragraph 2.4), and finally incubated in the dark at 28°C. Germination was observed every 24 h for 7 days in absence of metals and for 21 days in presence of metals.

For *in vitro* nodulation experiment, pre-germinated seeds were transferred to square plates (12×12 cm) with nitrogen-free BNM-agar medium (Ehrhardt et al., 1992), supplemented with sodium arsenite at a concentration of 30 μM. or in absence of arsenite. NH_4NO_3 was added to all plates at a concentration of 1 mM. 10 seeds were deposited in each plate and 5 replicates per treatment were performed. The seeds were inoculated or co-inoculated under the conditions described above using the selected bacteria, and non-inoculated seeds were used as controls. The plates were placed in a vertical position, incubated at 22°C with a cycle of 8 h of darkness and 16 h of light (120–130 μE $m^{-2} \cdot s^{-1}$) in a growth chamber (AGP-700-HR ESP; Radiber, Barcelona, Spain). Plants were harvested after 21 days and nodules from each condition were counted.

Experiments under greenhouse conditions

Soil was collected from the upper marshes of the Odiel River and sterilized in an autoclave at 121°C and 1 atm of overpressure for 30 min. This sterilization was repeated twice. To confirm soil sterilization, 3 samples of 1 g of soil were washed by shaking in 10 ml of sterile water. After decanting for 10 min, three samples of 100 μl of supernatant per tube were plated on TSA and no growth was observed on plates incubated 48 h at 28°C. Plastic pots (11 cm squared pots with 12 cm height) were filled with 1 kg of the previously sterilized soil (3,1, three parts of the soil from the marshes and one part of sterile perlite) and 2 pre-germinated seeds were placed per pot, 8 pots per condition. The experiment lasted 60 days and plants were irrigated with sterile water once a week (50 ml) and weekly inoculated with their respective inoculum (50 ml of cultures). The greenhouse had controlled light and temperature conditions; natural light was supplemented with fluorescent/incandescent lamps to get a photoperiod of 16 h light: 8 h dark; and the temperature was adjusted to 25°C during the day and 15°C during the night. At the end, the length of roots and shoots were measured, the number of nodules and leaves were counted, the diameters of the leaves were determined, and the nitrogen content in the plant was evaluated using an InfrAlyzer 300 (Technicon, Tarrytown, NY, United States), as described by Carrasco et al. (2005). Finally, samples were placed in an oven at 80°C for 48 h to determine the dry weight of both shoots and roots separately.

Determination of photosynthetic parameters and total chlorophyll content

For the determination of gas exchange, leaves were randomly selected from *M. sativa* plants and measured with an infrared gas analyzer (IRGA) LI-6400 (LI-COR Biosciences, Lincoln, NE, USA) equipped with a Li-6,400-02B leaf light chamber. Measurements were made between 9 am and 2 pm under a photosynthetic photon flux density of 1,500 μmol m^{-2} s^{-1}, a vapor pressure deficit of 2–3 kPa, at a temperature of approximately 25°C and an environment of CO_2 concentration of 400 μmol·mol^{-1} air. Gas exchange stabilization (120 s) was equilibrated, and measurements were recorded to determine the net photosynthetic rate (A_N). In addition, a fluorometric analysis was performed to study the energy use efficiency of photosystem II (PSII). The maximum quantum efficiency of PSII photochemistry (Fv/Fm) and the quantum efficiency of PSII (Φ_{PSII}) were determined using a

saturation pulse method described in Genty et al. (1989). Selected leaves were light- and dark-adapted for 30 min, followed by a saturated actinic light pulse of 10,000 μmol m^{-2} s^{-1} for 0.8 s at noon (1700 μmol photons m^{-2} s^{-1}) using a FMS-2 modulated fluorimeter (Hansatech Instruments Ltd., Pentney, United Kingdom). Details are described in Schreiber et al. (1986). With the recorded data, the electron transport rate (ETR) was calculated as described in Krall and Edwards (1992). Finally, the total content of chlorophyll described by Hiscox and Israelstam (1979) was determined. 50 mg of randomly selected leaves were ground using a mortar containing 100% acetone: 0.9% saline solution (4.1; v/v). The resulting extract was resuspended in acetone and measured at 652 nm using a spectrophotometer (Lambda 25; PerkinElmer, Walthmam, MA, United States) and the total chlorophyll content was determined using the formula described by Arnon (1949). Samples were run in duplicate.

Antioxidant enzyme analysis

Guaiacol peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzymatic activity were measured with leaves and roots randomly collected from each of the treatments at the end of the experiment. Immediately after the collection, the samples were deposited in nitrogen liquid and stored at −80°C until homogenized. 48 h after storage, 500 mg of plant material were homogenized in extraction buffer (50 mM sodium phosphate buffer at pH 7.6) and centrifuged at 4°C and 9,000 rpm for 20 min, and the plant extract was stored at −80°C. Total protein concentration was measured in the plant extract following the Bradford method (Bradford, 1976) using a BSA standard curve. In guaiacol peroxidase activity (GPX), guaiacol oxidation was measured at 470 nm. Catalase (CAT) was determined by measuring the disappearance of H_2O_2 at 240 nm. Superoxide dismutase (SOD) activity was assayed using autoxidation of pyrogallol at 325 nm. Finally, for ascorbate peroxidase (APX), the oxidation of L-ascorbate was measured at 290 nm. For the determination of the autoxidation of substrates, control tests were carried out without the presence of the enzyme extract. Enzymatic activities were expressed as units per μg of protein and were measured in triplicate.

Statistical analysis

Statistical analyzes were determined using Statistical version 12.0 software (Statsoft Inc., Tulsa, OK, United States). The normality of the results was verified using the Kolmogorov Smirnov test. The results of each treatment were compared using one-way ANOVA, and Fisher test was performed to determine statistical differences.

Results

Isolation and characterization of endophytic bacteria from nodules

Soil characterization

Bacteria were isolated from nodules of *Medicago* spp. that grows naturally in the high marshes of the Odiel River. The soil was composed of 72% sand, 0.9% organic matter, had low electrical conductivity and nutrients content, as well as moderate amounts of As, Cd, Cu and Zn. The soil characteristics are presented in Table 1.

Isolation of endophytes

A total of 33 endophytic bacteria were isolated from *Medicago* spp. nodules (13 strains grew in TY and 20 in TSA). They were identified based on colony morphology (color and shape of the colonies), cellular morphology (cocci, bacilli, coccobacilli) and type of cell wall by Gram staining (Gram positive or Gram negative). Other aspects such as grouping, if any (clusters, chains), or the presence of spores (central or terminal) were also observed. 85% of the strains were Gram negative bacilli and 15% were Gram positive bacilli, and only one of them presented spores (Supplementary Table 2). Subsequently, a Box-PCR of the 33 strains was performed in order to study the collection genotypically (Supplementary Figure 1). Several of the isolates showed identical band profiles, so the study was reduced to 24 strains for which the 16S rRNA genes were partially sequenced. The sequences showed similarity with *Achromobacter*, *Bacillus*, *Enterobacter*, *Ensifer, Lelliottia, Priestia* and *Pseudomonas* genera, being *Pseudomonas* and *Lelliottia* the most represented (Table 2).

Characterization of PGP properties, enzymatic activities and metals tolerance

PGP properties of the endophytes were determined, showing all of them four to five out of six analyzed properties, while two strains presented the six properties (Table 3; Supplementary Table 3). All the bacteria were able to produce siderophores and most of them (30) solubilized phosphate (Supplementary Table 3). N4 strain showed the highest production of siderophores according to the diameter of the halo in CAS medium (Table 3; Supplementary Table 3) and N15, N20, N30 and N31 were the best phosphate solubilizers (Supplementary Table 3). A high number of strains were able to grow in N free medium (30 isolates) and/or produced auxins (27 isolates). N8 strain produced the highest concentration of IAA, close to 18 mg L^{-1} (Table 3; Supplementary Table 3). 20 strains formed biofilm and 11 isolates showed ACC deaminase activity, presenting N8 strain the highest activity, close to 10 μmol α-ketobutyrate mg $protein^{-1}$ h^{-1} (Table 3; Supplementary Table 3).

The presence of enzymatic activities in the endophytes was studied (Supplementary Table 4). Cellulase and protease activities were the most abundant, since they were found in 22 and 17 strains, respectively (Supplementary Table 3). A low number of

10.3389/fmicb.2022.1005458

TABLE 2 Identification of cultivable entophytic bacteria isolated from nodules of *Medicago* spp. using the EzBiocloud database.

Strain	Sequence size (bp)	Related species	% ID	Accession number
N1	461	*Achromobacter piechaudii*	99.57	OP060607
N2	602	*Lelliottia jeotgali*	96.75	OP060608
N3	343	*Lelliottia jeotgali*	95.27	OP060609
N4	**1,154**	**Pseudomonas mucoides**	**97.47**	**OP060610**
N5	722	*Lelliottia jeotgali*	98.53	OP060611
N6	1,218	*Achromobacter spanius*	99.01	OP060612
N7	1,147	*Lelliottia jeotgali*	98.27	OP060613
N8	**1,127**	**Pseudomonas mucoides**	**99.13**	**OP060614**
N9	694	*Lelliottia jeotgali*	99.54	OP060615
N10	**1,111**	**Ensifer meliloti**	**98.83**	**OP060616**
N11	1,126	*Pseudomonas mucoides*	98.84	OP060617
N12	**863**	**Ensifer meliloti**	**99.88**	**OP060618**
N13	1,051	*Lelliottia amnigena*	97.71	OP060619
N14	865	*Priestia megaterium*	99.65	OP060620
N15	1,310	*Pseudomonas lactis*	97.10	OP060621
N16	1,035	*Pseudomonas lactis*	99.13	OP060622
N18	1,374	*Bacillus velezensis*	96.44	OP060623
N20	1,328	*Pantoea vagans*	95.41	OP060624
N21	678	*Pseudomonas lactis*	99.26	OP060625
N23	949	*Enterobacter huaxiensis*	96.63	OP060626
N25	1,257	*Bacillus proteolyticus*	95.83	OP060627
N30	1,314	*Pseudomonas capeferrum*	96.12	OP060628
N32	950	*Bacillus tequilensis*	99.58	OP060629
N33	1,209	*Pseudomonas capeferrum*	98.76	OP060630

Selected strains appear in bold.

TABLE 3 PGP properties and enzymatic activities showed by selected strains.

PGP properties	N4	N8	N10	N12
Phosphate solubilisation	11	12	15	14
Siderophores production	73	44	13	10.2
IAA production	1.562	17.939	1.266	1.00
Biofilm formation	+	+	+	+
N fixation	+	+	+	+
ACC deaminase activity	9.677	9.987	–	–
Enzymatic activities				
DNAse	–	–	+	+
Amylase	–	–	–	–
Cellulase	–	+	+	+
Lipase	–	–	–	–
Pectinase	–	–	–	–
Protease	+	+	–	–
Chitinase	–	–	–	–

N4: *Pseudomonas* sp. N4; N8: *Pseudomonas* sp. N8; N10: *Ensifer* sp. N10; N12: *Ensifer* sp. N12; +, presence of the activity; –, absence of the activity. Values of phosphate solubilisation and siderophores production express the diameter of the halo in mm. Values of IAA production are expressed in $mg{\cdot}L^{-1}$. Values of ACC deaminase activity are expressed in µmoles α-ketobutyrate·mg $protein^{-1}{\cdot}h^{-1}$.

isolates (2 to 5) showed pectinase, lipase, chitinase or DNase activities (Supplementary Table 4).

Tolerance of the strains towards As, Cd, Cu, and Zn were determined and expressed as the maximum tolerable concentration (Table 4; Supplementary Table 5). Endophytes showed good levels of metal/loids tolerance. They tolerated concentrations as high as up to 20 mM As, 2 mM Cd, 6 mM Cu and Zn.

Selection of endophytes

Among isolated endophytes, only N10 and N12 strains belonging to *Ensifer* genus were rhizobia able to nodulate *M. sativa*, so they were selected for assays *in planta*. N4 and N8 were selected as nodule enhancing bacteria (NEB) candidates based on their PGP properties and enzymatic activities, since they showed all the properties assayed and 2 enzymatic activities. They were also able to tolerate moderate levels of the assayed metal/loids (Table 4).

In vitro effect of endophytes in *Medicago sativa* germination and nodulation

The germination of *M. sativa* seeds was evaluated in the presence and absence of a mixture of metals/loids by inoculating them with the selected strains, performing individual inoculations (N4, N8, N10 and N12), co-inoculations combining each of the *Pseudomonas* and each of the rhizobia (N4+N10, N8+N10, N4+N12, and N8+N12) or inoculation with the four strains together (N4+N8+N10+N12). Inoculation improved seed germination and the consortium with the four strains reported the greatest increase, showing the highest percentage of germination both in absence and presence of metals (Figures 1A,B). The increase in the germination rate in absence or presence of metals, followed this pattern: CSN>N8+N10>N4+N10>N8+N12>N4+N12>N8>N10>N12>N4>C-. In absence of As, differences in germination among seeds inoculated with the four strains and those co-inoculated con *Pseudomonas* and *Ensifer* were not statistically significant ($p<0.001$), but these germination rates were significantly higher than those recorded in seeds inoculated with a single strain. Seeds inoculated with single strains did not show significant differences in germination rates among them. The same results could be observed in presence of As, with one single difference, since seeds inoculated with the consortium showed significantly higher germination rates than seeds co-inoculated with *Pseudomonas*

TABLE 4 Maximum tolerable concentration of metal/loid showed by selected strains.

Strain	Cd (mM)	As (mM)	Cu (mM)	Zn (mM)
N4	0.7	1	3	1.9
N8	0.4	2.5	1.9	1.8
N10	0.4	0.4	1.4	2.5
N12	0.1	0	1.8	1.4

N4: *Pseudomonas* sp. N4; N8: *Pseudomonas* sp. N8; N10: *Ensifer* sp. N10; N12: *Ensifer* sp. N12.

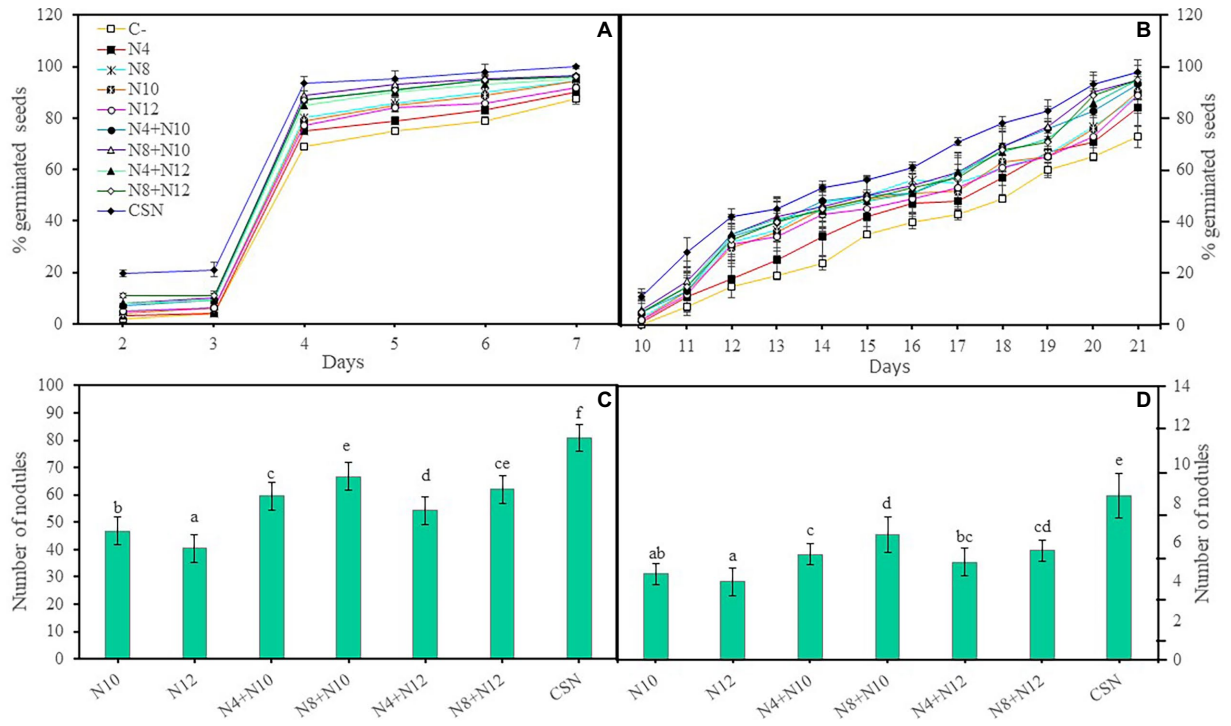

FIGURE 1
In vitro effects of inoculation of *M. sativa* with selected PGPNE. **(A)** Percentage of germinated seeds without metals and **(B)** with metals. Values are means±S.D. ($n=50$). **(C)** Number of nodules in plants without As and **(D)** with As. Values are means±S.D. ($n=5$). Different letters indicate statistical differences between means (One-way ANOVA, LSD test, $p<0.0001$). C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4+N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8+N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N10; N4+N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8+N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12.

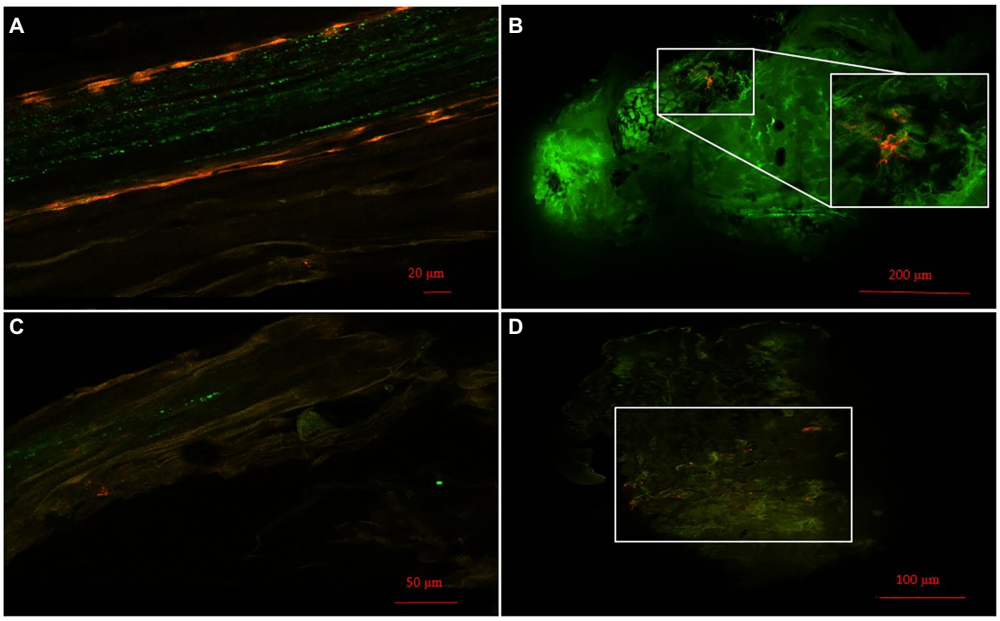

FIGURE 2
Bacterial colonization. **(A)** Images of roots and **(B)** nodules of colonized *M. sativa* 28days after inoculation with *Pseudomonas* sp. N4 marked with mCherry and *Ensifer* sp. N10. **(C)** Images of roots and **(D)** nodules of colonized *M. sativa* 28days after inoculation with *Pseudomonas* sp. N8 marked with mCherry and *Ensifer* sp. N10. The white square in **(B,D)** marks a group of *Pseudomonas* sp. N4 and *Pseudomonas* sp. N8, respectively, in the nitrogen fixation zone.

and *Ensifer* ($p < 0{,}001$). Non-inoculated seeds showed the lowest rates of seed germination, with significant differences, both in absence and presence of As. These significant differences were recorded at the end of the experiment.

To evaluate the ability of the *Pseudomonas* strains to improve *M. sativa* nodulation induced by *Ensifer*, plant seedlings were inoculated or co-inoculated with combinations of *Pseudomonas* and *Ensifer* strains in square plates with or without As. Co-inoculation with either of the *Pseudomonas* and a *Ensifer* strain significantly increased the number of nodules, both in absence and presence of As, compared with the single inoculation with *Ensifer* (Figures 1D,E). The combination *Pseudomonas* sp. N8 and *Ensifer* sp. N10 induced more nodules than any other couple, although the plants inoculated with the combination of the four strains (CSN) showed the highest number of nodules in both conditions, with significant differences. *Ensifer* sp. N10 showed a better behavior in nodulation than N12 and *Pseudomonas* sp. N8 seemed to be better as nodule enhancing bacteria than N4.

Pseudomonas behave as nodule endophytes in *Medicago sativa*

Localization of *Pseudomonas* strains was studied using confocal laser scanning microscopy (CLSM) and mCherry-labeled *Pseudomonas* sp. N4 and N8. Cells were visualized from 0.5 mm sections of roots and nodules of *M. sativa* co-inoculated with *Ensifer* sp. N10 and labelled N4 or N8. Examples of the results obtained are presented in Figure 2. The presence of labelled bacteria with red fluorescence could be observed both in root (Figures 2A,C) and nodule cells (Figures 2B,D). Groups of bacteria were clearly seen in the nitrogen fixation zone of *M. sativa* nodules, marked with a white square in the figures (Figures 2B,D).

Effect of inoculations under greenhouse conditions

Inoculation increased plant biomass

M. sativa plants were grown and inoculated under greenhouse conditions using soil from the marshes of the Odiel River (Huelva). Plant inoculation with any of the strains increased biomass, both shoots and roots, compared with non-inoculated control plants (Figure 3A). Single inoculation with *Pseudomonas* sp. N8 reported higher plant shoot biomass than single inoculations with *Ensifer* or N4, that showed the lowest shoot and root biomasses among single inoculated plants. No significant differences in root biomass were found among plants inoculated with N8, N10 and N12. Co-inoculation with *Pseudomonas-Ensifer* couples produced higher values of root and shoot biomasses than any of the single inoculation treatments, except for plants inoculated with N4-N10 couple, that did not show significant differences in shoot biomass compared with plants inoculated with N8. N8 + N10 combination showed the highest value in plant shoot biomass among the *Pseudomonas-Ensifer* co-inoculation treatments, with significant differences (Figure 3A). Nevertheless,

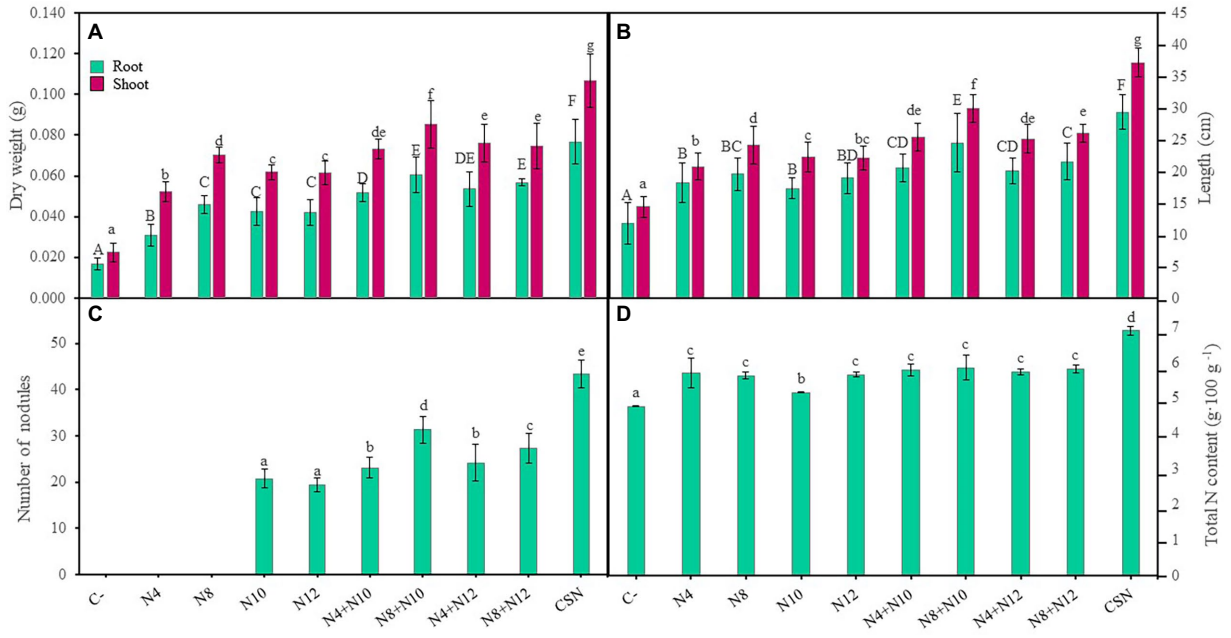

FIGURE 3
Effects of the inoculation of *M. sativa* plants with PGPNE. **(A)** Dry weight of shoot and roots, **(B)** length of shoot and roots, **(C)** number of nodules, and **(D)** nitrogen content after 60days in pots containing soil from the high marshes of the Odiel River. Values are means±S.D. (n=16). Different letters indicate statistical differences between means. Lowercase and uppercase letters are used to qualify different variables and are not comparable among them (One-way ANOVA, LSD test, p<0.001). C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4+N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8+N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N10; N4+N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8+N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12.

the highest values of root and shoot biomass were recorded in plants inoculated with the consortium of the four strains (CSN). Similitudes could be observed using plant root and shoot lengths as parameters to compare inoculation treatments (Figure 3B). Plants co-inoculated with *Pseudomonas* sp. N8 and *Ensifer* reported longer shoots than those inoculated with a single strain, while the combination N8 + N10 showed the longest roots and shoots among plants co-inoculated or inoculated with a single strain. Those inoculations including *Ensifer* and N4 showed no significant differences in root and shoot length compared with plants inoculated only with N8. The longest roots and shoots were observed in plants inoculated with the consortium (Figure 3B). These plants also presented the highest number of leaves with the widest diameter, with statistically significant differences compared to non-inoculated controls or any of the inoculation and co-inoculation treatments (Supplementary Figure 2).

Pseudomonas enhanced plant nodulation

Regarding the number of nodules, *Pseudomonas* increased the number of nodules induced by *Ensifer*, showing co-inoculated plants more nodules than plants inoculated only with *Ensifer*, with significant differences (Figure 3C). Although this result was independent of the *Ensifer-Pseudomonas* combination used, plants co-inoculated with strains N8 and N10 showed higher number of nodules than any other couple. The highest number of nodules was found again in plants inoculated with the consortium (Figure 3C), approximately 109 and 124% more nodules than plants inoculated with N10 and N12, respectively. Nitrogen content in stems and leaves of *M. sativa* plants was also evaluated (Figure 3D). Inoculation treatments increased the N content compared to non-inoculated plants, although no significant differences were found among single inoculation and co-inoculation treatments, except for single inoculation with N10, that reported lower values of N (Figure 3D). The highest content of N, with significant differences, was measured in plants inoculated with the consortium of the four strains.

Pseudomonas ameliorated the physiological state of the plants

Several photosynthetic parameters were measured in order to determine the physiological state of the plants (Figure 4; Supplementary Figure 3). Inoculated plants showed higher values in all the parameters recorded compared to non-inoculated plants and the highest values were always measured in plants inoculated with the consortium, with significant differences. Values in the net photosynthetic rate (A_N) could be described by the following pattern, with significant differences: CSN > N8 + N10 > N8 + N12 = N4 + N10 = N4 + N12 > N8 = N4 > N12 = N10 > C- (Figure 4A). With the exception of the inoculation with the consortium, no differences were found in the maximum quantum efficiency of the

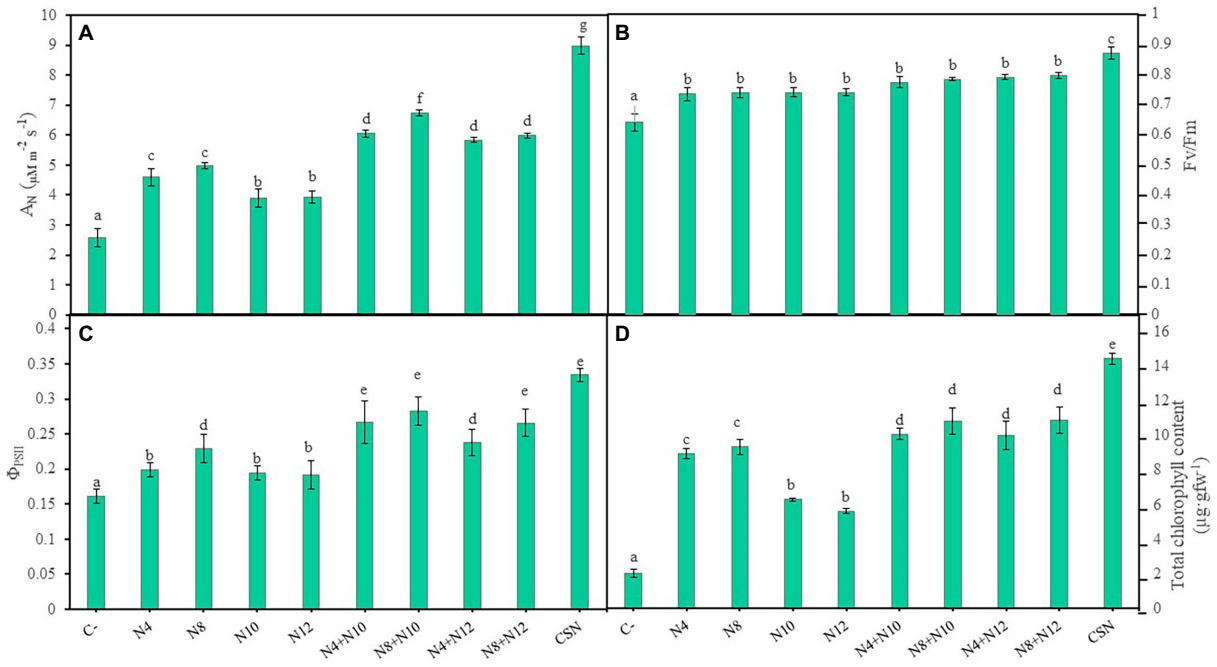

FIGURE 4
Photosynthetic parameters of *M. sativa* plants inoculated with PGPNE. **(A)** Net photosynthetic rate (A_N), **(B)** maximum quantum efficiency of PSII photochemistry (Fv/Fm), **(C)** quantum yield of PSII photochemistry (Φ_{PSII}), and **(D)** total chlorophyll content after 60days in pots containing soil from the high marshes of the Odiel River. Values are means±S.D. (n=16). Different letters indicate statistical differences between means (One-way ANOVA, LSD test, p<0.0001). C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4+N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8+N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N10; N4+N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8+N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12.

PSII photochemistry (Fv/Fm) among inoculation conditions (Figure 4B). Concerning the quantum yield of the PSII photochemistry (Φ_{PSII}), results followed the pattern: CSN > N8 + N12 = N8 + N10 = N4 + N10 > N4 + N12 = N8 > N12 = N10 = N4 > C- (Figure 4C). In the same way, plants inoculated with the consortium showed the highest values in electron transport rate (ETR), followed by plants co-inoculated with N8 + N10 (Supplementary Figure 3). Finally, values of total chlorophyll content in plants could be described by a pattern similar to the one observed for Fv/Fm: CSN > N8 + N12 = N4+ N12 = N8 + N10 = N4 + N10 > N8 = N4 > N12 = N10 > C- (Figure 4D).

Pseudomonas increased the activity of plant antioxidant enzymes

The influence of bacterial inoculations on plant response to stress was evaluated by determining the activity of different antioxidant enzymes in roots and leaves of *M. sativa* (Figure 5). All the enzymatic activities increased in response to inoculation, both in roots and shoots, compared to non-inoculated plants, with higher increases in roots than in shoots. The highest enzymatic activities were always recorded in plants inoculated with the consortium, with significant differences. Guaiacol peroxidase (Figure 5A) and ascorbate peroxidase (Figure 5D) activities presented similar results, showing plants inoculated with N8 + N10 the highest levels of activity in roots among plants co-inoculated or inoculated with single strains. Concerning catalase activity, plants co-inoculated with *Pseudomonas-Ensifer* couples had higher levels of activities in roots and shoots than those inoculated only with one strain, with the single exception of plants inoculated with N10, that showed the same level activity in shoots than co-inoculated plants (Figure 5C). Finally, plants inoculated with the combination N4 + N10 showed more superoxide dismutase activity in roots and shoots than any other inoculation condition, with the exception of plants inoculated with the consortium (Figure 5B).

Pseudomonas increased metal accumulation in roots

Accumulation of As and the most abundant metals in the high marshes of the Odiel river was determined in *M. sativa* tissues at the end of the greenhouse experiment (Figure 6). The highest levels of metal/loids found in shoots were 1.5 ppm of As, 0.04 ppm of Cd, 27.76 ppm of Cu and 61.2 ppm of Zn, without significant differences among inoculation conditions (Supplementary Table 6). Different behavior was observed in roots. Single inoculations with rhizobia (N10 and N12) reduced As accumulation in roots compared to control plants (Figure 6A). Plants inoculated with rhizobia also showed the lowest values of Cu and Cd accumulated in roots

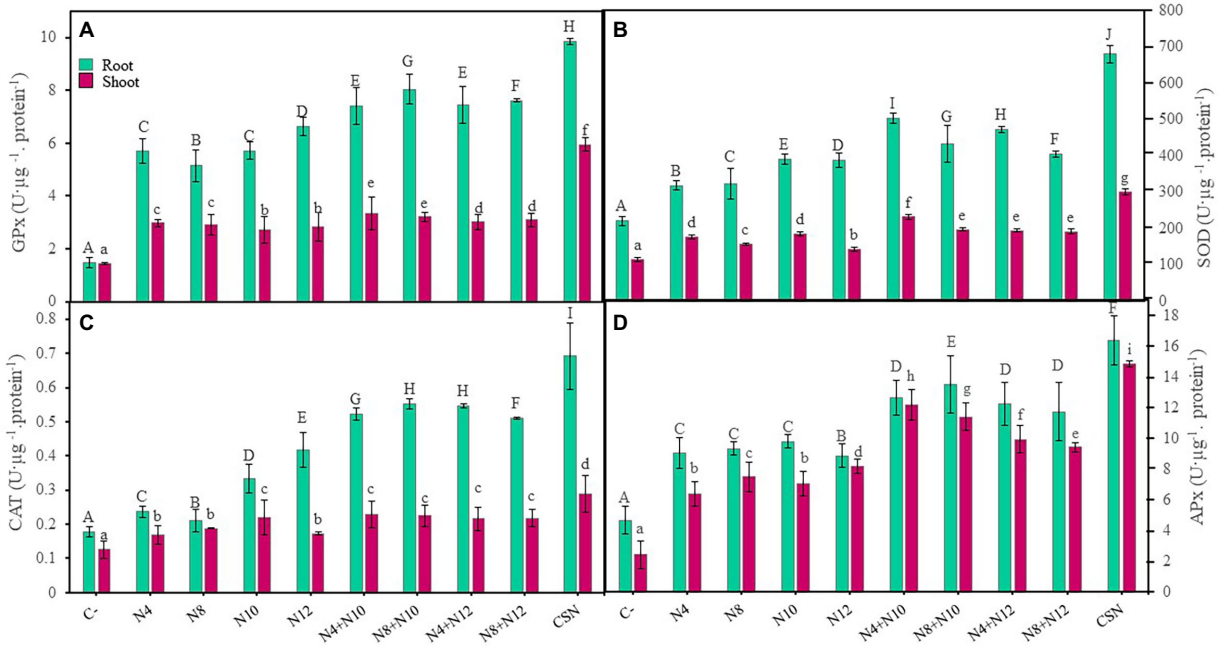

FIGURE 5
Antioxidant enzymes in *M. sativa* plants inoculated with PGPNE. **(A)** Guaiacol peroxidase, **(B)** superoxide dismutase, **(C)** catalase, and **(D)** ascorbate peroxidase activities after 60days in pots containing soil from the high marshes of the Odiel River. Values are means±S.D. (n=16). Different letters indicate statistical differences between means. Lowercase and uppercase letters are used to qualify different variables and are not comparable among them (One-way ANOVA; LSD test, p<0.001). C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4+N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8+N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N10; N4+N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8+N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12.

compared with the rest of inoculation conditions (Figures 6B,C). Concerning Zn, the lowest amounts were recorded in plants inoculated with N12 and the highest in plants inoculated with N8, while plants inoculated with the other strains (N4 and N10) accumulated the same amount of this metal (Figure 6D). On the contrary, the highest values of metal accumulation were found in roots of plants inoculated with the consortium of the four bacteria (Figure 6). In general, plants inoculated with *Pseudomonas-Ensifer* couples accumulated more As and metals in roots than those inoculated only with *Pseudomonas*, with the exceptions of Cu and Cd in plants inoculated with the couple N8-N10 (Figure 6).

Discussion

Climate change and human activities are causing abiotic stress in many soils previously suitable for crops. Use of agrochemicals, heavy metals release, drought or salinity cause a great loss of nutrients and native microorganisms in the soil, altering plant adaptation and growth (García-Martí et al., 2019; Du et al., 2021). Environmentally friendly solutions to regenerate soils should include the use of leguminous plants, since they are high biomass producers, reduce the use of nitrogen-based fertilizers and can be used for fed or feeder in agriculture (Graham and Vance, 2003; Ferreira et al., 2021). In addition, most legumes are metal phytostabilizers suitable for regeneration of metal contaminated soils (Jach et al., 2022). Legume adaptation and growth in nutrient-poor or degraded soils can be enhanced using adequate autochthonous rhizospheric or endophytic PGPB as inoculants (Monteoliva et al., 2022).

The objective pursued in this work was the isolation and characterization of endophytic bacteria from nodules of *Medicago* spp. plants growing in the estuary of the Odiel river (Huelva, Spain), capable of improving seed germination, nodulation and growth of *M. sativa* in soils with low nutrient content and moderate to high levels of heavy metals. 33 strains belonging to genera previously described as nodule endophytes, such as *Achromobacter, Bacillus, Enterobacter, Lelliottia, Pantoea, Priestia* or *Pseudomonas* (Aserse et al., 2013; Martínez-Hidalgo and Hirsch, 2017), were isolated. Among NRE, *Pseudomonas* was the most represented genus and only two isolated were nodule inducing rhizobia from *Ensifer* genus, the most common *Medicago* symbiont frequently isolated from wild legumes growing in arid soils (Mnasri et al., 2007; León-Barrios et al., 2009; Bessadok et al., 2020).

Characterization of the isolates revealed the presence of at least 4 out of 6 PGP properties studied, several enzymatic activities and moderate to high levels of As and metals tolerance in all of them. Strains *Pseudomonas* sp. N4 and *Pseudomonas* sp.

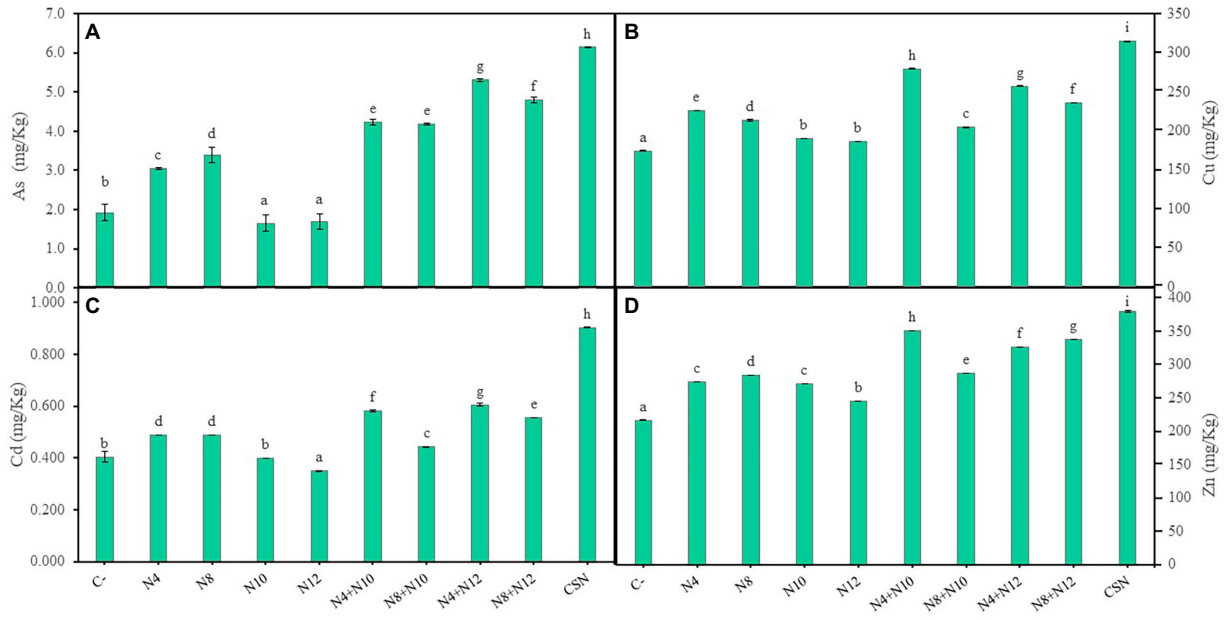

FIGURE 6
Accumulation of metal/loids in roots of *M. sativa*. **(A)** Accumulation of arsenic, **(B)** copper, **(C)** cadmium, and **(D)** zinc after 60days in pots containing soil from the high marshes of the Odiel River. Values are means±S.D. (n=16). Different letters indicate statistical differences between means (One-way ANOVA; LSD test, p<0.001). C-: non inoculation; N4: inoculation with *Pseudomonas* sp. N4; N8: inoculation with *Pseudomonas* sp. N8; N10: inoculation with *Ensifer* sp. N10; N12: inoculation with *Ensifer* sp. N12; N4+N10: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N10; N8+N10: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N10; N4+N12: co-inoculation with *Pseudomonas* sp. N4 and *Ensifer* sp. N12; N8+N12: co-inoculation with *Pseudomonas* sp. N8 and *Ensifer* sp. N12; CSN: inoculation with *Pseudomonas* sp. N4, *Pseudomonas* sp. N8, *Ensifer* sp. N10 and *Ensifer* sp. N12.

N8, showing all the PGP properties assayed, were selected for *in planta* experiments to determine their potential as nodule enhancing endophytes (NEE) in co-inoculation with *Ensifer* sp. N10 and *Ensifer* sp. N12. In this work, strains N4 and N8 have proved to be nodule endophytes, since fluorescent m-Cherry labelled strains were observed inside *M. sativa* roots and nodules induced by *Ensifer*.

Inoculation with the selected endophytes improved *M. sativa* seeds germination both in presence and absence of As and metals. Several properties may have contributed to this effect: (i) ACC deaminase activity, observed in N4 and N8, that regulates stress level in plants through ethylene degradation (Penrose and Glick, 2003; Chandwani and Amaresan, 2022), (ii) IAA produced by the selected strains, a hormone involved in plant development (Khan et al., 2020; Hu et al., 2021), and (iii) cellulase activity presents in strains N8, N10 and N12, a lytic enzyme that could participate in plant cell wall degradation during seed germination (Flores-Duarte et al., 2022b). The combined effect of these and other properties would also explain why co-inoculation experiments showed better results than single inoculation with one strain and the highest percentage of germination was observed in seeds inoculated with the consortium of the four strains.

The ability of the selected NEE to promote *M. sativa* growth and nodulation in greenhouse conditions was evaluated in soils collected from Odiel river marshes, with low levels of nutrients and moderate to high amounts of As and metals. Inoculation with any of the strains improved plant biomass, root and shoot length and number and diameter of the leaves. This improvement was generally higher in plants co-inoculated with *Pseudomonas-Ensifer* couples than in plants inoculated with one single strain. The highest plant weights and heights were recorded in plants inoculated with the four strains. The increase in the length of plant roots and shoots may be related to the production of IAA (Khan et al., 2020), that plays an essential role in the development and growth of plants, by increasing cell expansion and is an essential hormone in root development (Hu et al., 2021). Endophyte properties related with nutrient acquisition could also be involved in these results. P availability in the soil is a limiting factor for plant growth and development (Timofeeva et al., 2022). Endophytes could provide phosphorus through phosphate solubilization (Mei et al., 2021; Zhang et al., 2022). In legumes, P helps in the nodulation process, amino acid and proteins synthesis (Wang et al., 2020). Through siderophore production endophytes can provide essential metals such iron and Zn (Mahanty et al., 2017). Other related properties that influence legume growth could be biofilm formation, that is an important mechanism during bacterial attachment to the root, facilitating root colonization and endophyte entry and, at the same time, enhancing nutrient absorption (Das et al., 2012). Finally, the production of lytic enzymes by endophytes could benefit plant growth. These enzymes are essential in the symbiosis between plants and microorganisms, since they contribute to the degradation of organic matter, the colonization of bacteria, the acquisition of nutrients (Wang and Dai, 2010; Eid et al.,

2021), and the degradation of starch as an energy source in the germination stage (Walitang et al., 2017).

Concerning nitrogen, co-inoculation with *Ensifer* and *Pseudomonas* increased the number of nodules induced by *Ensifer* in single inoculations, both in presence and absence of metals, *in vitro* and in greenhouse conditions. Nevertheless, there were no significant differences in the amount of nitrogen accumulated in shoots among inoculation conditions, except in plants inoculated with the consortium that showed the highest levels of nitrogen with significant differences. ACC deaminase activity present in *Pseudomonas* could have facilitated the reduction of the stress levels in the the plants by reducing ethylene (Singh et al., 2022), which in excess causes defoliation, senescence and inhibit cell elongation, among other effects (Fatma et al., 2022). In that way, ACC deaminase favors germination, nodulation, and the development of plants (Singh et al., 2022). ACC deaminase activity and IAA production also play an interesting role in the nodulation process by delaying the senescence of the nodule, creating an interaction with the bacteroid (Alemneh et al., 2020). Strains N4 and N8 grew in a nitrogen-free medium, suggesting that they could fix atmospheric nitrogen. In a work in progress, nitrogenase genes (*nifKDH*) have been amplified in these strains (data not shown). This could explain the high levels of nitrogen measured in plant inoculated with *Pseudomonas*.

The positive effects of *Pseudomonas-Ensifer* co-inoculation on plant development were also reflected in the physiological state of the plants, since co-inoculations improved most of the photosynthetic parameters analyzed: A_N, Φ_{PSII}, ETR and total chlorophyll, except for plants co-inoculated with N4 + N12 plants, that showed the same values of Φ_{PSII} than plants inoculated with N8. In addition, plants inoculated with the consortium of the four strains showed the highest efficiency of photosystem II (Φ_{PSII}), the best balance of carbon and water assimilation, the greatest efficiency in the use of energy from the photochemical apparatus, and the biggest increase in total chlorophyll (Liu et al., 2020).

In this work, the levels of stress in *M. sativa* plants were measured by recording the activity of antioxidant defense enzymes, particularly catalase, guaiacol peroxidase, ascorbate peroxidase and superoxide dismutase, that act as protectors against reactive oxygen species (ROS). An excess of ROS production as well as a decrease in the antioxidant defense mechanism causes a breakdown of cell function, damaging the plant and reducing its development (Redondo-Gómez et al., 2011; Mesnoua et al., 2016). Our results showed higher enzymatic activities in plants co-inoculated with *Ensifer* and *Pseudomonas* than in single inoculated plants and the highest activities in plants inoculated with the consortium of the four bacteria. This pointed to the role of these bacteria in regulating stress mechanisms in plants. Induction of antioxidant enzymatic activities after inoculation with rhizospheric bacteria has been reported in plants of *M. sativa* under heavy metal stress (Raklami et al., 2019) and nutrient deficiency (Flores-Duarte et al., 2022b), and in crops such as maize, where inoculated plants showed an increase of catalase, peroxidase, and superoxide dismutase activities (Kukreti et al., 2020; Chaudhary et al., 2021; Agri et al., 2022). Concerning endophytes, increased enzymatic activities have been reported recently in *M. sativa* plants growing under stress conditions and inoculated with a bacterial consortium containing *Variovorax* nodule endophytes (Flores-Duarte et al., 2022a).

The use of legumes and associated rhizobia is an interesting tool to fight against soil metal contamination, since legume interacting with rhizobia have the ability to accumulate high concentrations of metals particularly in roots, with low levels of translocation to shoots, without disturbing plant growth (Jach et al., 2022). In this work, the highest levels of As, Cd, Cu and Zn measured in shoots of *M. sativa* were much below those allowed for human or animal consumption (Junta de Andalucía and Consejeria de Medio Ambiente, 1999), indicating that our strategy would not be dangerous for living beings in terms of metals mobilization. Regarding the accumulation of metals/loids in the roots, inoculation with *Pseudomonas* or *Pseudomonas-Ensifer* couples promoted metal accumulation and the highest levels of As, Cd, Cu and Zn were measured in *M. sativa* roots inoculated with the consortium of the four strains (CSN). In that way, inoculation with *Pseudomonas* strains enhanced the metal phytostabilization potential of *M. sativa* plants in soils with moderate to high levels of metals, without negative effects on plant growth.

Although combinations of *Pseudomonas* and *Ensifer* strains, and particularly those including N8, had a significant positive effect on *M. sativa* growth and nodulation, the best results were always recorded in plants inoculated with the consortium containing the four strains, demonstrating the advantages of using a consortium with several strains and the collaborative effects of their properties.

A consortium of three rhizospheric bacteria able to promote *M. sativa* growth and adaptation in estuarine soils with nutrients poverty has been recently reported (Flores-Duarte et al., 2022b). These PGPB inoculated as consortium in poor-nutrient soils increased plant shoot biomass (around 100%) and ameliorated *M. sativa* nodulation (around 50%) compared to plants inoculated only with rhizobia. Although we have to consider differences between soils to establish comparisons, particularly due to levels of metal contamination, in the current work our consortium of endophytes increased plant shoot biomass around 100% and also ameliorated *M. sativa* nodulation around 100% compared to plants inoculated only with the best performing rhizobia for each parameter. Despite these differences in nodulation improvement, both consortiums induced similar increases in plant N content (around 1 g/100 g). Inoculation of *M. sativa* with the rhizospheric consortium also induced positive effects on plant photosynthetic status under nutrient deficiency (Flores-Duarte et al., 2022b). All the photosynthetic parameters recorded showed increased values in plants inoculated with the consortium compared to plants inoculated only with rhizobia, even though, parameters such as A_N or Φ_{PSII} showed no significant differences (Flores-Duarte et al., 2022b). In this work, significant differences in the levels of all the photosynthetic parameters recorded, including A_N and Φ_{PSII}, were observed between plants inoculated with the consortium and those inoculated with

rhizobia, suggesting that endophytes were more efficient in enhancing the photosynthetic status of the plant than rhizospheric bacteria.

The presence of moderate to high levels of metals in the soil, increasing *M. sativa* stress conditions, required the isolation and characterization of specific bacteria able to help the plant to deal with these particular environmental conditions. Our results suggest that inoculants based on non-rhizobial nodule endophytes could be useful and efficient tools to enhance legume adaptation and growth in metal contaminated and nutrient-poor soils. At least under stress conditions, endophytes could provide some advantages as PGPB compared to rhizospheric bacteria, in addition to their narrower relationship with the plant and the lack of competence with soil bacteria (Adeleke and Babalola, 2021; Dwibedi et al., 2022). In that way, it looks necessary to investigate microbiomes of a wide diversity of nitrogen-fixing nodules to find useful inoculants to be applied in different environmental conditions (Martínez-Hidalgo and Hirsch, 2017).

Conclusion

Autochthonous *Pseudomonas* sp. N4 and *Pseudomonas* sp. N8 strains enhanced growth, nodulation and metal accumulation in roots of *M. sativa* plants inoculated with wild type *Ensifer* strains in soils with low nutrients content and moderate to high levels of metals, ameliorating the physiological state of the plants and helping to regulate plant stress mechanisms, thus facilitating plant adaptation to these abiotic stresses. Our results suggest that selected native PGPNE (plant growth promoting nodule endophytes) could be adequate biotools to promote legume adaptation, growth and phytostabilization potential in nutrient-poor and/or metal contaminated estuarine soils.

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/, OP060607-OP0606030.

Author contributions

IR-L and SN-T: conceptualization and writing—original draft preparation. NF-D and SC-D: methodology and investigation. SN-T: software. SN-T and NF-D: formal analysis. EM-N and SR-G: data curation. IR-L, EM-N, and EP: writing—review and editing and funding acquisition. IR-L and SR-G: supervision. EP: project administration. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by MCIN, project MCIN/AEI/10.13039/501100011033, UE "NextGenerationEU/PRTR (PDC2021-120951-I00) and Junta de Andalucía, I+D+I FEDER Andalucía project US-1262036 and PAIDI2020, project P20_00682.

Acknowledgments

We thank the personnel from the greenhouse and the microscopy service of the Central Services of the Universidad de Sevilla (CITIUS) for their help. NF-D also thanks Universidad de Sevilla for personal support through "Ayudas al estudio a refugiados" (Oficina de Cooperación al Desarrollo).

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.2022.1005458/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 26 January 2023
DOI 10.3389/fmicb.2022.1064055

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 07 October 2022
ACCEPTED 29 December 2022
PUBLISHED 26 January 2023

CITATION
Santra HK and Banerjee D (2023) Drought alleviation efficacy of a galactose rich polysaccharide isolated from endophytic *Mucor* sp. HELF2: A case study on rice plant. *Front. Microbiol.* 13:1064055. doi: 10.3389/fmicb.2022.1064055

Drought alleviation efficacy of a galactose rich polysaccharide isolated from endophytic *Mucor* sp. HELF2: A case study on rice plant

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Endophytes play a vital role in plant growth under biotic and abiotic stress conditions. In the present investigation, a Galactose-Rich Heteropolysaccharide (GRH) with a molecular weight of 2.98×10^5Da was isolated from endophytic *Mucor* sp. HELF2, a symbiont of the East Indian screw tree *Helicteres isora*. OVAT (One Variable at A Time) experiment coupled with RSM (Response Surface Methodology) study exhibited 1.5-fold enhanced GRH production (20.10gL^{-1}) in supplemented potato dextrose broth at a pH of 7.05 after 7.5days of fermentation in 26°C. GRH has alleviated drought stress (polyethylene glycol induced) in rice seedlings (*Oryza sativa* ssp. indica MTU 7093 swarna) by improving its physicochemical parameters. It has been revealed that spray with a 50-ppm dosage of GRH exhibited an improvement of 1.58, 2.38, 3, and 4 times in relative water contents and fresh weight of the tissues, root length, and shoot length of the rice seedlings, respectively "in comparison to the control". Moreover, the soluble sugars, prolines, and chlorophyll contents of the treated rice seedlings were increased upto 3.5 (0.7±0.05mg/g fresh weight), 3.89 (0.57±0.03mg/g fresh weight), and 2.32 (1,119±70.8μg/gm of fresh weight) fold respectively, whereas malondialdehyde contents decreased up to 6 times. The enzymatic antioxidant parameters like peroxidase and superoxide dismutase and catalase activity of the 50ppm GRH treated seedlings were found to be elevated 1.8 (720±53 unit/gm/min fresh weight), 1.34 (75.34±4.8 unit/gm/min fresh weight), and up to 3 (100ppm treatment for catalase – 54.78±2.91 unit/gm/min fresh weight) fold, respectively. In this context, the present outcomes contribute to the development of novel strategies to ameliorate drought stress and could fortify the agro-economy of India.

KEYWORDS

optimisation, drought stress alleviation, *Mucor* sp. HELF2, endophyte, hetero polysaccharide

Introduction

The foundation of the global food economy is agriculture, and in a nation like India, Gross Domestic Production (GDP) is heavily reliant on the agrarian model. Crop loss due to biotic and abiotic stressors is a widespread issue that requires effective management techniques to keep the agroecosystem in good shape. Biotic stress-related issues can be addressed with a variety of chemical formulations, such as pesticides, herbicides, fungicides, and biological techniques, such as biological control agents (BCA) and plant growth promotors (PGPs), but abiotic stress management strategies have received scant attention. As a result, issues with salt and drought stress are severely impeding crop development and yield, with dryness being the most detrimental. Agriculture production is significantly declining globally (Chen et al., 2017). The problem is getting worse as a result of cases

of global warming and water scarcity. The agrarian economy is struggling with production-related problems as well as severe financial constraints (Vurukonda et al., 2016). According to reports, to fulfil this ambitious goal by 2050, food production must increase by up to 60 to 110 percent. Drought-related challenges must be immediately resolved (Naumann et al., 2018; Dey et al., 2019; Paglia and Parker, 2021). Therefore, it is urgent to discover a new, long-lasting solution to this global dilemma (Coleman-Derr and Tringe, 2014). One of the initial answers to that problem is to create stress-resistant varieties, but doing so takes time, is rigorous, species-specific, and is expensive (Santra and Banerjee, 2022a). One approach might be to cultivate crops on reclaimed drought-affected land while using foliar plant growth-promoting/stress-resisting chemicals. Rhizobacteria that promote plant growth have already been evaluated for this purpose, and less-studied endophyte or endophytic fungal or bacterial polysaccharides are currently showing promise in this field (Chen et al., 2017; Sun et al., 2020a,b).

Endophytes are ubiquitous in occurrence and are procured from nearly all plants and plant parts studied to date across the globe (Coleman-Derr and Tringe, 2014; Chatterjee et al., 2022). The symbionts of plant tissues known as endophytes help the plant grow and give tolerance in challenging conditions. Due to their horizontal gene transfer as a result of the co-evolution of the host and microorganisms, they share crucial genes of essential metabolomes (Santra et al., 2022; Santra and Banerjee, 2022b; Santra and Banerjee, 2022c). They have a reputation for being bioactive chemical mines that are simple to access using contemporary biotechnological methods. Plant growth-promoting endophytic bacteria and fungi reside on different internal plant tissues and organs, i.e., in stems, roots, flowers, leaves, fruits, and seeds. Endophytes have recently drawn attention because they are an effective tool for teaching plants to tolerate lethal abiotic stressors like drought, salt, and heavy metal toxicity (Cherif et al., 2015; Mesa et al., 2015; Pinedo et al., 2015; Constantin et al., 2019; Moghaddam et al., 2021) through adopting various mechanisms. The two most prevalent and important methods of building stress resistance are decreasing the levels of the key gaseous hormone ethylene through the activity of ACC (1-aminocyclopropane−1-carboxylate) deaminase and increasing the content of prolines in the tissues (Blaha et al., 2006; Gamalero and Bernard, 2012; Marasco et al., 2012). In addition to these, accumulation of siderophores and osmolytes, increased antioxidant and photosynthetic rates, synthesis of phytohormones and organic acids, and emission of volatile organic compounds are other important mechanisms used by endophytes to increase host plant abiotic stress tolerance (Tiwari et al., 2016; Vurukonda et al., 2016). Recent reports include that microbial symbionts or fungal endophytes are the co-evolution partners of green plants and promote habitat-specific stress tolerance in host plants (; Rodriguez and Redman, 2008; Redman et al., 2011). A special type of long-chain polymeric secondary metabolite called exopolysaccharides (EPS) from endophytic sources holds immense agricultural utility especially in ameliorating drought and salt stress (Nadeem et al., 2014; Rolli et al., 2015).

In the current study, exopolysaccharide was extracted from the endophytic fungus *Mucor* sp. HELF2 (isolated from *Helicteres isora* flowers). The EPS was galactose-rich heteropolysaccharide (GRH) in composition. GRH was found to be effective in reducing drought stress conditions when applied to the foliar parts of the rice seedling *Oryza sativa* ssp. indica MTU 7093 swarna. GRH production by HELF2 was optimised by adopting statistical modelling using Minitab and the predicted model led to an enhancement of 1.5 times exopolysaccharide (GRH) production under optimised fermentation conditions. The application of 50 ppm GRH was discovered to be the most efficient dosage, and the physical/biochemical traits of the treated plants were discovered to be higher than those of the untreated ones. Root and shoot length, fresh weight, enzymatic antioxidant profiles, and proline contents were improved remarkably after treatment. The membrane damage caused by lipid peroxidation was also minimised when GRH was applied *in vivo*. MDA content was reduced and SOD, CAT, and POD values were elevated. The current study illuminates the agricultural potential of endophytic exopolysaccharide, which has the potential to expand the field of sustainable development and improve the agro-economy of our nation's indigenous population.

Materials and methods

Isolation and identification of GRH-producing endophytic fungi

Mucor sp. HELF2 was isolated as an endophyte from the flower of an ethnomedicinally valuable plant *Helicteres isora* collected from forests in the East Singbhum district, Jharkhand, West Bengal, India, and stored, maintained on PDA slants and Petri plates at 4 ± 2°C and 25 ± 2°C, respectively. In brief, plant parts were thoroughly washed by running tap water for 5 min, sodium hypochlorite (2–10%) for 2 min, and hydrogen peroxide (2%) for 1 min, respectively, and explants were incubated on water agar plates at 27°C on biological oxygen demand incubator for endophyte isolation. Water agar plates were supplemented with antibiotics (streptomycin and tetracycline- 50 mg L^{-1}) to avoid bacterial endophytes. The effectiveness of this sterilisation and isolation process was cross-checked by the explant imprintation technique described by Schulz et al. (1993). In brief, the aliquots used for explant sterilisation were spread on a water agar medium and incubated under the same conditions. After, 3–5 days of incubation, fungal hyphae emerged from the tissues and they were transferred to PDA plates for optimum growth (Schulz et al., 1993). Emerging fungal hyphal tips were transferred to PDA (Potato Dextrose Agar) medium and morphology (both macroscopic and microscopic) of the fungal isolate was recorded using light (Primo Star, Zeiss, Germany) and stereo microscope (Stemi 508, Zeiss, Germany).

The organism was identified by rDNA-based molecular technique as there was no reproductive structure produced by the endophytic fungi even in a medium with carnated leaves. In brief, genomic DNA of the fungal isolate was obtained (using DNeasy Plant Minikit-Qiagen, Germany) and a polymerase chain reaction was performed using the two universal primers named ITS1 (5'-TCCGTAGGTGAACC TTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′; Laich and Andrade, 2016; Landum et al., 2016). The PCR products were separated using 1% agarose gel in 1X TAE buffer (90 mM Tris-acetate and 2 nM EDTA, pH 8.0), stained with ethidium bromide (0.5 μg mL^{-1}), and documented using BIO-RAD Gel Doc EZ imager version 5.1 (United States). PCR products were sent for direct bi-directional sequencing using ABI 3730xl Genetic Analyzer (Applied Biosystems, United States) to Bioserve Biotechnologies (India) Pvt. Ltd., A Repro Cell Company, Hyderabad, India. The obtained consensus sequence of 620 bp was used for further study. Sequences were submitted to GenBank and were compared to the GenBank database using BLAST. Fifteen sequences along with HELF2 were selected and aligned using the multiple alignment software program Clustal W and the phylogenetic tree was prepared using MEGA 11 (Tamura et al., 2021).

Production of GRH and optimisation of culture conditions by OVAT technique

Endophytic fungi were grown in different 250 mL Erlenmeyer flasks with 50 mL potato dextrose broth in a shaker incubator at 120 rpm for 8 days. An initial medium pH of 6 and a medium temperature of 28°C were maintained.

To detect the optimum culture conditions for the maximum production of GRH, fungi were grown in varying fermentation times (4–10 days), then in different medium pH (5.5–7.9), and then at varying incubation temperatures (20–30°C) in separate Erlenmeyer flasks with separate PDB medium. To find out the requirement of additional nutrients for maximum GRH production and mycelial growth, various carbon sources (5 g%, w/v of fructose, glucose, maltose, starch, rhamnose, raffinose, glycerol), various organic and inorganic nitrogen sources (0.4 g% w/v of peptone, ammonium nitrate, urea, ammonium chloride, glycine and yeast extract) in different Erlenmeyer flasks were used with PDB as the basal medium. After the finalisation of the additional carbon and nitrogen sources, their optimum concentration was confirmed by using different concentrations of these products on a PDB medium and the respective biomass and GRH amounts were calculated. A variety of ionic salts (0.1 g%, w/v of $MgCl_2$, $FeCl_3$, KCl, NaCl) and phosphate sources (0.1 g%, w/v including NaH_2PO_4, K_2HPO_4, KH_2PO_4) were analysed separately to detect their role in fungal biomass and GRH production (Mahapatra and Banerjee, 2013, 2016).

To detect the O_2 requirement, fungi were grown with different medium volumes in 250 mL Erlenmeyer flasks. Headspace volume, medium volume, total volume, and medium depth in flask culture were measured for the indirect measurement of the organism's O_2 requirement (Wonglumsom et al., 2000).

BBD based optimisation

Further optimisation was performed with the RSM (Response surface methodology). The investigational design was a Box–Behnken experimental setup with the four most important factors obtained from the OVAT system. The four independent factors had three different levels (−1, 0, and + 1) each for the experiment. GRH production was set to a second-order polynomial equation by the means of multiple regression techniques. The model involving the most significant factors was derived. The system performance follows the subsequent second-order polynomial equation: $Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X^2_i$, where Y is the predicted response or dependent variable, x_i and x_j are independent factors, β_0 is the intercept of the regression equation, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient (Mahapatra and Banerjee, 2013, 2016).

Estimation of GRH

Fungal biomass was separated from the culture extract by centrifugation at 10,000 rpm. Mycelial biomass was dried at 55°C for 24 h and weighed. The supernatant was concentrated in a rotary evaporator under low pressure at 40°C. Chilled absolute ethanol was added to the concentrated supernatant (5:1 v/v), mixed thoroughly, and kept for 24 h at freezing conditions finally, the recovery of viscous precipitate was done by centrifugation at 10,000 rpm for 10 min. The recovered polysaccharide was dialyzed in a cellulose membrane (MW cut off 10,000) against distilled water for 24 h. It was tested for sugar and protein contents following the methods of Dubois et al. (1956) and Lowry (1951) with glucose and bovine serum albumin as the standard. Obtained EPS solution was concentrated in a rotary evaporator under low pressure at 40°C for characterisation.

Characterisation of GRH

GRH was purified by gel chromatographic technique using a Sepharose-6B gel filtration column (65 × 2 cm) and average molecular weight was determined following the methods of Mahapatra and Banerjee (2013). The dried polysaccharide was subjected to characterisation using GC–MS with some pre-treatments (Proestos et al., 2006). Using a water bath at 70°C for 15 min, 100 mg of dried exopolysaccharide was combined with 1 mL MeOH, 20 µl ribitol (which serves as an internal standard), and 20 µl nor-leucine. The entire mixture was then centrifuged for 5 min at 10,000 rpm, and the supernatant was immediately dried and dissolved in 20 µl of methoxy-amine HCL for 120 min at 37°C. The final 1 µl of the derivatized EPS sample was loaded onto the GC–MS for analysis of monosaccharide composition after 40 µl of TMS (Trimethyl siloxane) had been added. The instrument was set up with a 30 m × 0.25 mm DB-5 Ultra Inert column. With a split ratio of 25:1, the inlet temperature was 230°C, and the MS transfer line temperature was 250°C. A constant flow mode was used for the column's flow, which was 1.3 mL min^{-1} with an average linear starting velocity of 39 cm sec^{-1}. Helium was used as the carrier gas, and ZB − 1701 served as the guard column. The program was isothermal, holding at 70°C for 5 min, increasing by 10°C per minute to 180°C, holding for 2 min, increasing by 10°C per minute again to 220°C, holding for 1 min, and finally ramping up by 2.5°C per minute. Up to 265°C with a 1-min hold, then a ramp up to 285°C with a 1-min hold, and finally a climb of 1°C per min up to 290°C with a 0.6-min hold. The mass spectrum was obtained in scan mode from 40 to 650 amu with a detection threshold of 100 ion counts while the detector was in positive ion mode. Appropriate configurations (D-dextrorotatory and L-laevorotatory form) of the sugars were identified by matching them with the NIST library. To detect the sugar linkages, procedures of Ciucanu and Kerek (1984) and Das et al. (2009) were adopted using the GC–MS equipment.

Exopolysaccharide (GRH)-mediated plant growth promotion under drought stress

Initially, healthy and disease-free rice seeds (*O. sativa* ssp. indica MTU-7093 Swarna) were surface sterilised with a series of surface disinfectants: sodium hypochlorite (2.5%) for 20 min, deionised double-distilled water (3–5 times thoroughly) and then soaked in water for germination followed by storing at 22°C for 72–96 h. Uniformly seeds were transferred to a hydroponics box supplemented with Hoagland solution and were replaced at an interval of 3–4 days (Chen et al., 2011). Seedlings reaching an age of 15–20 days were divided into four separate groups with the control group (un-inoculated water), 20 ppm, 50 ppm, and 100 ppm GRH application, respectively, at a frequency of 3 times a day (morning, noon, and afternoon) for 45 days. Simultaneously, 20% polyethylene glycol (PEG)-6,000 (for 7 days) was mixed with a hydroponic solution as a drought-inducing component, and all the biochemical tests were performed from drought-induced seedlings. The

fresh weight of rice seedlings was measured and leaves were stored at −20°C for further biochemical estimations.

The relative water content (RWC) of the treated and control plants were calculated in percentage following the method of Arndt et al. (2015). Fresh leaves were plucked and fresh tissue weight (FW) was measured, then immersed in a 50 mL tube with distilled water, and placed in the dark at 4°C for 20 h. Further, the leaves were dried with filter paper and again weighed for turgid weight (TW) calculation. Lastly, the same leaves were incubated at 80°C for a period of 72 h and dry weight (DW) was measured immediately. Relative water content was calculated by the formula RWC (%) = (FW−DW)/ (TW−DW).

The chlorophyll content (mg g^{-1} of fresh weight) of the fresh leaves was measured according to the modified formula of Lichtenthaler and Wellburn (1983). Firstly, fresh leaves (0.5 g fresh weight) were split into small pieces and immediately dissolved in 50 mL methanol (80% v/v), covered with black paper, or kept in dark conditions for 24–36 h at 28–30°C. Centrifugation was performed and the supernatant was estimated (645 nm and 653 nm) for chlorophyll contents. Chlorophyll content (mg L^{-1} FW) = 8.05 A_{653} + 20.29 A_{645}.

To calculate the proline contents of the seedling methods proposed by Bates et al. (1973) were adopted. Leaves (0.5 g fresh weight) were split into small pieces and put in a test tube. Further treatment was done by mixing with 5 mL of 3% sulfosalicylic acid, incubated in a water bath for 10 min and 2 mL of the supernatant was mixed thoroughly with 2 mL of acetic acid, and 3 mL of 2.5% ninhydrin. Finally, the mixture was incubated in the water bath for a time period of 40 min^{-1} h and extracted using 4 mL methylbenzene, optical density was measured at 520 nm and compared with a proline standard curve.

Soluble sugar contents (in terms of mg g^{-1} fresh weight) were measured by the method of Watanabe et al. (2000). Fresh leaves (0.2 g fresh weight) were crushed in 80% v/v ethanol (10 mL) and centrifuged at 8000 g for 10 min at 4°C. We mixed 1 mL of supernatant thoroughly with 3 mL of anthrone reagent followed by heating at 100°C for 10–12 min, which was stopped by rapid cooling them on the ice. Finally, at 620 nm absorbance was estimated using glucose as a standard.

Malondialdehyde content (nmol g^{-1} fresh weight) was reported according to the method of Del Buono et al. (2011) 0.5 gm of fresh leaves were homogenised in 5% (w/v) trichloroacetic acid (TCA), centrifuged at 12000 g for a time period of approximately 15–20 min and then the supernatant was mixed with 5 mL of 0.5% thiobarbituric acid (TBA)-prepared with 20% TCA followed by incubation for 25 min and cooling at 100°C and room temperature, respectively. Finally, after centrifugation (7,500 g for 5 min), the supernatant was measured for its absorbance at 450, 532, and 600 nm. The amount of MDA was calculated by the following formula MDA content (nmol g^{-1}) = 6.45 (A_{532}-A_{600})−0.56A_{450}.

The methods of Lei et al. (2015) were followed for the measurement of peroxidase activity. The system contained multiple chemicals; 2.9 mL of 0.05 M phosphate buffer, 0.5 mL of 2% H_2O_2, 0.1 mL of 2% guaiacol, and also 0.1 mL of crude enzyme extract followed by the absorbance measurement at 470 nm. Lastly, POD activity was calculated as an amount of guaiacol oxidised per minute in nanomoles per minute per mg of protein. At the end of the reaction, the absorbance was measured at 470 nm. POD activity was defined as the amount of guaiacol oxidised per minute, and was expressed as nanomoles per minute per mg of protein.

Catalase activity was calculated following the protocols of Lei et al. (2015). O.1 mL H_2O_2 (2%) and 2 mL phosphate buffer (50 mM-pH 7.0) were mixed and the whole reaction was initiated by the addition of 0.1 mL of crude enzyme extract. Finally, the catalase activity was measured (at 240 nm) in terms of the decrease of values of H_2O_2 per minute, as nanomoles/min/gm of protein.

Superoxide dismutase activity was assayed following the protocols of Lei et al. (2015). The whole system contained a series of valuable freshly prepared reagents; 1.5 mL of 0.05 M phosphate buffer, 0.3 mL of 130 mM methionine solution, 0.3 mL of 750 µM nitroblue tetrazolium solution, 0.3 mL of 100 µM EDTA -Na_2 solution, 0.3 mL of 20 µM lactochrome solution, 0.5 mL of distilled water and finally 0.1 mL of crude enzyme extract. The complete reaction was initiated at 4000 Lx of illumination for a constant 20 min with no interruption. The control set comprises the same set of reagents and illumination but with no crude enzyme extract, rather replaced with a phosphate buffer. The third setup of control contains only phosphate buffer followed by incubation in dark conditions for the same time period of 20 min. Finally, after the completion of the reaction, the absorbance was estimated at 560 nm of wavelength. One unit of SOD activity was defined as the amount of enzyme which inhibits NBT reduction by 50%, also the results were expressed as unit/mg protein.

Statistical analysis

All experiments were performed in triplicate and the results are presented as means ± standard errors (SE). Data were analysed by Prism GraphPad version 9.2.0 (332) software (San Diego, California, United States). BBD experiments were done in Minitab (version 20.2).

Results

Identification of the isolate

The organism had enormously intertwining hyphae and septate hyaline whitish mycelium, and it lacked any sexual or asexual reproductive structures (Figures 1A–C). rDNA sequence data of the isolate was deposited in GenBank (ON146358). A BLAST search of the earlier existing database indicates a close genetic connection with other species of Mucor and the evolutionary history of the endophytic fungal isolate HELF2 was included using the neighbour-joining method (Saitou and Nei, 1987). The most appropriate phylogenetic tree with a total branch length of 0.00649585 is represented in Figure 1D. The tree was constructed to scale with branch lengths in similar units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances between the species were analysed using the maximum composite likelihood method (Tamura et al., 2021) and were in the units of the number of base substitutions per site. Gaps and missing data were removed from the dataset. There was a total of 620 nucleotides in the final dataset.

Optimisation of GRH production

Mucor sp. HELF2 was grown on a 250 mL Erlenmeyer flask in submerged condition for 10 days and the highest production of fungal GRH and biomass was detected after 8 days of fermentation (Table 1). A temperature of 26°C and a medium pH of 7.1 was found to be the most suitable one for GRH production. Glucose and peptone at a concentration of 11 g L^{-1} and 5.5 g L^{-1} were found to be the most

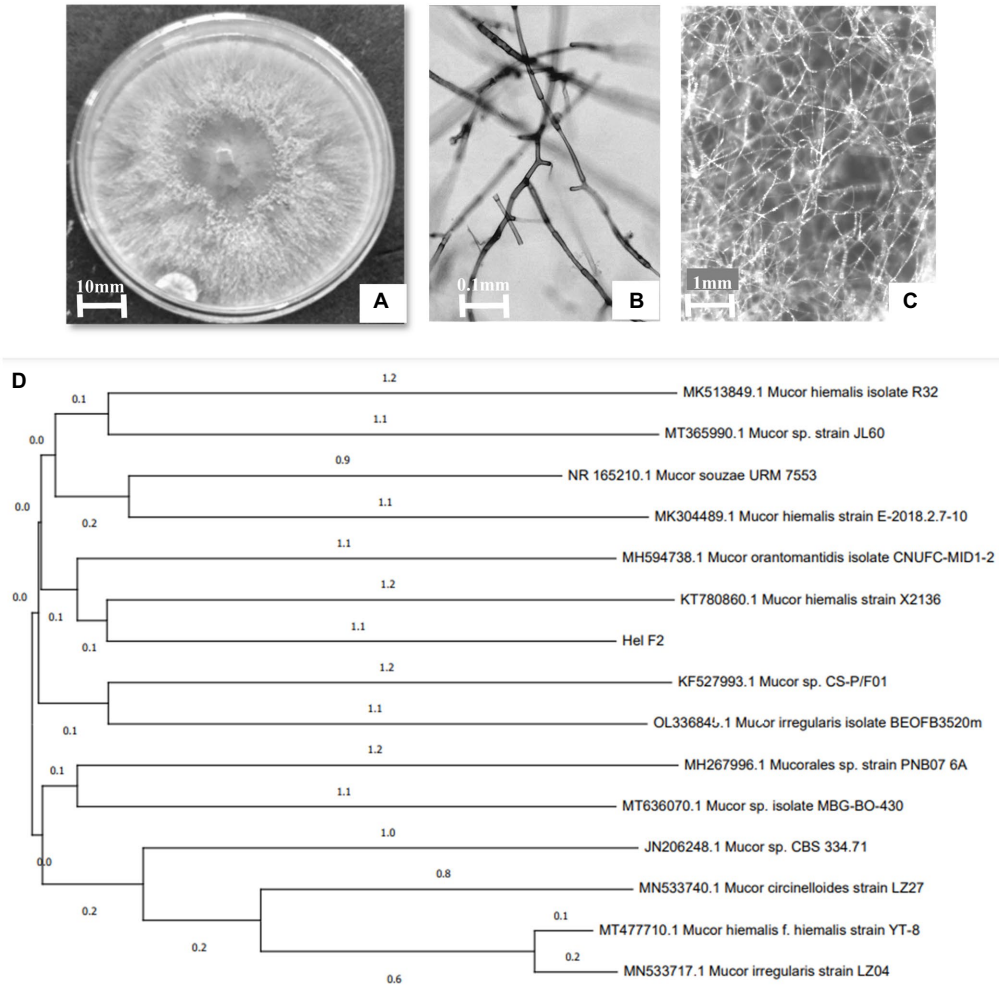

FIGURE 1
(A) 8-day old culture of HELF2 grown on PDA medium. Sterile hyphal aggregation without any sexual or asexual reproductive structures seen under a light **(B)** and stereo **(C)** microscope. **(D)** Phylogenetic tree shows the relationship of endophytic *Mucor* sp. HELF2 with other *Mucor* sp. strains.

appropriate ones for maximum GRH yield (Table 1). NaCl at a concentration of 0.1 g L^{-1} was the most effective salt (or source of metal ions) for GRH production. The detailed effect of different parameters on biomass and GRH production is summarised in Table 1.

The amount of dissolved oxygen in the fermentation medium affects EPS production. It depends on the medium volume, the headspace of the medium, and the medium depth. A medium volume of 75 mL in a 320 mL Erlenmeyer flask with 245 mL of headspace volume and 2.1 cm of medium depth and 2.53 cm of surface area was found to be the criteria for optimum GRH production (Table 2).

After OVAT optimisation RSM was adopted using a three-level Box Behnken Design. The most important four factors (glucose concentration, peptone concentration, medium pH, and fermentation time) with five replicates at the center points were established as a model for analysis of GRH production. The experimental design with variable predicted and measured values of GRH was presented in Table 3. Maximum GRH production was noted at the five replicated center points. The predicted response Y for GRH production by *Mucor* sp. HELF2 was described as coded factors in the following equation $Y_{GRH} = 5.9810 - 0.168024x_1 - 0.39671 x_2 - 0.09103x_3 - 0.37144x_4 - 0.25686x_1x_2 - 0.03737x_1x_3 + 0.09369x_1x_4 - 0.0367 x_2x_3 + 0.32736 x_2x_4 - 0.0337 x_3x_4 - 0.65663 x^2_1 - 0.6395 x^2_2 - 0.50414 x^2_3 - 0.85426 x^2_4$. Here Y_{GRH} is the predicted GRH yield and x_1, x_2, x_3, and x_4 are the four coded factors of glucose concentration, peptone concentration, medium pH, and fermentation time (day) respectively. A regression analysis with detailed statistical data related to the experiment is presented in Table 4. The F-test data of 1994.082 proved that the model was significant. The adjusted determinant coefficient (R^2 Adj) was found to be 0.9998 which represents that there is a high degree of correlation between the experimental and predicted values and there is more than 99% variation in response that could be predicted by second-order polynomial prediction equation. Adeq precision was reported to be 111.901 which indicates that the model is appropriate. The lack of fit *F*-value of 5.782 and *p*-value ($p < 0.0001$) was not at all valuable to the pure error and the fitness of the model was perfect. The high degree of precision and uniformity of the investigational outcomes were proved by the *p* value of lack of fit- 0.2830 (> 0.05) and a *p* value of probability (>F less than 0.05). The other linear and quadratic effects of glucose concentration, urea concentration, M-pH, and fermentation time were also significant ($p < 0.0001$). Finally, three-dimensional response surface plots and contour plots were constructed by Minitab (20.2) for a clear understanding of the effects of the parameters on GRH production (Figure 2).

TABLE 1 Effect of different fermentation influencing physical conditions and chemical supplements, on biomass and Galactose Rich Heteropolysaccharide production by endophytic fungi *Mucor* sp. HELF2 in submerged fermentation conditions.

Parameters tested	Effectors	The concentration of the effectors (gL^{-1})	Biomass (gL^{-1})	GRH (gL^{-1})
Fermentation time (h)	4 days	–	6.1 ± 0.04a	13.42 ± 0.03a
	6 days	–	5.99 ± 0.01a	13.96 ± 0.04b
	8 days	–	6.96 ± 0.8b	14.44 ± 0.03c
	10 days	–	5.76 ± 0.02c	13.11 ± 0.05a
Fermentation temperature (°C)	22	–	7.02 ± 0.03a	14.48 ± 0.02a
	24	–	7.18 ± 0.05a	15.51 ± 0.07b
	26	–	7.47 ± 0.01b	16.51 ± 0.08c
	28	–	6.96 ± 0.08a	16.11 ± 0.09b
Initial medium pH	6.9	–	7.7 ± 0.07a	17.51 ± 0.01a
	7.1	–	7.81 ± 0.06b	17.71 ± 0.04a
	7.3	–	7.73 ± 0.01a	17.41 ± 0.01a
	7.5	–	7.6 ± 0.05c	16.91 ± 0.04b
Additional carbon sources	Fructose	–	7.88 ± 0.03a	18.11 ± 0.01a
	Glucose	–	7.97 ± 0.05b	19.12 ± 0.05b
	Maltose	–	7.9 ± 0.06a	19.02 ± 0.03b
	Starch	–	7.82 ± 0.09c	18.9 ± 0.03c
	Rhamnose	–	7.87 ± 0.01a	18.93 ± 0.02c
	Raffinose	–	7.79 ± 0.02c	18.82 ± 0.02c
	Glycerol	–	7.82 ± 0.06c	18.51 ± 0.03d
Additional nitrogen sources	Peptone	0.4	8.71 ± 0.06a	19.86 ± 0.03a
	$(NH_4)_2SO_4$	0.4	7.99 ± 0.02b	19.28 ± 0.04b
	NH_4NO_3	0.4	8.1 ± 0.07b	19.41 ± 0.04c
	Urea	0.4	8.23 ± 0.04c	19.52 ± 0.05c
	NH_4Cl	0.4	8.16 ± 0.02b	19.7 ± 0.06d
	Glycine	0.4	8.2 ± 0.01c	19.74 ± 0.05d
	Yeast Extract	0.4	8.41 ± 0.09d	19.12 ± 0.06b
Glucose concentration	Glucose	7	9.18 ± 0.05a	19.9 ± 0.07a
		9	9.27 ± 0.06b	20.18 ± 0.02b
		11	9.47 ± 0.05c	20.23 ± 0.04b
		13	9.3 ± 0.06b	20.12 ± 0.03b
Peptone concentration	Peptone	4.5	9.59 ± 0.07a	20.24 ± 0.03a
		5	9.53 ± 0.08a	20.32 ± 0.04a
		5.5	9.7 ± 0.01b	20.5 ± 0.05b
		6	9.49 ± 0.06a	20.42 ± 0.02b
Different metal ions	$MgCl_2$	0.1	9.3 ± 0.01a	20.52 ± 0.06a
	$FeCl_3$	0.1	9.39 ± 0.06b	20.62 ± 0.03b
	KCl	0.1	9.51 ± 0.07c	20.81 ± 0.04c
	NaCl	0.1	9.8 ± 0.04d	20.92 ± 0.07d
	NaH_2PO_4	0.1	9.48 ± 0.03c	20.71 ± 0.08b
	K_2HPO_4	0.1	9.6 ± 0.07e	20.51 ± 0.09a
	KH_2PO_4	0.1	9.57 ± 0.08e	20.42 ± 0.10e

One-way ANOVA (Tukey's Multiple Comparison test) was performed to check the potential statistical differences in the case of the biomass ($g\,L^{-1}$) and GRH production ($g\,L^{-1}$) in different fermentation conditions (incubation time, temperature, the concentration of sugar and nitrogen sources, etc.). There were valid statistical differences in most of the cases ($p < 0.05$), the different letters a, b, c, d, e, and f indicate a significant difference, and the same letter at two positions indicates no statistical differences.

TABLE 2 Effect of medium volume, headspace volume, and medium depth on the dissolved oxygen level in the fermentation medium and their effect on GRH and biomass production.

Medium volume (mL)	The total volume of the flask (mL)	Headspace volume (mL)	Medium depth (cm)	Surface area (cm)	Biomass (gL^{-1})	GRH (gL^{-1})
25	320	295	1	2.5	11.66 ± 0.18a	21.01 ± 0.021a_1
50	320	270	1.5	2.58	11.8 ± 0.75a	21.42 ± 0.049b_1
75	320	245	2	2.53	12.99 ± 0.43b	22.04 ± 0.089c_1
100	320	220	2.5	2.41	11.69 ± 0.64a	21.28 ± 0.071a_1

One-way ANOVA (Tukey's Multiple Comparison test) was performed to check the potential statistical differences between the data (column-wise) of biomass ($g L^{-1}$), and GRH production ($g L^{-1}$) in different medium volumes (mL). There was a valid statistical difference in most of the cases. Different letters (a, b for biomass and a_1, b_1, c_1 for GRH yield) indicates valid statistical differences ($P < 0.05$).

TABLE 3 Experimental design and outcomes of the Box–Behnken Design (BBD) for optimisation of the GRH production from *Mucor* sp. HELF2.

Run	Independent variables				Response [GRH yield (gL^{-1})]	
	GC (x_1)	PC (x_2)	M-pH (x_3)	FT (x_4)	Measured	Predicted
1	−1(=10)	−1(=5)	0(=7.1)	0(=8)	21.189a_1	21.602a_1
2	1(=12)	−1	0	0	22.099a_2	22.177a_2
3	−1	1(=6)	0	0	21.984a_3	22.067a_3
4	1	1	0	0	21.684a_4	21.433a_4
5	0(=11)	0(=5.5)	−1(=6.9)	−1(=7)	22.083a_5	22.162a_5
6	0	0	1(=7.3)	−1	22.077a_6	22.123a_6
7	0	0	−1	1(=9)	21.733a_7	21.848a_7
8	0	0	1	1	21.779a_8	21.862a_8
9	−1	0	0	−1	22.139a_9	22.032a_9
10	1	0	0	−1	21.873a_{10}	21.921a_{10}
11	−1	0	0	1	21.789a_{11}	21.663a_{11}
12	1	0	0	1	21.686a_{12}	21.715a_{12}
13	0	−1	−1	0	22.206a_{13}	22.066a_{13}
14	0	1	−1	0	21.876a_{14}	21.918a_{14}
15	0	−1	1	0	22.164a_{15}	22.044a_{15}
16	0	1	1	0	21.853a_{16}	21.915a_{16}
17	−1	0	−1	0	22.114a_{17}	21.975a_{17}
18	1	0	−1	0	21.899a_{18}	21.94a_{18}
19	−1	0	1	0	22.083a_{19}	21.957a_{19}
20	1	0	1	0	21.88a_{20}	21.933a_{20}
21	0	−1	0	−1	22.307a_{21}	22.199a_{21}
22	0	1	0	−1	21.784a_{22}	21.824a_{22}
23	0	−1	0	1	21.801a_{23}	21.675a_{23}
24	0	1	0	1	21.749a_{24}	21.772a_{24}
25	0	0	0	0	22.57a_{25}	22.583a_{25}
26	0	0	0	0	22.621a_{26}	22.583a_{26}
27	0	0	0	0	22.583a_{27}	22.583a_{27}
28	0	0	0	0	22.581a_{28}	22.583a_{28}
29	0	0	0	0	22.562a_{29}	22.583a_{29}

One-way ANOVA (Tukey's Multiple Comparison test) was performed to check the potential statistical differences ($P < 0.05$) between the measured and predicted GRH production. There were no statistical differences between each data set (row-wise) and similar letters (a_1-a_{29}) in each row indicate the data are the same and lack statistical differences.

The model predicted a maximum response of 20.10 $g L^{-1}$ GRH yield when the necessary components are 5.2 $g L^{-1}$ of peptone, 10.5 $g L^{-1}$ of glucose, 7.05 MpH, and 180 (7.5 days) h of fermentation time. These predictions were authenticated by performing laboratory experiments in flask culture by triplicate with an outcome of 19.951 ± 0.091 $g L^{-1}$ of GRH.

TABLE 4 ANOVA for response surface quadratic regression model of Galactose Rich Heteropolysaccharide production by endophytic fungi *Mucor* sp. HELF2.

Source	DF	Sum of squares	Mean square	F-Value	*P*-Value prob.>F
Model	14	9.84399	0.19982	1994.082	<0.001
x_1 (GC)	1	0.38702	0.58191	1082.069	<0.001
x_2 (PC)	1	0.59729	0.81973	974.819	<0.001
x_3 (M pH)	1	0.49871	0.80132	908.019	<0.001
x_4 (FT)	1	0.29871	0.59713	801.011	<0.001
x^2_1 (GC*GC)	1	0.03152	0.57085	1109.039	<0.001
x^2_2 (PC*PC)	1	0.87199	0.87172	1339.760	<0.001
x^2_3 (M pH*M pH)	1	0.37189	0.57831	1039.93	<0.001
x^2_4 (FT*FT)	1	0.81923	0.79849	916.14	<0.001
$x_1 x_2$ (GC*PC)	1	0.39905	0.71280	919.82	<0.001
$x_1 x_3$ (GC*M pH)	1	0.41977	0.76862	808.015	<0.001
$x_1 x_4$ (GC*FT)	1	0.66890	0.92914	1139.19	<0.001
$x_2 x_3$ (PC*M pH)	1	0.90972	0.98132	996.02	<0.001
$x_2 x_4$ (PC*FT)	1	0.56491	0.89032	1138.091	<0.001
$x_3 x_4$ (M pH*FT)	1	0.69981	0.70237	767.091	<0.001
Residual	14	0.43781	0.20976		
Lack-of-Fit	10	0.53500	0.90493	5.782	0.2830
Pure Error	4	0.20778	0.96892		
Cor total	28	8.16903			
R^2		0.999209			
Adj R^2		0.999800			
Pred R^2		0.995062			
Adeq precision		111.901			

Characterisation of the exopolysaccharide

Exopolysaccharide produced by Endophytic *Mucor* sp. HELF2 was precipitated by applying chilled ethanol and crude EPS was then dialysed, and purified by gel filtration chromatography with a Sepharose-6B column. One major fraction obtained was eluted between 29 and 42 tubes (Figure 3C) and the colorimetric test confirms the absence of proteins in those fractions. The fraction was further investigated for monosaccharide analysis. The molecular weight of the homogeneous EPS was calculated from a calibration curve of standard dextran as ~2.98×10^5 Da (Figure 3D). Monosaccharide analysis of the derivatised EPS samples showed the occurrence of galactose, fucose, and glucose in a 13:2:1 ratio with D, L, and D configuration, respectively (Table 5). Each repeating unit of the fraction contained 13 galactose, two fucoses, and one glucose, which indicates that the studied EPS contained approximately 104 repeating units. We, therefore, considered that the Galactose Rich Heteropolysaccharide (GRH) could have been produced by endophytic *Mucor* sp. HELF2. FT-IR analysis of the EPS sample revealed the occurrence of strong absorption peaks at particular wavelengths of 3400.71, 2950.89, 1651.56, 1489.73 which represents C-H, O-H, C-O asymmetric stretching respectively, which indicates the basic characteristics and purity of the carbohydrate moiety. Figures 3A,B represent the FT-IR spectrum and GC–MS spectrum of the crude and derivatised EPS, respectively.

Plant growth-promoting traits of the GRH

GRH-sprayed rice seedlings were found to be much healthier, and more vigorous in terms of their fresh weight and relative water contents in comparison to the control (only drought-inducing agent-PEG treated). The control plants were characterised by low growth, chlorosis, and wilting of leaves. The rice seedlings exhibited maximum growth promotion after continuous 14 days of GRH treatment. The relative water content and fresh weight of the 50 ppm GRH treated plants were found to be higher than the plants of the control set, and plants treated with 20 ppm and 100 ppm GRH dosage. There was a 1.31, 2.38, and 1.74-time improvement in the fresh weight of seedlings in the 20, 50, and 100 ppm GRH treated plants compared to the control. The relative water contents were also increased by 1.14, 1.58, and 1.26 times in 20, 50, and 100 ppm GRH treated plants than the control one. There was a 3, and 4 times increase in root length and shoot length of the treated (50 ppm GRH) plant, respectively, compared to the control. The fresh weight of the seedlings were found to be improved after the GRH treatment and the 50-ppm GRH application was also found to be the most effective in comparison to the control. Table 6 represents the improved physical characteristics of the treated seedlings. Figure 4 represents the treated (20, 50, and 100 ppm) and control rice seedlings, showing their physical changes.

Not only physical but also biochemical characteristics were improved in the case of GRH-treated plants even after severe drought situations. The chlorophyll contents of the treated plants were found to be more elevated (2.32 times higher for 50 ppm GRH treated one) than the untreated control

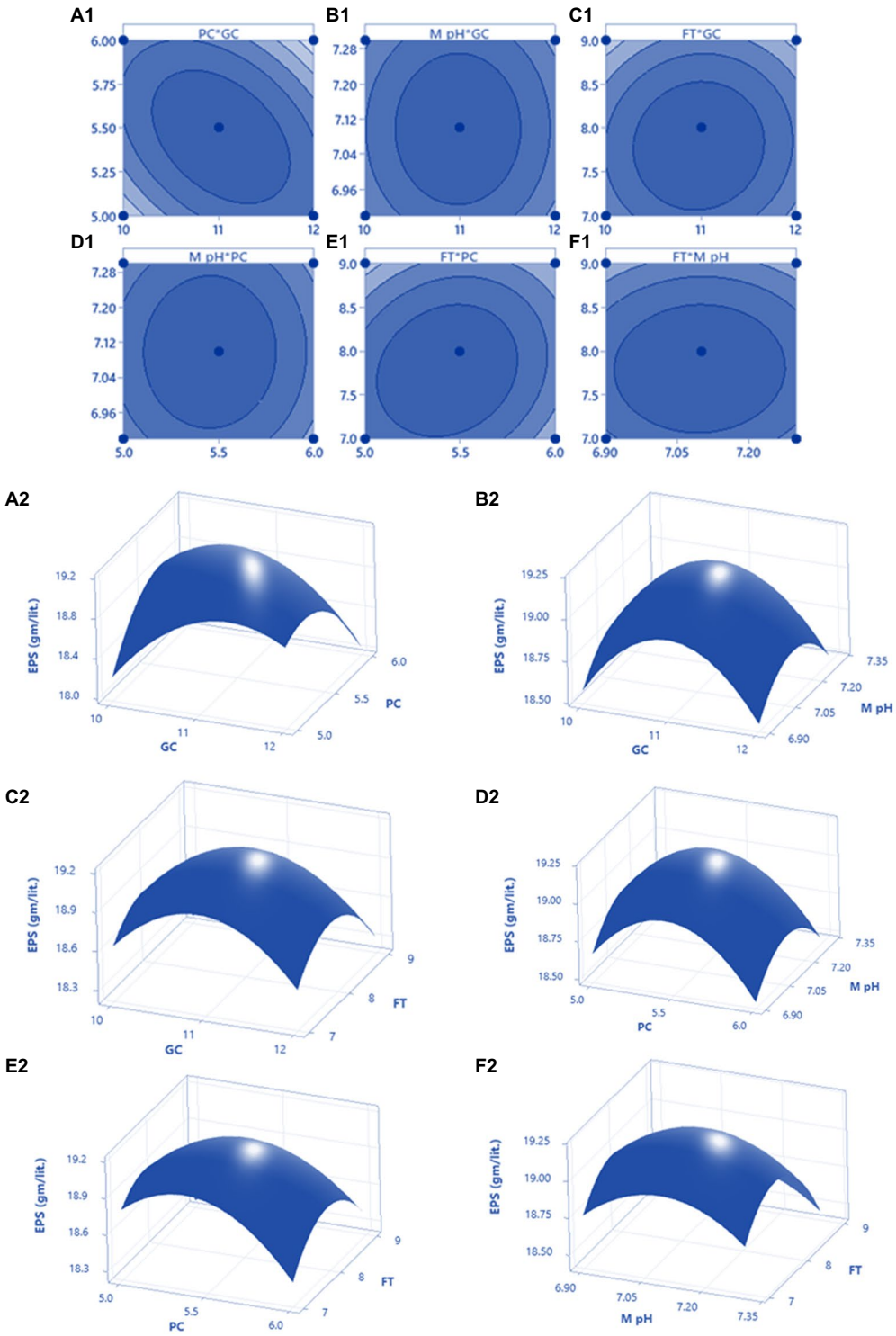

FIGURE 2
The 3D plot with 2D projection and contour plot showing the most important interactions of factors in RSM optimization of GRH production by HELF2. **(A1,A2)** between peptone concentration (PC) vs. glucose concentration (GC) at fermentation time (FT) 8days and medium pH 7.1 (MpH); **(B1,B2)** between MpH (7.1) and GC (11gL^{-1}) at FT (8days) and PC (5.5gL^{-1}); **(C1,C2)** between FT (8days) and GC (11gL^{-1}) at PC (5.5gL^{-1}) and MpH (7.1); **(D1,D2)** between MpH (7.1) and PC (5.5gL^{-1}) at FT (8days) and GC (11gL^{-1}); **(E1,E2)** between FT (8days) and PC (5.5gL^{-1}) at MpH (7.1) and GC (11gL^{-1}); **(F1,F2)** between FT (8days) and MpH (7.1) at GC (11gL^{-1}) and PC (5.5gL^{-1}).

(Figure 5A). The proline is a potent indicator of plant stress and higher proline contents indicate higher resistance towards stress and better adaptation to that stressful situation. Here the GRH-treated plant shows higher accumulations (approximately 3.89 times higher for 50 ppm GRH treatment) of proline contents than the control ones (Figure 5B). The presence of increased soluble sugar content in the plant tissues also indicates the higher survival ability of plants in drought-stress situations. In this experiment, we found almost 3.5 times higher accumulation of

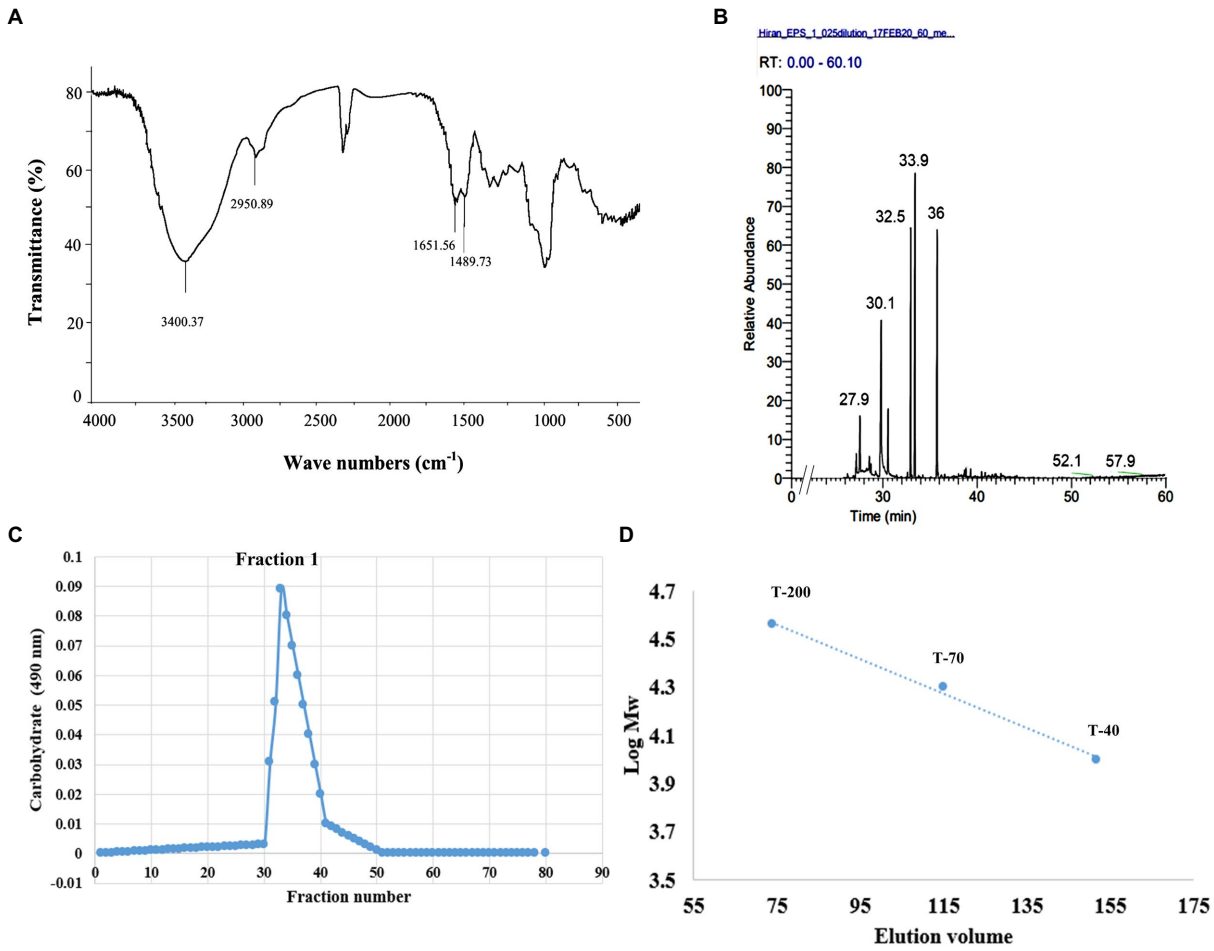

FIGURE 3
(A) FT-IR spectra of crude HELF2 GRH showing the necessary functional groups present in the sample. **(B)** GC–MS chromatogram of the derivatized fungal GRH showing the different peaks of monosaccharide compositions at different retention times. **(C)** Elution profile of the polysaccharide showing the occurrence of dominant fraction (F1) confirmed by carbohydrate test. **(D)** Standard curve of dextran needed for the detection of sugar concentration in the polysaccharide sample.

TABLE 5 Monosaccharide units present in the Galactose Rich Heteropolysaccharide (synthesised by endophytic fungi *Mucor* sp. HELF2) are represented here with their respective sugar linkages and molar ratios.

Methylated sugars	Linkage types	Molar ratio	Major fragments (m/z)
2,3,4-Me_3-Gal-	6)-Galp-(1-	5	40, 61, 72, 88, 103, 113, 131, 140, 159, 175, 191, 203, 219, 231
2,3-Me_2-Galp	-4,6)-β-D-Galp-(1-	4	44,46,59,69,72,83,85,97,99, 119,127,129,141,157,161,187,200
3,4-Me_2-Gal	-2,6)-α-D-Galp-(1-	4	44,72,89,100,131,157,173,189,233
2,3,4-Me_3-Fuc	α-L-Fucp-(1-	2	44,72,89,103,113,117, 131,161,175
2,4-Me_2-Glc	−3,6)-D-Glcp-(1-	1	39,43,58,74,88,99,100, 119,130,144,160,174,191,209,215,234,246

TABLE 6 Different physical parameters (fresh weight, root length, and shoot length) of GRH-treated and untreated drought-faced rice seedlings are represented here.

Treatment group (concentration of EPS)	Fresh weight (mg)	Root length (cm)	Shoot length (cm)	Relative water contents (%)
Control	97.81 ± 3.019a	2.01 ± 0.72a_1	9.6 ± 1.73a_2	69.02 ± 1.890a_3
EPS 20 ppm	128.93 ± 4.051b	3.13 ± 0.69b_1	13.36 ± 2.05b_2	79.11 ± 1.472b_3
EPS 50 ppm	232.93 ± 3.610c	6.61 ± 1.32c_1	33.4 ± 3.28c_2	109.31 ± 1.901c_3
EPS 100 ppm	171.11 ± 2.098d	4.53 ± 0.81d_1	23.41 ± 2.96d_2	87.39 ± 1.014d_3

One-way ANOVA (Tukey's Multiple Comparison test) was performed to check the potential statistical differences and the treated (20, 50, and 100 ppm EPS foliar spray) plants showed statistically valid differences ($P < 0.05$, the four different letters for each of the four parameters (column-wise) indicates significance differences) from the control plant.

soluble sugar contents in 50 ppm GRH-treated plants in comparison to the only PEG-treated one (Figure 5C). On the other hand, the MDA (Malondialdehyde) content is found to be correlated with lipid peroxidation and membrane damage. Higher MDA content in the plant tissues indicates the detrimental situation induced by the stress factors. In the case of treated seedlings, there was a sharp six time decrease in MDA contents compared to the control (Figure 6A). Other enzymatic antioxidative parameters were found to be also elevated after GRH treatment even in extreme drought situations. SOD, CAT, and POD activities increased up to 1.44, 2.09, and 1.79 times, respectively, in the case of GRH-treated rice seedlings compared to those treated only with PEG (Figures 6B–D).

Discussion

Agriculture is seen as the most important and crucial sector of the global economy, and it significantly affects our GDP (Gross Domestic Production). The increased explosion in population in recent years has increased the demands for global agricultural output or food production by 60–100% by the end of 2050 to meet these growing needs, but the main obstacles are the lack of suitable fertile croplands and the rising instances of soil desertification due to insufficient precipitation, random evaporation, and a lack of freshwater resources, among other factors (Naumann et al., 2018; Dey et al., 2019; Paglia and Parker, 2021). Therefore, the primary requirement for a successful solution is the restoration of land or the development of salt or drought stress varieties. The development of drought-tolerant plants could temporarily meet the world's food demand and protect crop plants, but the situation becomes severe when drought conditions (like the 2011–17 California drought and the 1997–99 Melbourne Millennium drought) occur on large scales around the globe. The food supply chain is hampered, and even the forest environment is impacted (Allen et al., 2010). Therefore, it is strongly recommended that deep ecological techniques that use non-toxic, natural substances be developed to address these issues. Exopolysaccharides produced by microbes, especially endophytes could have a significant impact (Chen et al., 2017). Even in situations with salt and drought challenges, endophytic fungi and bacteria are well known for their ability to promote plant growth (Azad and Kaminskyj, 2016; Bibi et al., 2019; Ali et al., 2021; Gupta et al., 2021). There are many reports on how microorganisms (both endophytes and rhizospheric) can reduce abiotic stress (Hammami et al., 2016), and endophytes often play an osmoprotective role in maintaining good water chemistry (managing Na^+/K^+ balance) within cells (Jha et al., 2011; Abdelaziz et al., 2017). Previous research has been undertaken, examining the role of endophytic fungi and bacteria in reducing the effects of salt stress in rice, maize, soybean, quinoa, barley, and barrel medic, as well as in the model plant *Arabidopsis thaliana* through endogenous hormone (abscisic acid) mediated morphological, biochemical (through ion balancing), and antioxidant defence-related pathways (Baltruschat et al., 2008; Bagheri et al., 2013; Jogawat et al., 2013; Li et al., 2017; Shahzad et al., 2017; Asaf et al., 2018; Fan et al., 2020; Ali et al., 2022; González-Teuber et al., 2022). Exo-polysaccharides and gamma-polyglutamic acid are also

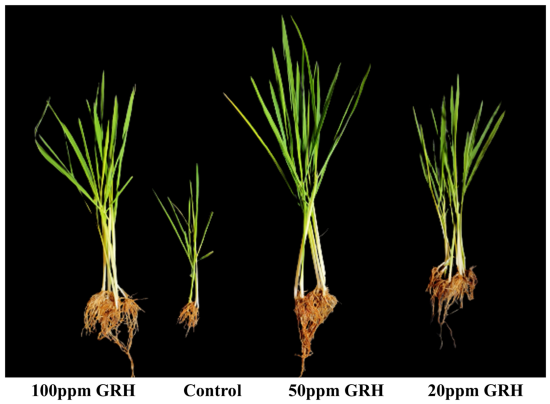

FIGURE 4
Phenotypes of rice seedlings *Oryza sativa* ssp. indica MTU 7093 Swarna under drought stress (induced by PEG treatment) sprayed with 20, 50, and 100ppm EPS of *Mucor* sp. HELF2 endophyte.

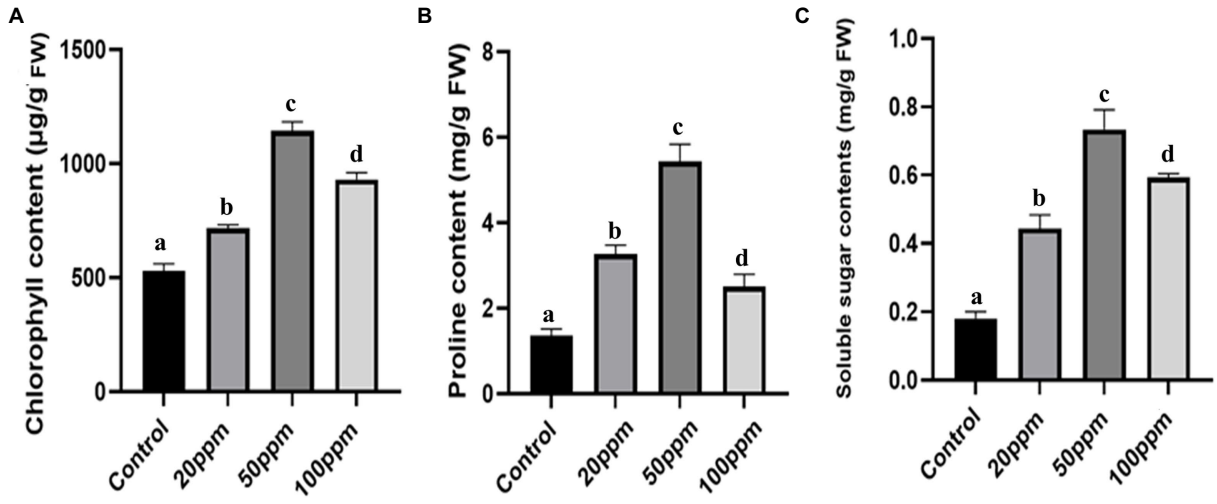

FIGURE 5
The effect of GRH foliar spray (20, 50, and 100ppm) on chlorophyll content **(A)**, soluble sugar content **(B)**, proline content **(C)** of *O. sativa* ssp. indica MTU 7093 swarna in comparison to control. Values on the graphs are the means ± Standard error (SE) of three replicates. Tukey's multiple comparison test was performed. The letters a, b, c, and d indicate significant differences compared to the control plant (At, $p<0.05$).

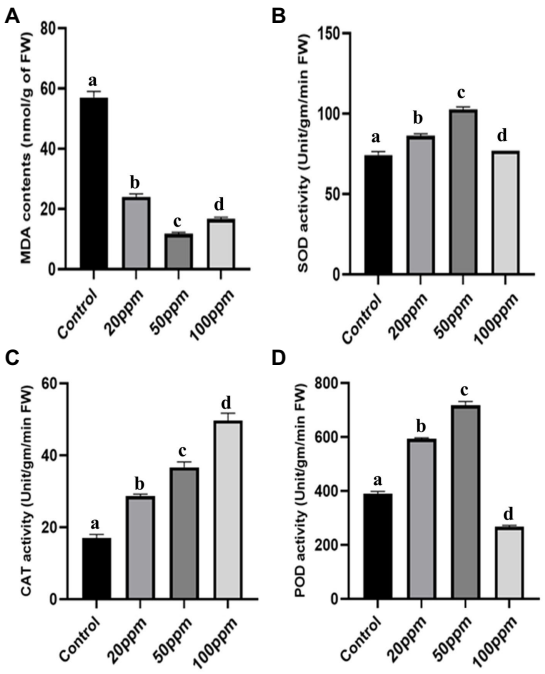

FIGURE 6
The effect of GRH foliar spray (20, 50, and 100ppm) on malondialdehyde (MDA) contents **(A)**, peroxidase (POD) activity **(B)**, catalase (CAT) activity **(C)**, superoxide dismutase (SOD) activity **(D)** of *O. sativa* ssp. indica MTU 7093 swarna in comparison to control. Values on the graphs are the means ± Standard error (SE) of three replicates. Tukey's multiple comparison test was performed. The letters a, b, c, and d indicate significant differences compared to the control plant (At, $p<0.05$).

discovered to be the most useful compounds released by plant growth-promoting microbes and have exceptional biotic and abiotic stress tolerance (Livingston et al., 2009; Lei et al., 2017). Here, drought stress ameliorating properties of GRH was evaluated on rice plants. Due to their widespread popularity around the world, rice seedlings (*Oryza sativa* ssp. indica MTU 7093 Swarna- Indian subcontinental cultivar) were taken into consideration for their studies on drought relief. It is a very demanding staple food, especially in China, India, and Japan (Uga et al., 2013; Zhu, 2016), and has greater irrigation water needs (Bouman et al., 2005, 2007). According to recent studies, around forty-two million hectares of rice farming face significant challenges because of a lack of water. To formulate an appropriate response, we reported on how Galactose Rich Heteropolysaccharide reduces the effects of drought stress on rice plants. Chen et al. (2017) and Santra and Banerjee (2022a) both found that the application of direct endophyte and EPS produced from endophyte alleviated salt and drought stress in wheat and rice plants, respectively. Due to their high polymeric configurations, effective water-holding capacities, and strong affinity to create bio-films or similar sorts of aggregations, polysaccharides are thought to have significant crop resistance (against both biotic and abiotic) enhancers and plant growth promotors (Muley et al., 2019). Chitosan, β-D-glucan, and other microbial polysaccharides have been found to have growth-stimulating and systemic disease resistance-inducing characteristics on cash crops such as *Solanum lycopersicum*, *Hordeum vulgare*, *Solanum tuberosum*, *Saccharum officinarum*, *Gossypium herbaceum*, and *Glycine max* (Uyen, 2014; Gandra et al., 2016; Blainski et al., 2018; Zhang et al., 2020).

The physical and biochemical traits of seedlings treated with GRH significantly improved. Proline levels and soluble sugar characteristics were also found to be altered, improving the stress-tolerating enzyme profile and enabling the plant to adapt to dry circumstances more successfully. The lower levels of MDA suggest that lipid peroxidation has significantly decreased and that membrane damage has become less frequent. The best concentration of EPS (i.e., Galactose rich heteropolysaccharide- GRH) for controlling the stressful condition was 50 ppm. Lower treatment doses are not sufficient to cause a noticeable change in the plantlets, whereas greater concentrations of GRH are likely to have a negative effect on the health of the plant. The findings of Sun et al. (2020b) are consistent with our findings because the 50 ppm EPS application was also determined to be the best-fitting one in that instance. Through a rise in endogenous ABA levels, polysaccharide treatments affect the stomatal physiology of the test plants' leaves and cause partial stomatal closure, which minimises water evaporation. To improve the internal water levels of the tissues, which are essential for the plant's proper growth and metabolism, these bioactive compounds function as anti-transpirant agents (Vishwakarma et al., 2017). Treatments with GRH increase the relative water contents of the tissues in this instance as well. The uniform build-up of rigid and highly water-soluble osmolytes (such as sugars, amino acids, and prolines) throughout the plant tissues, which provides subcellular stability and integrity, is another mechanism by which exopolysaccharide-mediated drought stress relief works (Hare et al., 1998; Krishnan et al., 2008). Increased levels of osmolyte aggregation raise osmotic pressure, which in turn induces higher water intake and insignificant water outflow. This keeps the cells' critical osmotic pressure constant needed for optimum cell growth and division (Kaur and Asthir, 2015). The proline and soluble sugar levels (osmolytes) are increased by 50 ppm of GRH treatment in the current study as well, balancing the ideal subcellular environment for a healthy water weight required for cell growth. Proline is found to be an important osmo-regulator, and its exogenous administration increases hosts' resistance to abiotic stress (Yoshiba et al., 1997; Ben Ahmed et al., 2010). For abiotically challenged plants, exogenous administration of water-soluble polysaccharides also demonstrates a similar response and causes a significant rise in proline levels (Yu et al., 2017; Zou et al., 2018). Thus, in this instance, GRH functions as a biological elicitor or priming agent that activates the cascades of biochemical processes required for water balance and antioxidant defence—ROS scavenging. Thus, as seen in the cases of rice, parsley, and tobacco, polysaccharides generate faster activation of transcription factors leading to the expression of defence-related genes, increasing the alleviation of drought stress (Conrath et al., 2002, Ortmann et al., 2006, Bozsoki et al., 2017). In drought-stricken areas, microbial exopolysaccharide enhances plant development by up- and down-regulating the expression of proline synthase and proline dehydrogenase, respectively (Sun et al., 2020a). By increasing SOD, POD, and CAT levels and fostering the effective operation of cellular biochemical machinery, which is essential for the survival of the plant, osmolytes also effectively eliminate harmful free radicals (reactive oxygen species) under drought stress (Sun et al., 2020b). The three key members of the antioxidant system SOD, CAT, and POD act in an integrated approach, where SOD acts as the first line of defence and converts superoxide free radicals to H_2O_2, which is further catalysed into water and oxygen by CAT and POD (Das and Roychoudhury, 2014). Last but not least, the MDA concentrations decrease, reducing the peroxidation of membrane proteins and lipids (Fu et al., 2010; Miller et al., 2010). Spraying potato and wheat with chitosan, polysaccharides from *Ganoderma lucidum*,

Lactobacillus plantarum, and *Pantoea agglomerans*, respectively, activates the antioxidant defence cascades (Ortmann et al., 2006; Blainski et al., 2018; Muley et al., 2019; Zhang et al., 2020). Here, foliar GRH spray applied at a dosage of 50 ppm enhanced the SOD, POD, and CAT levels while concurrently lowering the MDA contents.

The bio-active GRH produced by *Mucor* sp. HELF2 was a polymer of D-galactose, L-fucose, and D-glucose (molar ratio—13:2:1) with a molecular weight of 2.98×10^5 Da. Galacto-rhamnan and beta-glucan exopolysaccharides with molecular weights of 1.87×10^5 and 2×10^5 Da were found in endophytic *Fusarium* sp. SD5 and *Pestalotiopsis* sp. BC55, respectively, according to Mahapatra and Banerjee's reports from 2013 and 2016. Polysaccharides from edible mushroom *Termitomyces heimii*, and *Meripilus giganteus* also represents a similar type of monosaccharide compositions of L-fucose, D-galactose, and D-glucose, etc. (Maity et al., 2017, 2020). The EPS manufacturing process was optimised for carbon, nitrogen sources, a medium pH, and fermentation temperature to produce the greatest quantity of polysaccharides. The appropriate oxygen demand was also considered. The optimisation data makes it possible to quickly and affordably obtain the polysaccharides in large quantities. The results of the current inquiry on the optimization of GRH production broadly concur with those of Mahapatra and Banerjee (2013, 2016).

The present study examined endophytic exopolysaccharides (GRH) from an ecologically valuable plant and checked the drought tolerance action of the GRH on rice plants. Finally, varying concentrations of fungal EPS were used to reduce the effects of drought stress. Our research clarifies the idea of creating rice types resistant to drought through the external application of EPS, which supports environmentally friendly farming methods. This study provides the first evidence of the use of endophytic *Mucor* sp. HELF2 produced D-galactose-rich heteropolysaccharide to reduce drought stress in rice seedlings.

Conclusion

In the present study, rice seedling dehydration stress was lessened by a galactose-rich heteropolysaccharide derived from endophytic *Mucor* sp. HELF2. The outcome illustrated that treated plants had higher fresh weights, relative water contents, and chlorophyll levels. While the MDA concentration reduced, osmolytes such as soluble sugars, proline, as well as the antioxidant defence enzymes SOD, CAT, and POD, increased. The results support the conclusion that foliar spray of Galactose Rich Heteropolysaccharide efficiently promotes drought resistance in rice plants. GRH production was also optimised by adopting OVAT and RSM techniques and there was a 1.5-fold ($20.10\,g\,L^{-1}$) enhancement in GRH production in optimised fermentation conditions. The ability of GRH to alleviate the effects of drought stress on rice plants and the high yield of GRH makes it suitable for commercial exploitation. The current investigation's findings may encourage sustainable farming methods and have an impact on the cultivation of crops in drought-prone areas.

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/genbank/, ON146358.

Author contributions

HS designed and performed the experiments, and prepared the draft of the manuscript. DB designed the experiment and finalised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 14 March 2023
DOI 10.3389/fmicb.2023.1140378

OPEN ACCESS

EDITED BY
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University of Idaho,
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SPECIALTY SECTION
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

RECEIVED 09 January 2023
ACCEPTED 21 February 2023
PUBLISHED 14 March 2023

CITATION
Sehim AE, Hewedy OA, Altammar KA, Alhumaidi MS and Abd Elghaffar RY (2023) *Trichoderma asperellum* empowers tomato plants and suppresses *Fusarium oxysporum* through priming responses. *Front. Microbiol.* 14:1140378. doi: 10.3389/fmicb.2023.1140378

Trichoderma asperellum empowers tomato plants and suppresses *Fusarium oxysporum* through priming responses

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Plant-associated microbes play crucial roles in plant health and promote growth under stress. Tomato (*Solanum lycopersicum*) is one of the strategic crops grown throughout Egypt and is a widely grown vegetable worldwide. However, plant disease severely affects tomato production. The post-harvest disease (*Fusarium* wilt disease) affects food security globally, especially in the tomato fields. Thus, an alternative effective and economical biological treatment to the disease was recently established using *Trichoderma asperellum*. However, the role of rhizosphere microbiota in the resistance of tomato plants against soil-borne *Fusarium* wilt disease (FWD) remains unclear. In the current study, a dual culture assay of *T. asperellum* against various phytopathogens (e.g., *Fusarium oxysporum*, *F. solani*, *Alternaria alternata*, *Rhizoctonia solani*, and *F. graminearum*) was performed *in vitro*. Interestingly, *T. asperellum* exhibited the highest mycelial inhibition rate (53.24%) against *F. oxysporum*. In addition, 30% free cell filtrate of *T. asperellum* inhibited *F. oxysporum* by 59.39%. Various underlying mechanisms were studied to explore the antifungal activity against *F. oxysporum*, such as chitinase activity, analysis of bioactive compounds by gas chromatography–mass spectrometry (GC–MS), and assessment of fungal secondary metabolites against *F. oxysporum* mycotoxins in tomato fruits. Additionally, the plant growth-promoting traits of *T. asperellum* were studied (e.g., IAA production, Phosphate solubilization), and the impact on tomato seeds germination. Scanning electron microscopy, plant root sections, and confocal microscopy were used to show the mobility of the fungal endophyte activity to promote tomato root growth compared with untreated tomato root. *T. asperellum* enhanced the growth of tomato seeds and controlled the wilt disease caused by the phytopathogen *F. oxysporum* by enhancing the number of leaves as well as shoot and root length (cm) and fresh and dry weights (g). Furthermore, *Trichoderma* extract protects tomato fruits from post-harvest infection by *F. oxysporum*. Taking together, *T. asperellum* represents a safe and effective controlling agent against *Fusarium* infection of tomato plants.

KEYWORDS

***Trichoderma asperellum*, tomato, *Fusarium oxysporum*, IAA, biological control, root anatomy, confocal microscopy**

Introduction

Tomato (*Solanum lycopersicon* L.) is one of the most essential and widespread horticultural crops worldwide (Pritesh and Subramanian, 2011; Bawa, 2016), and it is a rich source of vitamins A and C (Pramanik and Mohapatra, 2017; Boccia et al., 2019). Egypt is a top-five tomato producer, accounting for 7.2 to 8.6 million tons annually (Nakai, 2018). However, there is a significant concern regarding food security, as demographic projections indicate that the global population will rise to 9.5 billion by 2050 (Attia et al., 2022). Food security and safety are increasingly threatened as the human population continues to expand due to soil-borne fungal pathogens emerging worldwide. In addition, such pathogens can reduce crop productivity in greenhouse and field conditions (e.g., vegetable crops), consequently causing severe losses to global food security (Almeida et al., 2019). Fungal diseases cause over 1.6 million deaths annually, and over one billion people suffer from severe fungal infections. Moreover, fungal pathogens are considered the highest threat to plant-host species, representing the main reason for reducing plant growth (approximately 65%) (Zhang and Ma, 2017; Almeida et al., 2019; Hewedy et al., 2020a,b). In addition, toxigenic fungi are the most important plant pathogens, causing diseases such as *Fusarium oxysporum* species complex (FOSC) (Zhang and Ma, 2017; Hewedy et al., 2020a,b), *Rhizoctonia solani* (Ghazala et al., 2022), *Fusarium solani* (Saengchan et al., 2022), and *Fusarium graminearum* (de Chaves et al., 2022). The plant root is the key site for interacting with the host plant, microbial pathogens, and the rhizosphere microbiome (Zhalnina et al., 2018). Furthermore, plants have become more susceptible to disease due to continuous exposure to stress and climatic changes worldwide (Attia et al., 2022). Tomato plants are affected by numerous infections caused by many different agents, including fungus, bacteria, viruses, and physiological disorders, responsible for symptoms including fruit spots, rots, wilts, and leaf spots/blights (Jones et al., 2014; Elshafie et al., 2017). *F. oxysporum* f. sp. *lycopersici* (Fol) is a potent fungal pathogen infects tomato crops by penetrating the roots. This pathogen causes yellowing of lower leaves, browning of vascular tissues, and wilting symptoms in tomato seedlings above the soil line with substantial yield losses (Borrero et al., 2012; Li et al., 2018). Moreover, infection of tomato plants is achieved by spore germination or mycelium, leading to higher plant transpiration and lower nutrient translocation, causing wilting crown and root rot and, eventually, death of the plant (Akbar et al., 2018; Manikandan et al., 2018). *Fusarium oxysporum* is a widely distributed and phylogenetically diverse species known as a mycotoxin producer (Irzykowska et al., 2012) and is ranked fifth out of the 10 most lethal (death-causing) plant pathogens. Two main formae speciales are *F. oxysporum*f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) (Ribeiro et al., 2022). FOL is responsible for *Fusarium* wilt, and FORL causes *Fusarium* crown and root rot, among the most studied plant diseases. In Egypt, the damage to tomato crop production due to *F. oxysporum* wilt occurred in 67% of the total planted area (Selim and El-Gammal, 2015). In addition, some fungal species produce mycotoxins (e.g., aflatoxin) on plants which negatively affects the post-harvest treatments and helps to spread the causal agent between the next generation of seeds (Marshall et al., 2020). Thus, various methods, such as chemicals and pesticides, are employed to control and alleviate plant diseases (Ristaino et al., 2021). However, using fungicides to control *Fusarium* wilt is ineffective because fungal conidia stay viable for a long time, and pesticide residues are harmful to human health (Attia et al., 2022). Therefore, new control strategies must be developed with the aim of economic and environmental sustainability in plant and crop protection (Hernández-Aparicio et al., 2021). Biological control is a desirable alternative method to control the *Fusarium* wilt infection by using antagonistic nonpathogenic microorganisms to minimize the harmful effects in numerous crops (Glick, 2012). Chemical fungicides and soil fumigation have been widely used for controlling FWD disease. *Trichoderma* strains are vital anti-pathogen biocontrol agents (Pal and Gardener, 2006; Contreras-Cornejo et al., 2016; Zhang et al., 2022). This genus is a ubiquitous filamentous fungi that grow in the rhizosphere and colonizes plant roots as an opportunistic, avirulent plant symbiont (Harman, 2006). As *Trichoderma* species is a known aggressive wide range of soil fungal pathogens worldwide to suppress soil fungal diseases and plant pathogen invasion. They are frequently applied as biofungicides against pathogens such as *Botrytis cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, and *Sclerotium rolfsii* on crops of economic importance (Mohiddin et al., 2010; Olowe et al., 2022; Sharma et al., 2022). *Trichoderma* fungi indirectly exert their biological control machinery toward fungal pathogens by competition for nutrients and space, antibiosis, production of growth-promoting substances (e.g., IAA, nutrient uptake) (Lei and Zhang, 2015; França et al., 2017) or by secretion of bioactive metabolites, some cell wall-degrading enzymes (CWDEs), such as chitinases and β-1,3-glucanases and mycoparasitism (Elshahawy and El-Mohamedy, 2019; Sallam et al., 2019). Mycoparasitism is the most efficient antifungal mechanism of *Trichoderma* spp. (e.g., *T. virens*, *T. harzianum*) *via* recognizing the pathogen and growing alongside the pathogen hyphae, then dissolution and death of the pathogen (Benítez et al., 2004; Sharon et al., 2007; Hewedy et al., 2020a,b; Contreras-Cornejo et al., 2021; Taylor et al., 2021; Mukherjee et al., 2022). Various factors such as native environmental habitats, strains, pathogen species, and laboratory findings for testing their antifungal activities affect the *Trichoderma* biocontrol machinery. *Trichoderma asperellum* is a promising strain based on *in vitro* assays against plant fungal phytopathogens. For example, *T. asperellum* GDFS1009 has been shown tosuppress *F. oxysporum f.* sp. *cucumerinum* (*Fusarium* wilt of cucumber) and *Fusarium graminearum* infections (Wu et al., 2017; Ketta and Hewedy, 2021), with 98 and 91% inhibition of colony radial growth, respectively, to cope with biotic stresses (Kamaruzzaman et al., 2021). In this context, the discovery of a sustainable solution is the main aim of this study to select and test a potent fungal strain to reduce the negative influence of fungal pathogenicity with the aim of controlling plant disease as well as improving plant growth and plant health to sustain food production. Thus, in this study, we sought to isolate *T. asperellum* from the rhizosphere of garlic (*Allium sativum*), identified using the ITS gene, test the antifungal activities against different fungal pathogens, and focus on the causal agent that causes FWD. Moreover, exploring the antagonistic mechanisms such as chitinase activity and bioactive secondary metabolites, as well as the role of *T. asperellum* for tomato growth promotion (e.g., IAA, P solubilization). Moreover, we present a new trend regarding post-harvest applications using fungal secondary

metabolites against *F. oxysporum* mycotoxins to achieve sustainable food production in an eco-friendly environment. Finally, we used confocal microscopy as a throughput technique to explore the mobility of *Trichoderma* in the tomato roots.

Materials and methods

Phytopathogens and antagonistic strain

Five phytopathogenic fungi (*F. oxysporum*, *F. solani*, *F. graminearum*, *Alternaria alternata*, and *Rhizoctonia solani*) were obtained from Agriculture Research Center, Giza, Egypt. *Trichoderma* isolate (Biocontrol agent) was isolated from the garlic (*Allium sativum*) rhizosphere soil by serial dilution method. Briefly, the rhizospheric soil sample (10 g) was suspended in 90 mL of sterile distilled water, and the mixture was shaken at (200 rpm) for 30 min. Next, the soil suspension was serially diluted up to (10^{-3}) dilution, then 100 µl was spread on Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (50 mg/L) and streptomycin (15 mg/L) to suppress bacterial growth (Woldeamanuale, 2017). Subsequently, the inoculated plates were incubated at 28°C for 5–7 days. At the end of the incubation period, the fungal isolate was purified, kept on slants at 4°C, and subcultured every 4 weeks (López Errasquín and Vázquez, 2003). The pure culture of the fungal isolate was characterized using morphological and microscopical characteristics (Harman and Kubicek, 2002) and confirmed by molecular identification.

Molecular identification

Genomic DNA was extracted with the QIAamp DNeasy Plant Mini kit according to the manufacturer's instructions. Amplification of the fungal ITS region was carried out with forward ITS1 (5′ TCC-GTA-GGT-GAA-CCT-GCG-G 3′) and reverse ITS4 (5′TCC-TCC-GCT-TAT-TGA-TAT-GC 3′) primers (White et al., 1990). The polymerase chain reaction program was performed as previously described (Hewedy et al., 2020c). The homology of the ITS rDNA sequence of the isolate was analyzed using the BLAST program from the GenBank database.[1]

In vitro antagonistic activity of *Trichoderma asperellum* against phytopathogenic fungi

The antagonistic activity of *T. asperellum* against fungal pathogens was evaluated by dual culture assay (Awad et al., 2018). One mycelial disk of the pathogen (6 mm diameter) was deposited on one side of the Petri plate, and another disk of (antagonist) was deposited equidistantly on the other side. PDA plates containing only disks from the pathogen mycelium were used as controls. All the experiments were conducted in triplicates. After 7 days of incubation at 28°C, the radial growth of the phytopathogens in the control and treatment plates was measured, and the percentage of inhibition of radial mycelial growth (PIRG) was calculated using the following equation:

$$\%PIRG = R1 - R2 / R1 \times 100$$

where $R1$ = radial growth of the phytopathogen in the control plate; $R2$ = radial growth in the presence of an antagonist (Fishal et al., 2022).

Effect of *Trichoderma asperellum* culture filtrates on *Fusarium oxysporum*

Based on the dual culture assay results, the maximum growth inhibition was recorded against *F. oxysporum*. Thus, different concentrations of the culture of *T. asperellum* filtrate were tested against *F. oxysporum*. *T. asperellum* was inoculated in 50 mL of potato dextrose broth and incubated at 28°C for 14 days on a rotary shaker at 150 rpm. Cell-free supernatant was obtained by filtration through Whatman filter paper No. 1. The filtrate was sterilized using a 0.2 µm pore biological membrane filter (Jangir et al., 2019). Each sterilized filtrate was mixed into a PDA medium to obtain 5, 15, and 30% (v/v). Finally, 20 mL of the medium was amended with different filtrate concentrations and poured into 90 mm Petri plates. The secondary metabolites in the filtrate were tested for their efficacy against the test pathogens. The test pathogen was centrally inoculated with an individual equal disk (5 mm) of seven-day-old culture. PDA plates inoculated with pathogens without culture filtrates served as a control. Three replicates were maintained for each treatment and incubated at 28°C. The percentage inhibition of mycelial growth was calculated as mentioned above.

Scanning electron microscopy

The confrontation of hyphal interaction between *F. oxysporum* and *T. asperellum* was also examined by SEM. Briefly, a mycelial disk (5 mm) of both microbes was cultured on PDA for 4–5 days of incubation. The plate cultures were examined under a light microscope to check the early touch stage. The contact interactions were labeled, and 1 cm agar blocks were removed for SEM preparation, fixed with osmium tetroxide, and then dehydrated using a serial dilution of the ethyl alcohol and acetone. A critical point drier (Tousimis Autosamdri–815 Coater) was then used to dry the processed samples with gold using a sputter coater. Then, the mycoparsatism and the hyphae interactions were examined by SEM (JEOL JSM 6510 lv). The microscope was operated at 30 KV at EM Unit, Mansoura University, Egypt. Three replicates were included for each sample (Su et al., 2010).

Assessment of plant growth-promoting and antifungal properties of *Trichoderma asperellum*

Indole-3- acetic acid

Trichoderma asperellum was grown on potato dextrose broth (PDB) supplemented with L-Tryptophan (0.1 g/L) for 4–5 days at

1 http: www.ncbi.nlm.gov/BLAST/

28 ± 2°C on a rotary shaker at 150 rpm (Nandini et al., 2021). Next, both mycelium and debris were separated by filtration and centrifugation, respectively. Briefly, 1 mL of the culture filtrate and 4 mL of Salkowski reagent (1 mL of 0.5 M ferric chloride in 50 mL of 35% perchloric acid) were mixed and kept for 30 min in the dark for color change. The generation of pink indicated the production of IAA. The optical density was detected and measured at 530 nm using a spectrophotometer (JENWAY 6315). A standard curve of IAA concentrations was designed to evaluate the corresponding concentration of IAA-released *T. asperellum* in the bioassay media.

Phosphate solubilization efficacy of *Trichoderma asperellum*

A plug of *T.asperellum* (5 mm) was grown on a modified NBRIP medium (Nautiyal, 1999) supplemented by Bromophenol blue as an indicator for 7 days at 28°C. The medium contained the following composition (gL^{-1}: 10.0 glucose), 0.5$MgCL_2.6H_2O$, 0.25$MgSO_4.7H_2O$, 0.2KCL, 0.1 $(NH_4)_2SO_4$ and 15.0 agar, with the addition of 50 mL of K_2HPO_4 (10% w/v) and 100 mL of $CaCL_2$ (10% w/v) to precipitate insoluble calcium phosphate ($CaHPO_4$). The pH of the medium was adjusted to 7.0. After incubation, a yellow halo zone around the fungal colony indicated phosphate solubilizing ability. The solubilization zone and colony diameters were measured and calculated using the equation below to indicate the solubilization efficiency (Pande et al., 2017; Aloisio et al., 2019).

SE = (Solubilization diameter)/(Growth diameter) ×100.

Chitinase activity of *Trichoderma asperellum*

Chitinase detection medium with ingredients/per liter: 0.3 g of $MgSO_4.7H_2O$, 3.0 g $(NH4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µl of Tween-80, 4.5 g of colloidal chitin and 0.15 g of bromocresol purple pH (4.7), was prepared and autoclaved at 121°C for 15 min. Then, the plates were inoculated with a fresh culture plug of *T. asperellum*, incubated at 25 ± 2°C for 7 days, and were observed for colored zone formation (Agrawal and Kotasthane, 2012).

Trichoderma asperellum promotes tomato plant growth

Inoculum preparation

Seven-day-old pure culture of *T. asperellum* was used. After cultivation on PDA slants and incubation at 28°C, sterile water was poured into the slants, and the spores were scraped with a sterile glass rod. The spore suspension was filtered and put into tubes, and the spore concentration of *T. asperellum* was adjusted at 1×10^6 spore/mL (Scudeletti et al., 2021; Natsiopoulos et al., 2022).

Effect of *Trichoderma asperellum* on tomato seeds germination and growth promotion

Tomato seeds of (Hybrid Madera F1) were used in all experiments. The tomato seeds were surface sterilized with 2.5% (v/v) sodium hypochlorite for 10 min and rinsed three times with distilled water before seed inoculation. Afterward, the seeds were immersed for 24 h in fungal suspensions prepared at 1.0×10^6 spore mL^{-1}. The control treatment consisted of seeds immersed only in sterile distilled water for the same period. The seeds were deposited in Petri dishes containing autoclaved tissue papers moistened with sterile water (16 seeds per dish) that were sealed with parafilm to prevent evaporation and then incubated at 28°C for 7 days (Kipngeno et al., 2015; Barroso et al., 2019). Each treatment was replicated three times. The germination rate (%) was estimated by counting the number of seeds germinating after one week of cultivation.

Three sterilized tomato seeds were transferred to one side of the plate prepared with water agar containing 0.2% (w/v) glucose (Yoo et al., 2018). The other side contained a *T. asperellum* disk (5 mm) grown on PDA. Plates without inoculum were considered a control, and the plates were incubated for 3–4 days in darkness at 28°C. After the incubation period, the germination percentage (GP) was evaluated according to the following equation:

$$GP(\%) = n / N \times 100$$

where: n = number of germinated seeds; N = total number of seeds.

Next, treated and untreated (control) seeds were sown in pots (8 cm) in diameter containing a mixture of sterilized peat moss and soil (1:2 w/w) at 28 g/pot, with a density of five seeds per pot. Pots were maintained under controlled conditions in the growth chamber (22 ± 3°C). Experiments were performed using a completely randomized design (CRD) with two treatments and three replicates. The pots were rotated three times a week to ensure uniform growth conditions in the growth chamber. After 21 days, plant samples were taken to measure the shoot height (cm), the root length (cm), and plant biomass. The seedling vigor index (VI) was calculated as described by Buriro et al. (2011).

$$\text{Seedling vigor index} = \left[\text{seedling length}\,(\text{cm}) \times GP\,(\%)\right]$$

Confocal microscopic assay

The interaction between *T. asperellum* strain and the tomato-root system during the early stages of root colonization was examined by confocal microscopy. SYTOX Green (Thermo Fisher Scientific-United States) was used for *Trichoderma* staining. Briefly, tomato roots were carefully removed from the sterilized soil and gently swirled 4–5 times in autoclaved water to wash the clay particles. Then, the primary root was stained using 10 µM propidium iodide (PI) for 10 min to label the plant cell wall and washed by ddH_2O 4–5 times. Subsequently, the root was placed on a glass slide to facilitate the visualization of the localization of *T. asperellum* (green) in the tomato root (red) surface as a plant growth promoter (Chacón et al., 2007).

Estimation of the biocontrol efficiency of *Trichoderma asperellum* against *Fusarium* wilt disease

Sterilized tomato seeds were sown in pots (8 cm) in diameter containing 28 g of sterilized peat moss and soil (1:2, w/w). Each pot contains five seeds, and the experiment included four treatments as follows:

1. Control (distilled water) only.
2. Pathogen (*F. oxysporum*) only.

3. Bioagent (*T. asperellum*) only.
4. *Trichoderma* + *Fusarium*.

Tomato plants were inoculated and kept at 25°C for 21 days, and the parameters of plant phenotype were measured, including the root length, shoot, and fresh weight. The shoots and the roots were dried in an oven at 70°C until constant weight (Mwangi et al., 2011).

Tomato root anatomy under biotic stress

The roots of tomato plants were sectioned and studied anatomically. The wax method was used in this experiment (Johansen, 1940). Briefly, plant roots were fixed for 72 h using FAA (formalin: acetic acid: alcohol 90:5:5) fixative. Then, roots were washed several times using distilled water and dehydrated using serial concentrations of alcohol 50, 70, 90, 95, and 100% (v/v), respectively. For clearing, they were transferred every 3 h from a mixture of 1:1 cedarwood oil: and absolute alcohol into pure cedarwood oil, followed by a mixture of cedarwood oil and xylene, and left overnight in pure xylene. Wax embedding was carried out in an oven adjusted at 60°C, embedded in clear wax, and sectioned using a rotatory microtome. Staining was done using safranine and fast green stains. Sections were mounted in a drop of Canada balsam, covered, and left to dry. The prepared slides of each root treatment were repeatedly examined under the light microscope (LEICA DM750) at the Microscopy Department, Faculty of Agriculture, Cairo University.

Trichoderma asperellum protects tomato fruits against FWD

Preparation of *Trichoderma asperellum* crude extract

Bioactive secondary metabolites of *T. asperellum* were extracted using ethyl acetate solvent, as previously described by Chen et al. (2018). *Trichoderma* was grown in a PDB medium and incubated on a rotary shaker (150 rpm) for 21 days at 28°C. After the incubation period, the fermented broth was filtered through Whatman filter paper No. 1, and the metabolites produced by the fungus were extracted from an equal volume of ethyl acetate. An equal volume of ethyl acetate was added to the filtrate and vigorously shaken for 5 min at room temperature. The mixtures were transferred to separating funnels, and the organic layers of ethyl acetate were allowed to separate from the aqueous layers. Then, the ethyl acetate layer was allowed to dry at room temperature, and the dried extracts were stored at 4°C for further use.

Assessment of extracted secondary metabolites against mycotoxigenic *Fusarium oxysporum* in tomato fruits

The antifungal activity of the *T. asperellum* extract was evaluated against *Fusarium* on tomato fruits, which were obtained from the supermarket, were initially washed under running water, sterilized for 2 min in 2% (v/v) sodium hypochlorite, and then rinsed with sterile water. Watery fruits were placed in plastic trays (sterilized with 70% (v/v) ethanol and under UV) and dried for 2 h under a laminar flow cabinet. The fruits were wounded with a sterile needle, and 20 µl of the pathogen spore suspension at 10^4 spores/mL was inoculated onto the wound. Subsequently, the drop was dried for 1 h. Finally, tomato fruits were treated with 20 µl of *Trichoderma* crude extract in the same wound where the pathogen inoculum had been applied. The treated tomato fruits were dried under a laminar flow cabinet until the droplet was completely dry, and the closed trays were incubated at room temperature (Stracquadanio et al., 2021; Maliehe et al., 2022). The untreated positive control was inoculated with the spore suspension of the pathogen, and the negative control was inoculated with *Trichoderma* extract. Fruits were monitored daily, and results were evaluated on the 11th day of incubation. The diameter of the lesions was also observed.

Mycotoxin analysis

Two grams of ground tomato fruits were mixed with 8 mL (80 acetonitrile (ACN): 20 H_2O) and shaken for 20 min. Then, the mixture was centrifuged at 3500 rpm for 10 min and filtered through a 0.45 µm nylon filter. After filtration, the extract was diluted (1:4) with water containing 5 mM ammonium acetate. Analysis of mycotoxins (FB1, FB2, DON, and ZEN) was performed using liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with an ExionLC AC system for separation and SCIEX Triple Quad 5,500+ MS/MS system equipped with electrospray ionization (ESI) for detection. The instrument data were collected and processed using the SCIEX OS 1.6.10.40973 software. The targeted analytes were separated with an Agilent Zorbax Eclipse Plus C18 Column (4.6 × 100 mm, 1.8 µm) (Waskiewicz et al., 2010). The mobile phases consisted of two eluents containing 10 mM ammonium formate, eluent A was 0.1% (v/v) formic acid in the water, and eluent B was 0.1% (v/v) formic acid in methanol (LC grade). The mobile phase gradient was programmed as follows: 10% B at 0 min, 10–30% B from 0.0–2.0 min, 30–100% B from 2.0–11.0 min, 100% B from 11.0–11.5 min, 100–10% B from 11.5–12.0 min, 10% B from 12.0 to 15.0 min. The flow rate was 0.6 mL/min, and the injection volume was 10 µl. For MS/MS analysis, positive ionization mode (+MRM) was applied with the following parameters: curtain gas: 20 psi; collision gas: 9 psi; nebulizer current: 3; source temperature: 600°C; ion source gas 1 (nebulizer gas): 60 psi.

GC–MS analysis

The chemical composition of *T. asperellum* crude extract was assessed to detect the active constituents exhibiting antifungal activity. GC–MS was performed using a Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, United States) with a direct capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased by 5°C/min to 230°C for 2 min, then increased to the final temperature of 290°C by 30°C /min and held for 2 min. The injector and MS, transfer line temperatures, were kept at 250°C and 260°C; Helium was used as a carrier gas at a 1 mL/min constant flow rate. The solvent delay was 3 min, and diluted samples of 1 µl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. In full scan mode, EI/MS were collected at 70 eV ionization voltages over m/z 40–1,000. The ion source temperature was set at 200°C, and the components were identified by comparing their retention times and mass spectra with those of

the WILEY 09 and NIST 11 mass spectral databases (Mulatu et al., 2022).

Statistical analysis

Analysis of variance (ANOVA) was assessed using IBM SPSS Statistics Version 28. The experiments were conducted using a completely randomized design with three replicates. The growth of the fungal strains was compared by t-test, and their biocontrol activities were evaluated by Fisher's least significant difference (LSD) test at the 5% significance level.

Results

Isolation, morphological and molecular identification

Trichoderma asperellum was isolated from garlic rhizosphere soil by plate dilution, and the pure culture was maintained on a Potato dextrose agar (PDA) medium. Macroscopic morphology of *T. asperellum* revealed the rapid growth of the colony (3–4) days with 1–2 concentric rings. The mycelium, initially of a white color, acquired green and yellow shades or remained white due to the abundant production of conidia (Figure 1A). Regarding microscopic observations, *T. asperellum* showed globous, subglobous, or ovoid conidia, ampuliform phialides, and branched conidiophores, as shown in Figures 1B,C. The identification of the fungal isolate was further confirmed by analysis of the ITS sequence. One band of 531 bp was amplified using ITS primers, and the sequence alignments were performed using the Basic Local Alignment Search Tool (BLAST) to determine the phylogenetic positions of our strain with other *Trichoderma* strains on the GenBank. The fungal strain had (100%) matched identify with *T. asperellum* (Figure 1D). Moreover, the ITS sequence of *T. asperellum* was submitted to GenBank under the accession number (OQ130157).

Evaluation of the antagonistic activity of *Trichoderma asperellum* against phytopathogens

Trichoderma asperellum was tested against several plant pathogenic fungi (*F. oxysporum*, *F. solani*, *F. graminearum*, *A. alternata*, and *R. solani*) to assess the antifungal activity using dual culture assay. Results showed that *T. asperellum* could inhibit the radial mycelial growth of all tested pathogens. The maximum growth rate inhibition (53.23%) was recorded against *F. oxysporum* followed by *F. graminearum* (45%), *F. solani* (43.19%), and *R. solani* (30.89%). In contrast, the lowest inhibitory activity percentage was exhibited against *A. alternata* (20.67%) (Table 1; Figure 2A). Based on the dual culture assay results, the highest growth rate inhibition was displayed against *F. oxysporum*. Therefore, different concentrations of sterilized culture filtrate of *T. asperellum* were tested against *F. oxysporum* to evaluate the optimum inhibiting concentration. Results showed that the inhibitory activity increased by increasing the sterilized culture filtrate concentration, and the maximum inhibitory activity percentage (59.38%) was observed at a concentration of 30% (Table 2; Figure 2B).

Scanning electron microscopy

The mycoparasitic nature of *T. asperellum* on *F. oxysporum* as a dual culture was examined by SEM (Figure 3). Interestingly, *T. asperellum* hyphae grew over the hyphae of *F. oxysporum*, followed by quick and excessive coiling. Finally, *F. oxysporum* lysis was eventually observed (Figures 3D–F).

Plant growth-promoting and antifungal properties of *Trichoderma asperellum*

To assess the growth-promoting effects of *T. asperellum* on plant development, the plant growth hormone IAA was measured using a colorimetric assay. Results showed that the amount of IAA produced by *T. asperellum* was ($12.5 \pm 0.5742 \mu g mL^{-1}$). Moreover, *T. asperellum* could solubilize phosphate, exhibiting phosphorus solubilization indices of 100% (Figures 4E,F). Additionally, *T. asperellum* showed high chitinase activity in a colloidal chitin medium supplemented with bromocresol purple (with final acidic pH of 4.7). *Trichoderma*, breakdown chitin to N- acetyl glucosamine shifting pH toward the alkalinity, which changes the medium color to purple (Figures 4B,C).

Effect of *Trichoderma asperellum* on tomato seeds germination and growth promotion

Data illustrated in Figure 5; Supplementary Figure S1 indicated that *T. asperellum* treatment enhanced seed germination, root and shoot length, and vigor index in tomato seedlings. After 7 days, seeds treated with spore suspension of *T. asperellum* (10^6 spore mL^{-1}) resulted in the highest seed germination rates (100%), as compared to the control (81.25%). Also, it can be seen that in the presence of *T. asperellum* grown on PDA in split interaction, the germination rate of seeds was enhanced (100%) as compared to an un-inoculated plate (66.6%) as presented in Figures 5C,D. In addition, *T. asperellum* inoculation promoted tomato growth parameters (Figures 5E,F). We observed that the Average shoot length of plants inoculated with *T. asperellum* simultaneously was 14.5% longer than the control. The average root length was also 17.6% longer than the control treatment (Figures 5G,H).

Confocal microscopy

The capacity of the fungus *T. asperellum* to colonize tomato roots and stimulate plant growth was observed using a confocal imaging assay. Sterilized tomato seeds were inoculated with *Trichoderma* spores before germination on Petri dishes (150 mm) and kept in the dark for 3 days to study the early stages of the fungal root colonization. Interestingly, the root elongation of tomato seedlings and intercellular hyphal growth were observed compared with the uninoculated treatment (control) (Figures 5I,J). To obtain more insight, an *in vitro*

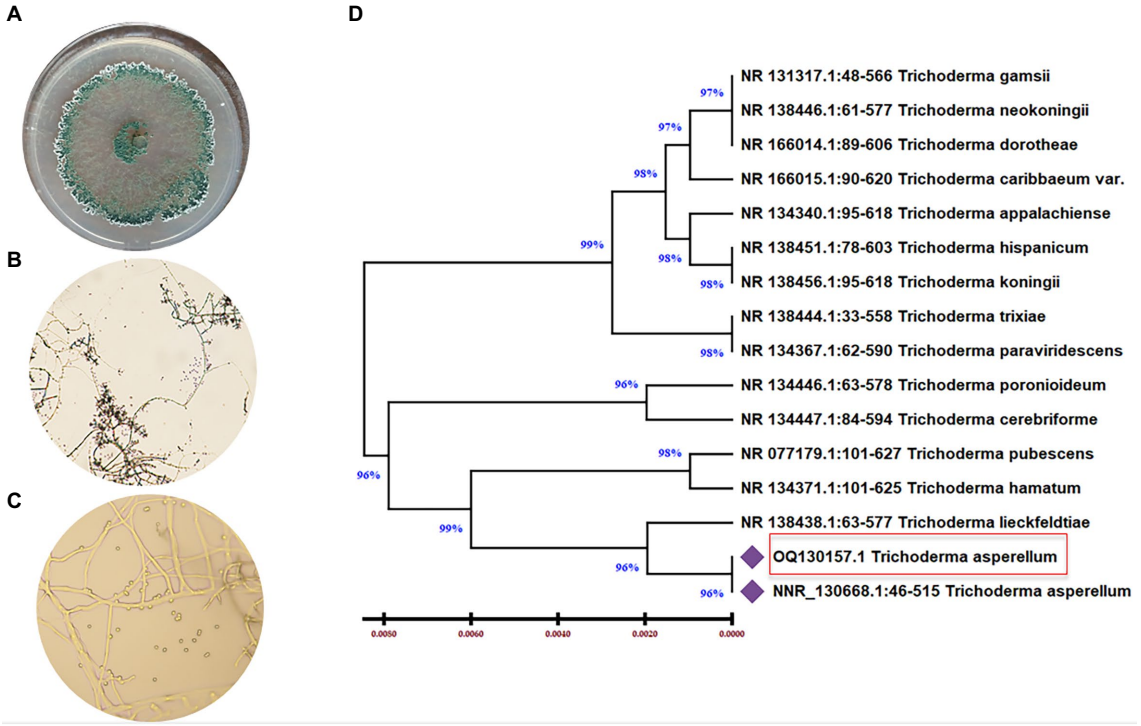

FIGURE 1
Morphological and molecular identification of *T. asperellum*. **(A)** The cultural view of T. *asperellum* grown on synthetic nutrient agar (SNA) media at 28°C for five days, which shows a ring around the original inoculum, **(B,C)** Microscopic view of conidia and conidiophores. **(D)** Molecular phylogenetic based on rDNA internal transcribed spacers (ITS) analyzes of *T. asperellum* by maximum Likelihood Model of MEGA11.0. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

TABLE 1 Antagonistic activity of *T. asperellum* against some phytopathogenic fungi.

Bioagent	Phytopathogenic fungi									
	F. oxysporum		*F. solani*		*F. graminearum*		*A. alternata*		*R. solani*	
	RMG (cm)	PIRG (%)	RMG (cm)	PIRG (%)	RMG (cm)	PIRG (%)	RMG (cm)	PIRG (%)	RMG (cm)	PIRG (%)
Control	6.43 ± 0.03	0.00	5.63 ± 0.15	0.00	6.00 ± 0.05	0.00	4.16 ± 0.03	0.00	7.18 ± 0.11	0.00
T. asperellum	3.01 ± 0.04	53.23	3.20 ± 0.17	43.19	3.30 ± 0.11	45	3.30 ± 0.05	20.67	4.96 ± 0.12	30.89

RMG, Radial Mycelial growth; PIRG, Percent inhibition of Radial Mycelial growth; ± SE.

experiment was carried out to study the effect of a biocontrol agent at the initial stage of root colonization by *T. asperellum* to understand its influence on tomato growth promotion. After inoculation, tomato roots were covered with conidia. Initial contact between conidia and the root was primarily at the region of root hairs.

Effect of *Trichoderma asperellum* on the growth of tomato plants under biotic stress of *Fusarium oxysporum*

There was a notable variation between the tomato plants inoculated with *Trichoderma* and *Fusarium* individually, as shown in Figure 6A. Data presented in Figures 5K,L showed that *T. asperellum* improved the growth of tomato plants under *Fusarium* infection. In addition, Figures 6B–D showed a significant increase in the root length and the fresh weights of shoots between the control (untreated) and the inoculated tomato plants with *Trichoderma* under biotic stress. The inhibitory effects of *Trichoderma* inoculation against *Fusarium* invasion were demonstrated in Figure 6A. *Fusarium* pathogen invaded the root system and significantly decreased roots and shoot dry weights, the number of leaves, and root length. In addition, there was a marked increase in the root length and shoot fresh weight during the *Trichoderma-Fusarium* interactions (Figures 6C–D). *T. asperellum* controlled the FWD caused by *F. oxysporum* by enhancing the plant phenotypes, including shoot and root length (cm), shoot and fresh root weight (g), shoot and root dry weight (g), as well as the number of leaves. It can be seen that number

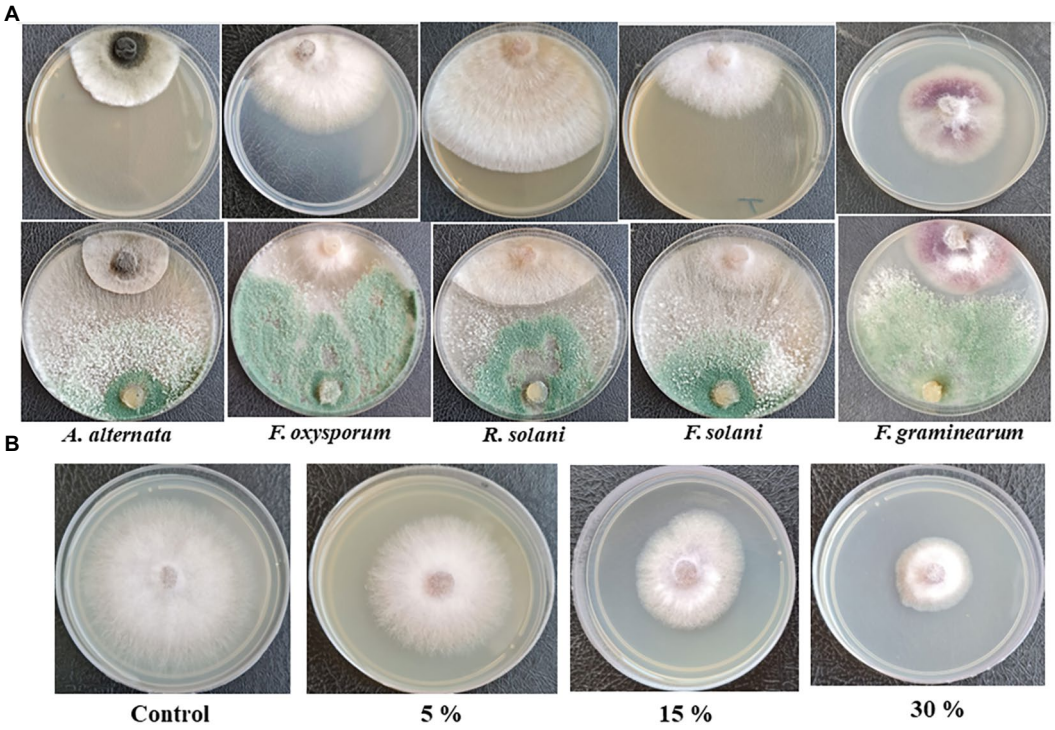

FIGURE 2
(A) Antagonistic activity of *T. asperellum* against plant pathogenic fungi in dual culture assay, **(B)** Effect of different concentrations of *T. asperellum* filtrate on *F. oxysporum* growth.

TABLE 2 Effect of different concentrations of *T. asperellum* culture filtrate against *F. oxysporum*.

Culture filtrate concentrations (%)	*F. oxysporum* (RMG) (cm)	PIRG (%)
Control	7.63 ± 0.03	0.0
5	5.34 ± 0.08	30
15	4.65 ± 0.02	39.03
30	3.10 ± 0.05	59.38

RMG, Radial Mycelial growth; PIRG, Percent inhibition of Radial Mycelial growth; ± SE.

of leaves in plants inoculated with *Trichoderma* was (45%) higher than the number of leaves in the treated plants with *Fusarium* alone.

Root anatomy

Transverse sections of tomato roots treated with *T. asperellum* and the interactions between bioagent and the fungal pathogen were studied to evaluate their impact on root health. Interestingly, the inoculation of tomato roots with *Trichoderma* increased whole root thickness and the number of xylem vessels compared to the control (Figures 7A,D,G). The highest degradation area of the root cortex was observed in plants infested with *F. oxysporum*. Lateral root (LR) development was also detected in the treated tomato roots with *T. asperellum* (Figure 7F). However, the combination of *T. asperellum* and *F. oxysporum* enhanced the root thickness in the cortex of the tomato tap root (Figure 7I). Light microscope sections showed that *Trichoderma* enhanced the cortex and xylem diameters. It was also noted that the xylem and the endodermis diameters were thinner in control roots than in other treatments; this trend was the same in both hybrids (Figures 7C,F,I).

Biocontrol efficiency on tomato fruits against FWD

The disease control efficacy of *T. asperellum* against *F. oxysporum* is shown in Figures 8A–C. After 11 days of storage, tomato fruits inoculated with *F. oxysporum* and treated with *T. asperellum* extract showed an increase in shelf-life compared with the untreated control (*Fusarium* only). Moreover, tomato fruits inoculated with *Trichoderma* metabolites showed a significant reduction in lesion diameter compared to the pathogen-treated fruits (Figure 8). Results indicated that *T. asperellum* exhibited a significant inhibitory effect against *F. oxysporum*. In addition, the mycotoxins typically produced by *Fusarium* species (FB1, FB2, ZEN, and DON) were not detected by LC–MS/MS analysis, even in the untreated controls.

GC–MS analysis

The GC–MS analysis of the ethyl acetate extract of *T. asperellum* revealed the presence of different bioactive compounds (Figure 8D). The compound name, molecular formula (MF), molecular weight (MW), concentration (peak area %), and retention time (RT) are present in Table 3. Interestingly, the most predominant compounds were 1,2-Benzenedicarboxylic acids (32.15%), Palmitic Acid, TMS

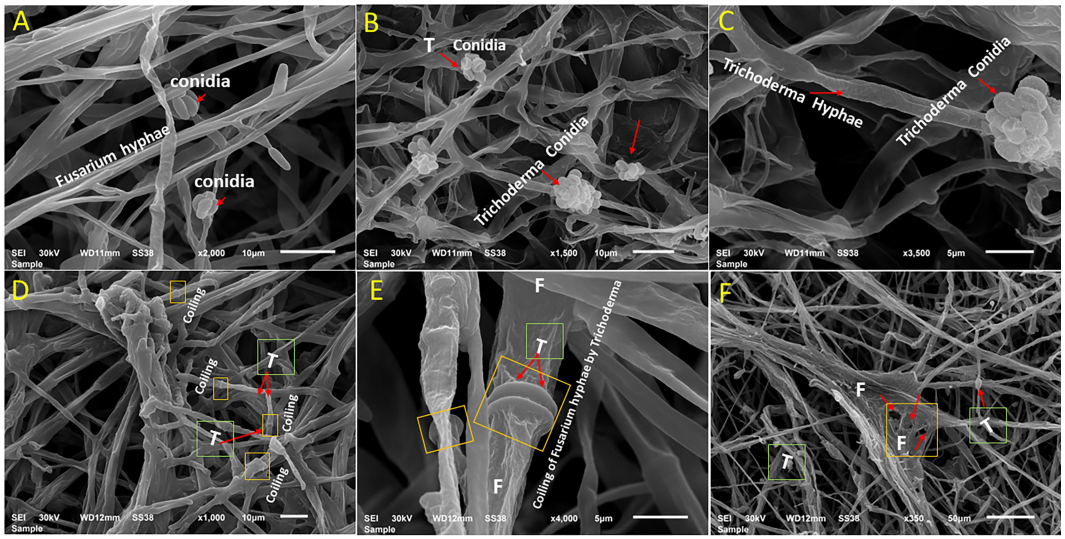

FIGURE 3
Scanning electron microscopy interpretations of the mycoparasitic of *T. asperellum* on *F. oxysporum*. **(A)** *Fusarium* pathogen alone, **(B,C)** *T. asperellum* alone (endophytic hyphae and conidia), **(D)** Mycelium of both fungi in contact *via* growing of *Trichoderma* hyphae (T) over *Fusarium* hyphae, **(E)** Coiling of *Fusarium* hyphae (F) by *Trichoderma* (T) as one of the antagonistic mechanisms (arrows), **(F)** Deformation of *F. oxysporum* by *T. asperellum*.

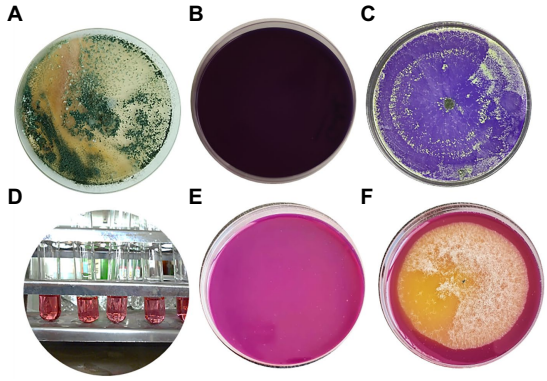

FIGURE 4
(A) *Trichoderma asperellum* strain grown on PDA at 28°C for seven days. **(B,C)** Screening of *T. asperellum* for chitinase activity on medium supplemented with colloidal chitin and control without inoculum. **(D)** IAA production by *T. asperellum* (5 replicates). **(E,F)** Phosphate solubilization of *T. asperellum* on NBRIP media supplemented with bromophenol blue as an indicator. **(E)** Uninoculated (control), **(F)** inoculated with *T. asperellum*.

derivative (17.05%), D-Glucopyranose, 5TMS derivative(7.04%), Myo-Inositol, 6TMS derivative(4.55%), Oleic Acid, (Z)-, TMS derivative (2.82%), 9,12,15-OCTADECATRIENOIC ACID, 2-[(TRIMETHYLSILYL) OXY]-1-[[(TRIMETHYLSILYL) OXY] MET HYL]ETHYL ESTER, (Z, Z, Z)-(1.35%), 4H-1-Benzopyran-4-one,2-(3,4-Dihydroxyohenyl)-6,8-DI-á-D-Glucopyranosyl-5, 7-Dihydroxy (1.35%) and Stearic acid, TMS derivative (0.82%).

Discussion

Various antagonistic activities were studied against different fungal pathogens using *T. asperellum,* as shown in Figure 3. This was sufficient evidence to conclude that the *Trichoderma* strain could be a bio-control agent and stimulate plant health *via* secondary metabolites and hormone production. In addition, this strain has the capability to sustain beneficial interaction with the host plant under stressful biotic conditions. Several *Trichoderma* isolates have been reported to suppress FWD (Srivastava et al., 2010; Marzano et al., 2013). *Trichoderma* species also have the potential of antagonistic interactions with different plant pathogens, which determines the biocontrol efficiency of the fungal pathogens. Mycoparasitism, antibiosis, competition for nutrients, and induced systemic resistance in plants are diverse antifungal activities of *Trichoderma* spp. (Druzhinina et al., 2011; Modrzewska et al., 2022). Therefore, these studies showed the successful application of *Trichoderma* in the field, which could impair plant health and productivity and suppress the *Fusarium* wilt disease. Additionally, this study investigated the contribution of *T. asperellum* to P solubilization and IAA production. Experiments were conducted in duplicate to investigate the ability of *Trichoderma* to promote plant growth. *Trichoderma* produced a clear halo around its colonies, which solubilized soluble P *in vitro*. Interestingly, *T. asperellum* released an auxin-like phytohormone that significantly increased the total root length of tomato plants. Since the tomato is one of the most produced crop plants growing in fields and greenhouses worldwide, this study evaluated the mechanism by which *Trichoderma* assisted tomato roots in response to *Fusarium* infection during plant growth. Plant roots exploit morphological plasticity to adapt and respond to different soil environments (Alaguero-Cordovilla et al., 2018). However, most of the investigated *Trichoderma* species colonize the root surface or inhabit inside root tissues as endophytes (Bailey et al., 2006). *Trichoderma* application with tomato seedlings shows adequate protection against *F. oxysporum*. Interestingly, the *T. asperellum* also benefitted plant growth promotion *via* root elongation during the pathogen attacks (Figure 5G). This is consistent with the dual cultural assay data between the biological agents and the pathogen infection, which decreased the hyphal growth. The results showed that the highest growth rate inhibition was displayed against *F. oxysporum*, causing FWD.

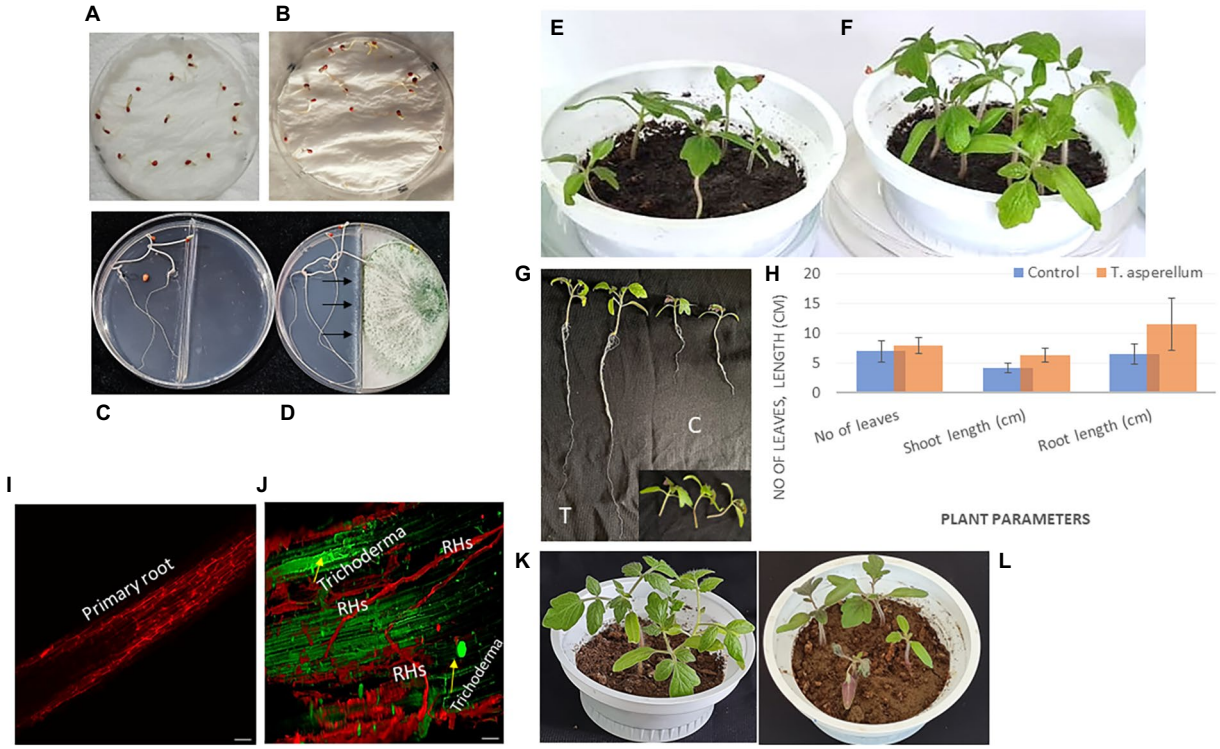

FIGURE 5
Trichoderma asperellum colonizes a tomato plant's root system and promotes root elongation. **(A)** Seeds treated with distilled water. **(B)** Seeds treated with a spore suspension of *T. asperellum*. **(C)** Split interaction of seeds growing on water agar medium without *Trichoderma* treatment (control). **(D)** Split interaction with *Trichoderma* treatment. **(E,F)** Tomato plants are grown in the presence of *Trichoderma* after seed coating (pot at right) and without microbial inoculation (control only) (pot at left). **(G)** Promotion of root and shoot length *via Trichoderma* inoculation (T) compared to untreated plants (C). **(H)** Plant parameters include the number of leaves, shoot length, and root length with *Trichoderma* inoculation and control treatment. *In vivo* tests indicated the growth-promoting potential of this fungal endophyte. *Trichoderma* could boost plant growth by activating either an individual or numerous mechanisms. **(I,J)** Representative confocal laser scanning microscopical analysis illustrating the early and robust colonization of tomato roots/root hairs (RHs) by *T. asperellum* compared with the control (without microbial inoculation) using CLSM with a Leica TCS SP2. **(K,L)** Tomato plants are grown in the presence of *T. asperellum* after three weeks from seed coating (pot at left) and plants infected with *Fusarium* (pot at right).

A precise *in vitro* experimental design was established to explore the impact of *Trichoderma* inoculation on the tomato root system during early growth (Figure 5D). Our results revealed that the biocontrol agent led to observed differences during early growth. The treatments were categorized into tomato roots with no microbial inoculation (control) and root inoculation with *Trichoderma*. The effect of *Trichoderma* strain on plant growth under abiotic stress was initially evaluated by measuring the root and shoot length and the number of leaves. In addition, the fungal inoculation significantly increased the plant parameters compared to untreated plants (control), as shown in Figure 5H. In that context, some studies have revealed that the inoculation of *T. harzianum* SQR-T037 improved the growth of tomato plants under greenhouse and field conditions (Cai et al., 2013, 2015).

The root system is essential for plant growth because of its basic functions in the selective absorption of water and nutrients (Karthika et al., 2018; Das et al., 2022), storage organ, and a selective barrier against pathogens (Wiedemeier et al., 2002; Atkinson et al., 2014). Characterization of root morphology and cellular development of two tomato species, *S. pennellii* and *S. lycopersicum* 'M82' during early growth were studied by Ron et al. (2013). This study provided significant differences in an extensive range of root traits with developmental significance. In addition, the newly emerged lateral roots (LRs) were significantly correlated with *Trichoderma* inoculation in tomato plants (Figure 7F). In addition, *Trichoderma* (green) colonized tomato roots (red), which visualized its mobility by the confocal imaging microscope (Figure 5J). It is well known that *Trichoderma* strains are able to colonize plant roots and stimulate the growth *via* the production of phytohormones, thus playing a crucial role in regulating the plant root system (Benítez et al., 2004; Harman et al., 2004; Cai et al., 2013). These findings agreed with our observations which suggested that improving plant growth occurs through a direct effect of *Trichoderma* species on root development.

Furthermore, *Trichoderma* can regulate plant growth through other mechanisms, such as mineral solubilization, to sustain plant health. These findings were consistent with the results found in this study when inoculating tomato seeds with *T. asperellum* in healthy conditions or under pathogenic stress. Tomato root colonization and *Trichoderma* mobility were visualized using confocal laser imaging (Figure 5J) in the rhizosphere to discover the tomato root-*Trichoderma* interactions. Tomato seeds were sterilized, germinated, and inoculated, and seedlings were grown under the conditions described (Simons et al., 1996). *Trichoderma* and *Fusarium* were labeled with autofluorescent protein (AFP) markers to visualize their interactions

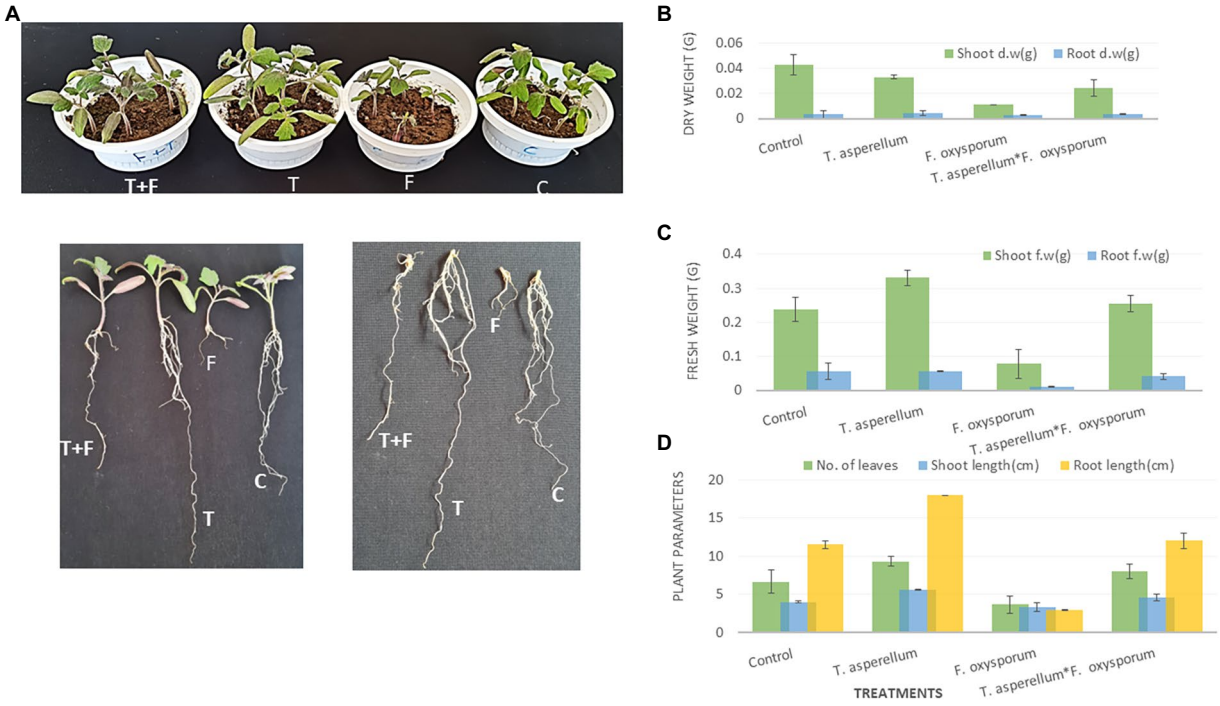

FIGURE 6
The Effect of *T. asperellum* on the growth of tomato plants under biotic stress of *F. oxysporum* including **(A)**; T-*Trichoderma* enhances both root and shoot of tomato seedlings, F-*Fusarium* can penetrate tomato roots and cause wilting or root rotting diseases, T+F-the interaction between the fungal endophyte and the pathogen, C-tomato plants without microbial treatment. Shoot and root dry weight **(B)**. Shoot and root fresh weight and **(C)**. The number of leaves, shoot, and root length (cm) **(D)**.

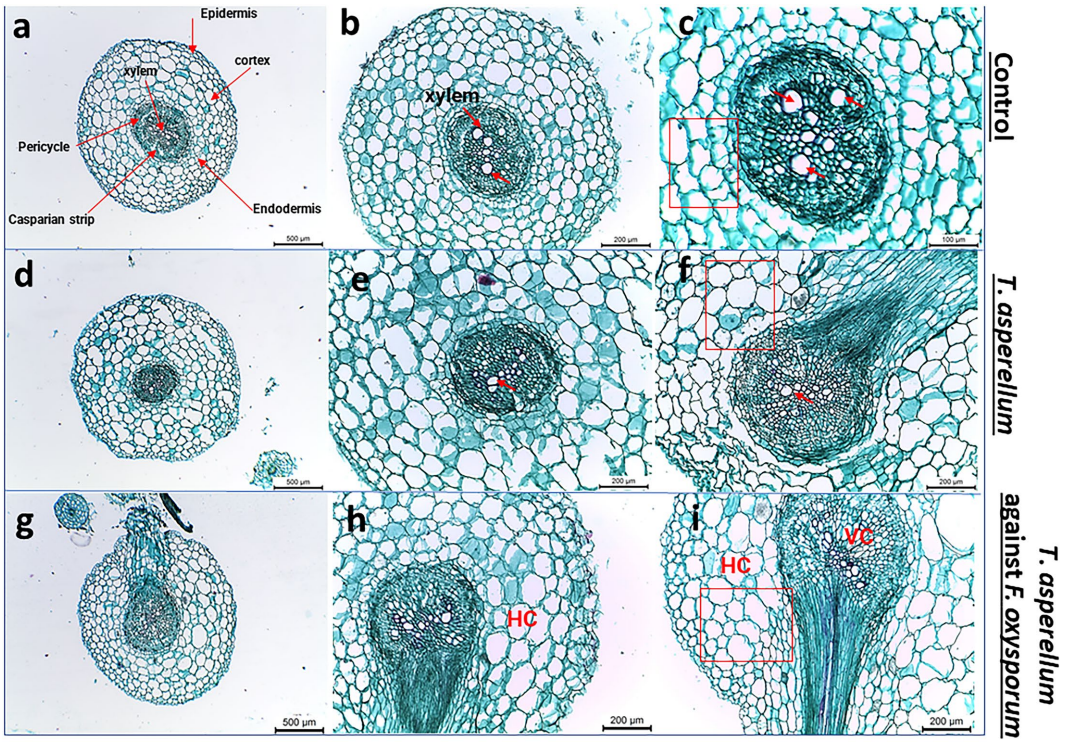

FIGURE 7
Light microscope images of tomato root cross sections near the root tip taken from plants grown under biotic stress. **(A–C)** Healthy tomato root, **(D–F)** Transverse sections of tomato roots plant treated with *Trichoderma*, **(G–I)** shows the root cross-sections after applying *Trichoderma* against *Fusarium* pathogen. Red arrows indicate the xylem vessels.

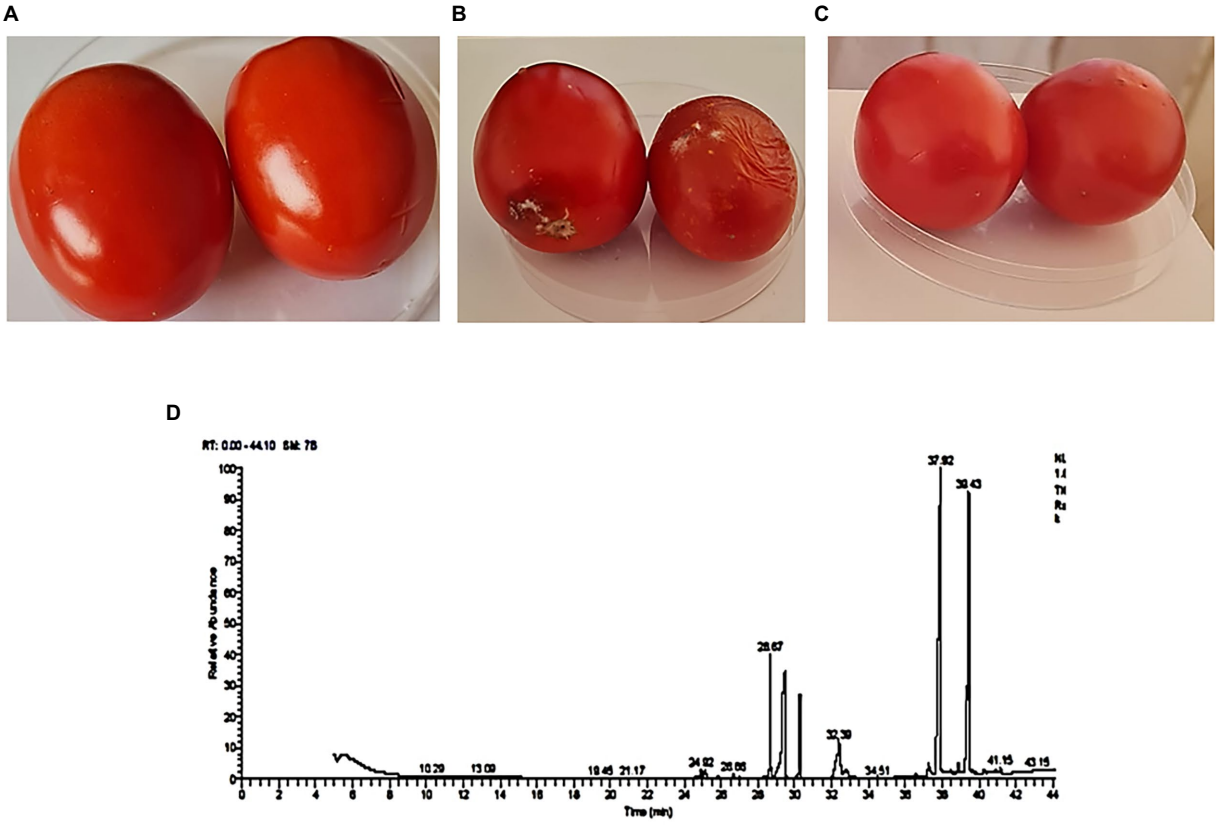

FIGURE 8
Biocontrol efficacy of *T. asperellum* crude extract *against F. oxysprum*. **(A)** Tomato fruit inoculated with *T. asperellum* extract. **(B)** Tomato fruit inoculated with a spore suspension of *F. oxysporum only*. **(C)** Tomato fruit inoculated with *T. asperellum* extract and *F. oxysporum*. **(D)** The fraction of bioactive secondary metabolites were detected using *a GC–MS* chromatogram obtained from *T. asperellum*.

using confocal laser scanning microscopy (CLSM). This study better understood the biocontrol interaction and mycoparasitic activity (Bloemberg, 2007).

Similarly, our study showed the fungal mobility in the tomato rhizosphere, which stimulates root growth promotion (Bolwerk and Lugtenberg, 2005), as shown in Figures 5I,J, which illustrates the early and robust stage of tomato root colonization. In addition to direct parasitism of plant pathogens, interactions with *Trichoderma* enhance plant fitness in response to biotic and abiotic stresses (Bolwerk et al., 2005; Hermosa et al., 2012). All microbes generally hunt for food, of which the root supplies a substantial amount of exudate. Visualization of plant-microbe and microbe-microbe interactions during the plant developmental stages is essential to understanding the underlying mechanisms behind the root exudates/signaling and microbial colonization *via* tracking microbial mobility. In addition, it explores the various mechanisms of biological control by increasing tolerance to abiotic stress and stimulating defense strategies against fungal pathogens (Hermosa et al., 2012). Tomato seedlings inoculated with *Burkholderia tropica* strain MTo-293 exhibited some activities in plant growth promotion and biological control under greenhouse conditions (Bernabeu et al., 2015). They studied the performance of the microbial colonization of different vegetal tissues. A later confocal microscopy study also revealed that *T. harzianum* could colonize the epidermis and cortex of tomato roots (Chacón et al., 2007). In a recent study, *T. harzianum* T-78 enhanced the resistance of tomato plants against the root-knot nematode Meloidogyne incognita through priming for salicylic acid (SA)-and jasmonic acid (JA)-regulated defenses (Martínez-Medina et al., 2017). Root colonization by *Trichoderma* requires a complex molecular dialog between the fungus and plant. This colonization is limited to the outermost layers of the root and does not penetrate the plant vascular bundle, which increases the resilience of abiotic stresses (i.e., salinity and drought). In addition, improving the capacity to absorb nutrients and actively stimulates plant growth (Contreras-Cornejo et al., 2021). *T. atroviride* AN35 and *T. cremeum* AN392 colonized the roots of wheat plants. The microscopic imaging showed that the hyphae of the *Trichoderma* grew on the root surface of wheat and corn (*Zea mays*) seedlings (Contreras-Cornejo et al., 2018). This behavior was also observed in *T. atroviride* during the colonization of *Arabidopsis* roots (Salas-Marina et al., 2011). A study by Ruano-Rosa et al. (2016) showed the root colonization process between *T. harzianum* and olive crops.

Trichoderma species are known to produce a wide range of bioactive secondary metabolites that are known to have antifungal, antibacterial, and toxic properties to control a wide range of phytopathogens, such as *Fusarium* species, *Botrytis cinerea*, *Pythium* species, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Ustilago maydis* (Hermosa et al., 2014; Khan et al., 2020). Analysis by GC –MS is essential for the identification of natural compounds of the microbe to explore the underlying mechanisms of the antifungal activity (Siddiquee et al., 2012; Qualhato et al., 2013; Meena et al., 2017). Usually, volatile compounds are identified, such as aromatic compounds, fatty acids, general hydrocarbons, and hydroxy or amino

TABLE 3 Bioactive compounds identified in *T. asperellum* crude extract using GC–MS analysis.

T No.	R.T.	Area %	Name of the compound	Formula	Molecular weight
1	5.46	1.03	Methanol, TBDMS derivative	C7H18OSi	146
2	24.92	0.51	D-(−)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 2)	C21H52O6Si5	540
3	25.18	0.31	D-(−)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 1)	C17H42O5Si4	438
4	26.66	0.23	D-(+)-Galactopyranose, 5TMS derivative (isomer 2)	C21H52O6Si5	540
5	28.67	7.04	D-Glucopyranose, 5TMS derivative	C21H52O6Si5	540
6	29.46	17.05	Palmitic Acid, TMS derivative	C19H40O2Si	328
7	30.28	4.55	Myo-Inositol, 6TMS derivative	C24H60O6Si6	612
8	32.40	2.82	9-Octadecenoic acid, (E)-, TMS derivative	C21H42O2Si	354
9	32.40	2.82	11-cis-octadecenoic acid 1TMS	C21H42O2Si	354
10	32.40	2.82	13-Octadecenoic acid, (E)-, TMS derivative	C21H42O2Si	354
11	32.40	2.82	Oleic Acid, (Z)-, TMS derivative	C21H42O2Si	354
12	32.83	0.82	Octadecanoic acid, trimethylsilyl ester	C21H44O2Si	356
13	32.83	0.82	10,12-Docosadiynedioic acid, 2TMS derivative	C28H50O4Si2	506
14	32.83	0.82	Stearic acid, TMS derivative	C21H44O2Si	356
15	32.83	0.82	5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester	C26H42O2Si	414
16	37.28	1.35	4H-1-Benzopyran-4-one,2-(3,4-Dihydroxyohenyl)-6,8-DI-á-D-Glucopyranosyl-5, 7-Dihydroxy	C27H30O16	610
17	37.28	1.35	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-{[(trimethylsilyl)oxy] methyl}ethyl ester, (Z,Z,Z)-	C27H52O4Si2	496
18	37.91	32.15	1,2-Benzenedicarboxylic acid	C24H38O4	390

R.T, Retention time.

compound metabolites (Siddiquee et al., 2012). In the present study, 18 bioactive compounds were detected in the crude extract of *T. asperellum* by GC–MS analysis. The most commonly identified compounds were fatty acids and their derivatives, esters such as 1,2-Benzenedicarboxylic acid, Palmitic acids, Oleic acid, 9-Octadecenoic acid (E), methyl ester, and 9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z), sugars (D-Glucopyranose) and sugar alcohol (Myo-Inositol). It was investigated that sugars are essential to fuel the energy required for defenses and serve as signals for regulating defense genes in plant-microbe interactions (Roitsch et al., 2003; Bolton, 2009). Myo-inositol plays essential roles in stress responses, development, and many other processes (Michell, 2008; Valluru and Van den Ende, 2011). Moreover, the volatile metabolites play essential roles in mycoparasitic interactions between *Trichoderma* and plants (Altieri et al., 2009) 1,2 Benzenedicarboxylic acid has antifungal activity (Chen et al., 2020). Liu et al. (2008) emphasized the antifungal activity of palmitic acid against four economically important phytopathogenic fungi (*Alternaria solani*, *Colletotrichum lagenarium*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *F. oxysporum* f. sp. *lycopersici*). Walters et al. (2004) examined the antifungal ability of oleic acid against plant pathogenic fungi. They verified that a 1,000 μM could reduce the mycelial growth of all tested fungi (*Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avenae*, and *C. perniciosa*). The primary mechanism of the antifungal action of fatty acids states that fatty acids insert themselves into the lipid bilayers of fungal membranes compromising membrane integrity, resulting in an uncontrolled release of intracellular electrolytes and proteins, eventually leading to cytoplasmatic disintegration of fungal cells (Avis and Bélanger, 2001). Thus, these fatty acids play a vital role in the extract of *T. asperellum* by controlling the growth of *F. oxysporum*. Therefore, it protects tomato plants from *Fusarium* diseases.

Surprisingly, the mycotoxins naturally produced by *Fusarium* species (FB1, FB2, ZEN, and DON) were not detected by LC–MS analysis, even in the untreated controls. Our results agree with Stracquadanio et al. (2021), who showed that *F. graminearum* had no mycotoxin production in tomato fruits. Likewise, in the studies by Lori et al. (2003) and Haidukowski et al. (2005) on the same strain, *F. graminearum* ITEM 126 showed mycotoxin production in contaminated wheat kernels. This could be because this strain does not produce mycotoxins when infecting tomatoes but only in small grain cereals and maize (Munkvold, 2017).

Conclusion

Tomato is a global economically essential vegetable crop. However, FOL is the causal agent of the FWD of tomato crops. Biological control offers a promising eco-friendly method to manage this disease. The *Trichoderma* strain is successfully used as a bio-control agent because it stimulates the plant immune system against pathogen attacks. Its growth-promoting ability in soil provides an additional benefit in the agricultural application of fertilizers and antifungal activity. Furthermore, a combination of the *in vitro* and *planta* experiments will improve the role of this fungus on plant performance. In addition, the fungus successfully promotes plant growth in controlled conditions. The plant growth-promoting traits and biocontrol efficiency of the *Trichoderma* strain were also evaluated based on the recurring role of *T. asperllum* as a biocontrol

agent against various fungal pathogens. *T. asperellum* displayed the highest antagonistic activity against *F. oxysprum* at 53.24%, and 30% free cell filtrate inhibited *F. oxysporum* at 59.39% as well. This study provides vital insights into the plant-microbe interaction and the microbe-microbe interactions, including diverse antagonistic mechanisms. For instance, chitinase activity, IAA, and P solubility were observed *in vitro* assays, indicating *Trichoderma's* capability to control the fungal pathogen and enhance plant growth. Additionally, using confocal microscopy, tomato root colonization was visualized to understand the mobility of the *Trichoderma* strain in the host plant. These data suggest microbial application in seed coating or foliar spraying, which may improve food safety by applying beneficial microbes. In sum, improving the efficacy and development of biocontrol agents to help small farmers will increase their crop productivity in an accessible and economical way. Thereby positively impacting farmers' profits and sustaining the food safety approaches. In addition, developing sustainable crops that could be grown with little to no pesticides and/or chemical fertilizers reduces costs for farmers in developing areas. Moreover, developing novel and environmentally friendly approaches is essential to reduce the FWD incidence and yield loss in tomato crops. Therefore, more field applications with this *Trichoderma* strain are required. In addition, the transcriptome profiles of the root system following microbial treatment and the influence of endophytic fungi on tomato metabolism.

Data availability statement

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

Author contributions

AS, RA, KA, and OH designed the study. AS, RA, and OH wrote the original manuscript. AS and RA conducted all the in vitro experiments, confocal work, and collected the data. AS, RA, and OH conducted the in planta assays. OH performed the ITS sequencing to confirm fungal genus taxonomy. OH, KA, and MA performed the statistical analysis and edited the manuscript. All authors contributed to the article and approved the submitted version.

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.2023.1140378/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 04 December 2023
DOI 10.3389/fmicb.2023.1149004

OPEN ACCESS

EDITED BY
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RECEIVED 20 January 2023
ACCEPTED 15 November 2023
PUBLISHED 04 December 2023

CITATION
Zahra ST, Tariq M, Abdullah M, Zafar M, Yasmeen T, Shahid MS, Zaki HEM and Ali A (2023) Probing the potential of salinity-tolerant endophytic bacteria to improve the growth of mungbean [*Vigna radiata* (L.) Wilczek].
Front. Microbiol. 14:1149004.
doi: 10.3389/fmicb.2023.1149004

Probing the potential of salinity-tolerant endophytic bacteria to improve the growth of mungbean [*Vigna radiata* (L.) Wilczek]

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Soil salinity is one of the major limiting factors in plant growth regulation. Salinity-tolerant endophytic bacteria (STEB) can be used to alleviate the negative effects of salinity and promote plant growth. In this study, thirteen endophytic bacteria were isolated from mungbean roots and tested for NaCl salt-tolerance up to 4%. Six bacterial isolates, TMB2, TMB3, TMB5, TMB6, TMB7 and TMB9, demonstrated the ability to tolerate salt. Plant growth-promoting properties such as phosphate solubilization, indole-3-acetic acid (IAA) production, nitrogen fixation, zinc solubilization, biofilm formation and hydrolytic enzyme production were tested *in vitro* under saline conditions. Eight bacterial isolates indicated phosphate solubilization potential ranging from 5.8–17.7 μg mL^{-1}, wherein TMB6 was found most efficient. Ten bacterial isolates exhibited IAA production ranging from 0.3–2.1 μg mL^{-1}, where TMB7 indicated the highest potential. All the bacterial isolates except TMB13 exhibited nitrogenase activity. Three isolates, TMB6, TMB7 and TMB9, were able to solubilize zinc on tris-minimal media. All isolates were capable of forming biofilm except TMB12 and TMB13. Only TMB2, TMB6 and TMB7 exhibited cellulase activity, while TMB2 and TMB7 exhibited pectinase production. Based on *in vitro* testing, six efficient STEB were selected and subjected to the further studies. *16S rRNA* gene sequencing of efficient STEB revealed the maximum similarity between TMB2 and *Rhizobium pusense*, TMB3 and *Agrobacterium leguminum*, TMB5 and *Achromobacter denitrificans*, TMB6 and *Pseudomonas extremorientalis*, TMB7 and *Bradyrhizobium japonicum* and TMB9 and *Serratia quinivorans*. This is the first international report on the existence of *A. leguminum*, *A. denitrificans*, *P. extremorientalis* and *S. quinivorans* inside the roots of mungbean. Under controlled-conditions, inoculation of *P. extremorientalis* TMB6, *B. japonicum* TMB7 and *S. quinivorans* TMB9 exhibited maximum potential to increase plant growth parameters; specifically plant dry weight was increased by up to 52%, 61% and 45%, respectively. Inoculation of *B. japonicum* TMB7 displayed the highest potential to increase plant proline, glycine betaine and total soluble proteins contents by 77%, 78% and 64%, respectively, compared to control under saline conditions. It is suggested that the efficient STEB could be used as biofertilizers for mungbean crop productivity under saline conditions after field-testing.

KEYWORDS

mungbean, biofertilizer, salt-tolerance, endophytic bacteria, *Bradyrhizobium japonicum*

Introduction

Mungbean [*Vigna radiata* (L.) Wilczek] is a highly nutritious food and considered as the most important pulse crop worldwide. It is preferred in our daily diet due to the presence of sulfur comprising amino acids and high phosphorus content. The global mungbean cultivated area is approximately 7.3 million hectares with an average yield of 721 kg ha^{-1} (Nair and Schreinemachers, 2020). This crop has a strategic position in Asian countries for its nutritional security, being rich in carbohydrates, proteins, vitamins and minerals. In Pakistan, the total area under cultivation of mungbean is approximately 302,000 hectare with a production of 264,000 tonne (Javed et al., 2021; Economic Survey of Pakistan, 2021–2022). Mungbean seeds are highly nutritious, containing 59–65% carbohydrates, 24–28% proteins, 3.5–4.5% fibers, 1–1.5% fats and 334–344 kcal energy (Sehrawat et al., 2021). Mungbean is used as a staple food in different Asian countries including Pakistan, Thailand, India and the Philippines (Delic et al., 2009).

The production of legume grains retards due to numerous abiotic stresses, particularly salt stress, which impairs the activity of symbiotic bacteria and reduces the plant growth (Pataczek et al., 2018; Kartik et al., 2021). Salinity negatively impacts plant physiological activities by plant dehydration, disrupting ionic and osmotic balance, which ultimately causes plant death (Shahzad et al., 2017; Majeed and Muhammad, 2019). Mungbean is highly sensitive towards salinity with a threshold level of electrical conductivity (EC) of 1.8 dS m^{-1} (Pataczek et al., 2018). Plants adopt different strategies such as antioxidant synthesis, osmosensing and maintaining the ionic-homeostasis to cope with salt stress (Chauhan et al., 2022). Ecofriendly salt-tolerant plant growth-promoting bacteria (PGPB) are promiscuous to improve these mechanisms of plants to tolerate salinity.

The agriculture sector largely relies on the synthetic fertilizers, specifically urea and diammonium phosphate (Economic Survey of Pakistan, 2021–2022). Chemical fertilizers are made up of salts of nitrate, ammonium, phosphorus, and potassium, as well as a variety of heavy metals and regular nucleosides (Sabry, 2015). Chemical fertilizer use has increased dramatically in recent years. Careless use of chemical fertilizer results in the accumulation of heavy metals in plant structures, which then infiltrate our food chain (Savci, 2012; Alsafran et al., 2022). It can pollute our environment by contaminating water, soil and air, which entails huge environmental costs and pose serious threats to human health. Extensive use of chemical fertilizers has distorted the nitrogen cycle and other biological processes; prompting global concerns about increased emission of nitrogen oxides, soil acidification and water eutrophication (Fox et al., 2007; Conway and Pretty, 2013; Singh et al., 2019). Widespread application of fertilizers, urbanization, large scale farming and improper farming practices are some of the major causes of soil salinity. The soil salinization is increasing day by day and contaminates agricultural land (Upadhyay and Chauhan, 2022). Alternative methods are required to meet the food demand in a sustainable manner.

Biofertilizers are environment-friendly alternatives to chemical fertilizers. Biofertilizers contain PGPB, which can be applied to the soil or seed surfaces to promote plant growth by improving nutrient availability to plants and controlling phytopathogens (Agri et al., 2022; Valle-Romero et al., 2023). Biofertilizers are host specific, so the nutrients provided by them are less prone to leaching and volatilization, making them ideal for sustainable agriculture (Bhardwaj et al., 2014; Simarmata et al., 2016; Imran et al., 2021). PGPB improve plant growth directly by a variety of mechanisms, primarily including nitrogen fixation, phosphate solubilization and phytohormone production; and indirectly by bioantagonism and inducing systemic resistance (Upadhyay et al., 2022). These beneficial bacteria are mostly present in the plant rhizosphere, root interior and inside nodules. Efficiency of biofertilizers reduces due to the salt stress, as salinity impairs the bacterial cell metabolism and reduces the production of plant growth-promoting substances (Deshwal and Kumar, 2013). The high salt concentration adversely affects the important processes such as decomposition, nitrification, denitrification, soil biodiversity and microbial activity (Kumawat et al., 2022). Salt-tolerant PGPB produce phytostimulants, plant defense-related enzymes including catalases, superoxide dismutases, peroxidases and glucanases, upregulate the expression of Na^+/K^+ ion channel proteins, which helps to maintain ionic homeostasis and increase plant growth (Chauhan and Upadhyay, 2023; Singh et al., 2023).

Endophytic bacteria have magnanimous potential to promote plant growth, since they live in the closer proximity or inside the plant (Chanway et al., 2000; Dalal and Kulkarni, 2013; Afzal et al., 2019). They are better protected from the challenging environment as they invade plant roots and reside in the root cortical region (Compant et al., 2005; Ryan et al., 2008; Zhang et al., 2020). Endophytic bacteria have a better ability to symbiotically associate with their host plants compared to rhizospheric bacteria (Bacilio-Jiménez et al., 2003). Endophytes regulate plant defense mechanisms by producing antioxidants and to mitigate the oxidative damage caused by salt stress and help plants to tolerate the stress (Jhuma et al., 2021; Chaudhary et al., 2022; Kamran et al., 2022). Moreover, they upregulate the expression of SOS1 Na^+/ K^+ antiporter which control the Na^+ and K^+ efflux to maintain ionic-homeostasis inside the plant cell (Tyagi et al., 2022). Several endophytic bacteria well-known for improving plant growth are *Burkholderia, Herbaspirillum, Pantoea, Gluconobacter, Klebsiella, Rahnella, Pseudomonas, Bacillus, Xanthomonas, Stenotrophomonas, Variovorax* (Riggs et al., 2001; Rosenblueth and Martínez-Romero, 2006; Doty et al., 2009; Rat et al., 2021).

In this study, salinity-tolerant endophytic bacteria (STEB) were isolated from the mungbean root and characterized *in vitro* for the plant growth-promoting properties under saline conditions. Potential bacteria from the *in vitro* testing were phylogenetically identified by *16S rRNA* gene sequence analysis and evaluated under controlled-conditions for plant growth-promoting properties under saline conditions.

Materials and methods

Sample collection and isolation of endophytic bacteria

A 8-week-old Mungbean [*Vigna radiata* (L.) Wilczek] plants were collected from the cultivation site of Government College University Faisalabad, Pakistan (GPS coordinates at 31°23′42.5″ N and 73°01′45.5″ E). Intact roots were washed with water, and surface sterilized by dipping in 5% bleach for 2 min and 70% ethanol for 30 s. Roots were washed with sterilized water to remove the effect of chemicals. One-gram roots were separated from the plants using sterilized forceps and crushed in a sterilized mortar pestle within 3 mL saline solution (0.85% NaCl). Each root suspension was serially diluted up to 10^{-5} dilution. An aliquot of 100 µL from each dilution was spread on yeast extract mannitol (YEM) plates and incubated at 28 ± 2°C for 48 h (Shahid et al., 2015; Tsegaye et al., 2019). Bacterial colonies showing different morphology were selected and purified by sub-culturing (Adamu-Governor et al., 2018). Size and shape of bacterial cells were observed under light microscope. Gram's reaction was also performed according to Wang et al. (2017).

Screening of salt-tolerant endophytic bacteria

Salt-tolerance ability of isolated bacteria was evaluated according to Verma et al. (2020), at varying levels of NaCl concentrations. YEM broth (20 mL) in a 50 mL flask was prepared containing different concentrations of NaCl, i.e., 0.5, 0.1, 1.5, 2, 3 and 4% (w/v). Bacterial culture (0.1 mL) was inoculated in each flask and incubated at 28 ± 2°C for 42 h. Bacterial culture without salt was used as control. Optical density (OD) of bacterial growth was recorded after every 6 h at 600 nm using spectrophotometer (Patil et al., 2014).

Phosphate solubilization

Screening of phosphate solubilizing bacteria was performed according to Oo et al. (2020) with some modifications. A single colony of bacterial isolate was spotted on Pikovskaya's agar plate supplemented with 2% NaCl (w/v) and incubated at 28 ± 2°C for 7 days. Halo zone formation was observed around colonies to identify phosphate solubilization potential (Linu et al., 2019; Nacoon et al., 2020). Phosphate solubilization was quantified by the Phospho-molybdate blue color method according to Khan et al. (2022). Bacterial cultures were grown in Pikovskaya's broth supplemented with 2% NaCl (w/v) and incubated at 28 ± 2°C for 7 days. After incubation, bacterial cultures were centrifuged for 10 min at 13,000 rpm and 1 mL of supernatant was mixed with 0.2 mL Phospho-molybdate regent, blue color production was observed, and absorbance was recorded at 882 nm using spectrophotometer. A phosphate standards curve was prepared to quantify phosphate concentration of samples (Behera et al., 2017).

Indole-3-acetic acid production

Indole-3-acetic acid (IAA) production of bacterial isolates was determined by Salkowski's calorimetric assay. Bacterial cultures were grown in YEM broth, supplemented with L-tryptophan (100 µg mL^{-1}) and 2% NaCl (w/v), incubated at 28 ± 2°C for 48 h and centrifuged at 12,000 rpm for 10 min. Salkowski's reagent (4 mL) was mixed with 1 mL of supernatant, gently mixed and incubated for 30 min at room temperature (Bhattacharyya et al., 2020). Pink coloration was taken as indication of IAA production and its absorbance was measured at 530 nm using spectrophotometer. An IAA standards curve was prepared to quantify IAA concentration of samples (Myo et al., 2019; Hyder et al., 2020).

Nitrogen fixation

The ability of bacterial isolates to fix nitrogen was tested by inoculating a single colony on solid nitrogen free media [containing (g L^{-1}) mannitol 20 g, K_2HPO_4 0.2 g, NaCl 0.2 g, $MgSO_4$ 0.2 g, K_2SO_4 0.1 g, $CaCO_3$ 5.0 g, agar 20 g] supplemented with 2% NaCl (w/v) and incubated at 28 ± 2°C for 48 h. After incubation, nitrogen fixation was determined based on the bacterial growth and recorded as arbitrary values weak (+), moderate (++), strong (+++) or negative (−) (Hardarson and Danso, 1993; Mirza and Rodrigues, 2012).

Zinc mobilization

In vitro qualitative screening of zinc solubilizing bacterial isolates was measured by adopting the protocol of Ramesh et al. (2014), with some modifications. Tris-minimal agar (TMA) medium supplemented with 2% NaCl (w/v), containing insoluble 0.1% zinc source, i.e., ZnO and $ZnCO_3$, separately. Supplemented TMA plates were spot inoculated with freshly grown bacterial cultures and incubated in the dark at 28 ± 2°C for 7 days. Halo zone formation around the bacterial colony was observed and zinc solubilization efficiency (ZSE) was calculated according to the formula (Rezaeiniko et al., 2022; Upadhayay et al., 2022).

$$\mathrm{ZSE} = \begin{pmatrix} \text{diameter of solubilization halo} / \\ \text{diameter of the colony} \end{pmatrix} \times 100.$$

Cellulase and pectinase activity assay

Cellulolytic activity of bacterial isolates was assessed by spot inoculating individual colonies onto carboxymethyl cellulose (10 g L^{-1}) agar plates supplemented with 2% NaCl (w/v) and incubated at 28 ± 2°C for 3 days (Islam and Roy, 2018). Plates were stained with 0.2% Congo red dye for 15 min and washed with distilled water. The appearance of a halo zone around the colony indicates cellulase activity of bacteria (Suárez-Moreno et al., 2019).

Pectinase production was determined by inoculating bacterial colonies onto pectin (10 g L^{-1}) agar plates, supplemented with 2% NaCl (w/v) and incubated at 28 ± 2°C for 7 days (Devi et al., 2022). Plates were stained with 1% iodine solution for 15 min and washed with distilled water. The formation of a halo zone around the colony indicated pectinase activity of bacterial cultures (Tsegaye et al., 2019).

Biofilm formation assay

Biofilm formation by bacterial isolates was tested according to Zahra et al. (2023), by using a microtiter plate. Bacterial isolates were grown up to an optical density ($OD_{600\ nm}$) of 2 in YEM broth medium, supplemented with 2% NaCl (w/v). Bacterial cultures were centrifuged at 6,000 rpm for 2 min. The supernatant was discarded and the pellet was washed with sterile water. The bacterial cells were resuspended in fresh YEM broth and diluted to an $OD_{600\ nm}$ of 0.2. An aliquot (150 µL) of each bacterial cell suspension was added to 96-well polyvinyl chloride (PVC) plate in six replicates and incubated at 28°C for 48 h. After incubation, each bacterial culture was removed from wells and gently washed with sterilized water. Wells were stained with 150 µL of crystal violet (0.001%) for 15 min. After staining, crystal violet was removed, and wells were washed with sterilized water and air dried. Crystal violet dye absorbed by the wells was solubilized by adding 150 µL of 95% ethanol. Biofilm formation was quantified by measuring the amount of absorbed dye at $OD_{570\ nm}$ in a microtiter plate reader (Sampedro et al., 2020; Jhuma et al., 2021).

Microbial compatibility assay

Microbial compatibility tests of bacterial isolates were assessed according to Tariq et al. (2014) by pour plate technique to determine the compatibility of isolates with each other. In this assay, a pairs of bacterial isolates was designated as A and B, and checked for compatibility. Log phase grown culture of isolate A was diluted to 10^4 cfu mL^{-1} and 3 mL of the culture was mixed in 25 mL hand-cool molten nutrient agar medium. It was poured into Petri plates and incubated at 28 ± 2°C for 24 h. Concentrated culture (3 µL) of isolate B was inoculated in the center of the plate and incubated at 28 ± 2°C for 48 h. The zone of inhibition that developed around bacterial isolate B was recorded. The appearance of a zone of inhibition represented that the bacterial pair was not compatible with each other, represented by a red box. If no zone of inhibition appeared, the bacterial pair was considered compatible and represented by a green box. Each isolate pair was tested in this manner, and compatibility and non-compatibility was presented as green and red box, respectively (Zul et al., 2022).

Molecular identification and phylogenetic analysis of efficient endophytic bacteria

Six efficient STEB were identified phylogenetically by sequencing the *16S rRNA* gene, according to La Pierre et al. (2017). The *16S rRNA* gene was amplified using universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg et al., 1991). A 25 µL reaction mixture was prepared for *16S rRNA* gene amplification by using the PCR recipe [10X Taq polymerase buffer 2.5 µL, 2 mM dNTPs 2.5 µL, (10 pmoles 100 μL^{-1}) primers fD1 & rD1 2 µL, 25 mM MgCl 2 µL, (5 U µL) Taq polymerase enzyme 0.3 µL, H_2O 11.7 µL, (20 ng μL^{-1}) template DNA 2 µL]. The reaction mixture was placed in a thermocycler for amplification and adjusted initial denaturation to 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 60 s, primer annealing at 55°C for 50 s, primer extension at 72°C for 1 min 40 s and final extension at 72°C for 5 min. After amplification, the amplicons were examined in a gel documentation system on 1% agarose gel. The amplified products were purified through ThermoScientific GeneJET PCR Purification Kit and Sanger sequenced using the commercial service of Macrogen, Korea. Forward and reverse sequences were assembled manually and compared with database sequences by using NCBI BLAST tool (Altschul, 1990). Closely related authentic sequences were retrieved from databases, and pairwise sequence comparisons were performed using Sequence Demarcation Tool (SDT) v.1.2 (Zhang et al., 2000; Muhire et al., 2014). A phylogenetic tree was constructed using the maximum likelihood method as implemented by MEGA 11 with 1,000 bootstrap values (Kumar et al., 2016; Noori et al., 2021).

Controlled-conditions experiment and biochemical analysis

The controlled-condition experiment was conducted on mungbean cultivar NM-2021 with eight treatments (TMB2, TMB3, TMB5, TMB6, TMB7, TMB9, consortia and water as control) in completely randomized design (CRD) with four replicates. Freshly grown bacterial culture was centrifuged (6,000 rpm) and bacterial pellet was resuspended in sterilized water adjust OD 0.5 (Mishra et al., 2009). Seeds were surface sterilized with 5% bleach for 2 min and washed with sterilized water. Surface-sterilized seeds were placed on Petri plates containing moist filter paper and incubated at 25 ± 2°C in a dark room for 2 days. Uniformly sized seedlings were transferred into pots containing sterilized soil, supplemented with 1% NaCl. Plants were placed in a growth chamber at 35 ± 2°C during the day and 25 ± 2°C at night. Bacterial culture (100 µL) of each treatment was applied to the roots of each plant. Plants were watered with 10 mL of quarter-strength nitrogen-free Hoagland's solution and sterilized water on alternating days. Plants were harvested after 6 weeks of germination and agronomical parameters including root length, shoot length, plant fresh weight, plant dry weight and number of nodules per plant were recorded (Tounsi-Hammami et al., 2022). The agronomical data was statistically analyzed using CoStat window version software (Cardinali and Nason, 2013).

Proline contents

Proline contents were determined according to Bates et al. (1973) with some modifications. Leaf samples (0.5 g) were ground in liquid nitrogen and 10 mL chilled K-P buffer was added. The mixture was centrifuged at 13,000 rpm for 5 min. Supernatant (0.5 mL) was transferred in a test tube containing 1 mL of 3% sulphosalicylic acid and incubated at 95°C for 5 min in a water bath. After incubation, the mixture was cooled down at room temperature and 1 mL of glacial acetic acid and ninhydrin was gently added, mixed and incubated at 95°C for 20 min in a water bath. The mixture was immediately cooled down on ice. Toluene (2 mL) was added in the mixture, vortexed and incubated at room temperature for 20 min. After incubation, two layers were developed. The upper layer was carefully collected and absorbance was recorded at 520 nm using spectrophotometer. The proline contents were measured by comparing the absorbance with standard curve (Sapre et al., 2022).

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TABLE 1 Colony and cell morphology of mungbean root endophytic bacteria.

Isolate	Colony morphology	Cell morphology	Gum production	Gram staining
TMB1	Medium, double ringed, light pink, smooth, flat	Small rod	+	+
TMB2	Large, circular, milky white, smooth, flat	Rod	+	–
TMB3	Very small, circular, white, smooth, flat	Rod	+	–
TMB4	Very small, circular, pink, wavy, flat	Circular	–	+
TMB5	Small, circular, light yellowish, smooth, flat	Small rod	+	–
TMB6	Large, irregular, yellowish, wavy, flat	Rod	+	–
TMB7	Small, circular, light pink, smooth, flat	Small rod	+	–
TMB8	Medium, circular, milky white, wavy, flat	Circular	+	+
TMB9	Small, circular, pink, wavy, flat	Rod	–	–
TMB10	Small, irregular, white, wavy, flat	Circular	+	–
TMB11	Small, circular, milky white, smooth, flat	Rod	–	–
TMB12	Very small, circular, white, smooth, flat	Small rod	–	+
TMB13	Very small, circular, yellowish white, smooth, flat	Rod	+	–

Total soluble proteins

Total soluble proteins were quantified according to Bradford (1976) modified method. Leaf samples (0.5 g) were ground in chilled K-P buffer. After grinding, the mixture was centrifuged at 13,000 rpm for 5 min. Supernatant (0.1 mL) was collected, Bradford reagent (1 mL) was added and incubated at room temperature for 30 min in dark. After incubation, absorbance was recorded at 595 nm using spectrophotometer. Total soluble proteins were measured by comparing the absorbance with standard curve.

Glycine betaine

Glycine betaine in leaf tissues was estimated by following the modified protocol of Nawaz and Wang (2020). Fresh leaf samples (0.5 g) were ground in 10 mL chilled K-P buffer, vortexed and centrifuged at 13,000 rpm for 5 min. Supernatant (0.5 mL) was collected in a separate test tube and 1 mL of H_2SO_4 was added. KI_3 (0.2 mL) was added into the reaction mixture and incubated at −4°C for 90 min. After incubation, 2.8 mL chilled dH_2O and 6 mL of 1–2 dichloroethane was added into the mixture and incubated at room temperature for 30 min. Two layers were formed. The lower layer of red color was collected carefully, and absorbance was measured at 365 nm using a spectrophotometer. Quantify of glycine betaine contents was measured by comparing the absorbance with the standard curve.

Results and discussion

Isolation of endophytic bacteria

Bacterial colonies were observed on the plates after incubation. Based on colony size, shape, color, edges, surface and gum production, thirteen bacterial morphotypes were selected. Cell morphology of all bacterial isolates was rod shaped, except TMB4, TMB8 and TMB10, which showed circular cell shape. Only four bacterial isolates, TMB1, TMB4, TMB8 and TMB12, were Gram's positive, while the rest of the bacteria were Gram's negative (Table 1). Legume roots contain a large array of endophytic bacteria, which may play an important role in plant growth promotion directly and indirectly (Bhutani et al., 2018a). Our results are in agreement with several studies that confirmed the occurrence of bacteria in the legume root samples. Chaudhary et al. (2021) isolated *Rhizobium pusense* from the roots of mungbean and evaluated its plant growth-promoting properties. Bhutani et al. (2021) also isolated endophytic bacteria from surface sterilized roots of mungbean that demonstrated high potential to improve plant growth. Abedinzadeh et al. (2019) also isolated endophytic bacteria from roots of maize and reported that these bacteria have the ability to tolerate salinity and increase plant growth. Similarly, Hung and Annapurna (2004) also isolated 65 endophytic bacteria from soybean root and nodules.

Screening of salt-tolerant endophytic bacteria

Salinity tolerance was examined in mungbean isolates at different NaCl concentrations ranging from 0.5–4%. There was significant inhibition in bacterial growth at 3 and 4% NaCl concentration. Six bacterial isolates, TMB2, TMB3, TMB5, TMB6, TMB7 and TMB9, were able to tolerate salinity level up to 2% NaCl concentration (Figure 1), whereas three isolates, TMB1, TMB8 and TMB10, showed minor growth inhibition at 2% NaCl. The remaining four isolates, TMB4, TMB11, TMB12 and TMB13, showed significant growth inhibition at 2% NaCl salinity concentration (Supplementary Table S1). Salinity is one of the major problems for crop productivity in Pakistan due to the presence of salt contents in the soil and water (Vaishnav et al., 2019; Kartik et al., 2021). Adaptability of the bacterial inoculants to the stressed environment of any cultivation region is considered as a promiscuous feature for its use as biofertilizers (Pérez-Rodriguez et al., 2020). Recently, Kumar et al. (2021) screened salt tolerant bacteria at different concentrations of NaCl and further characterized them for plant growth promotion. Khan et al. (2015) also isolated rhizospheric and endophytic bacteria, and reported that these bacterial isolates tolerate higher concentrations of NaCl. Salinity affected soil is defined as a soil that has electrical conductivity (EC) value greater than 4 dS m^{-1} (Munns and James, 2003). EC value of 4

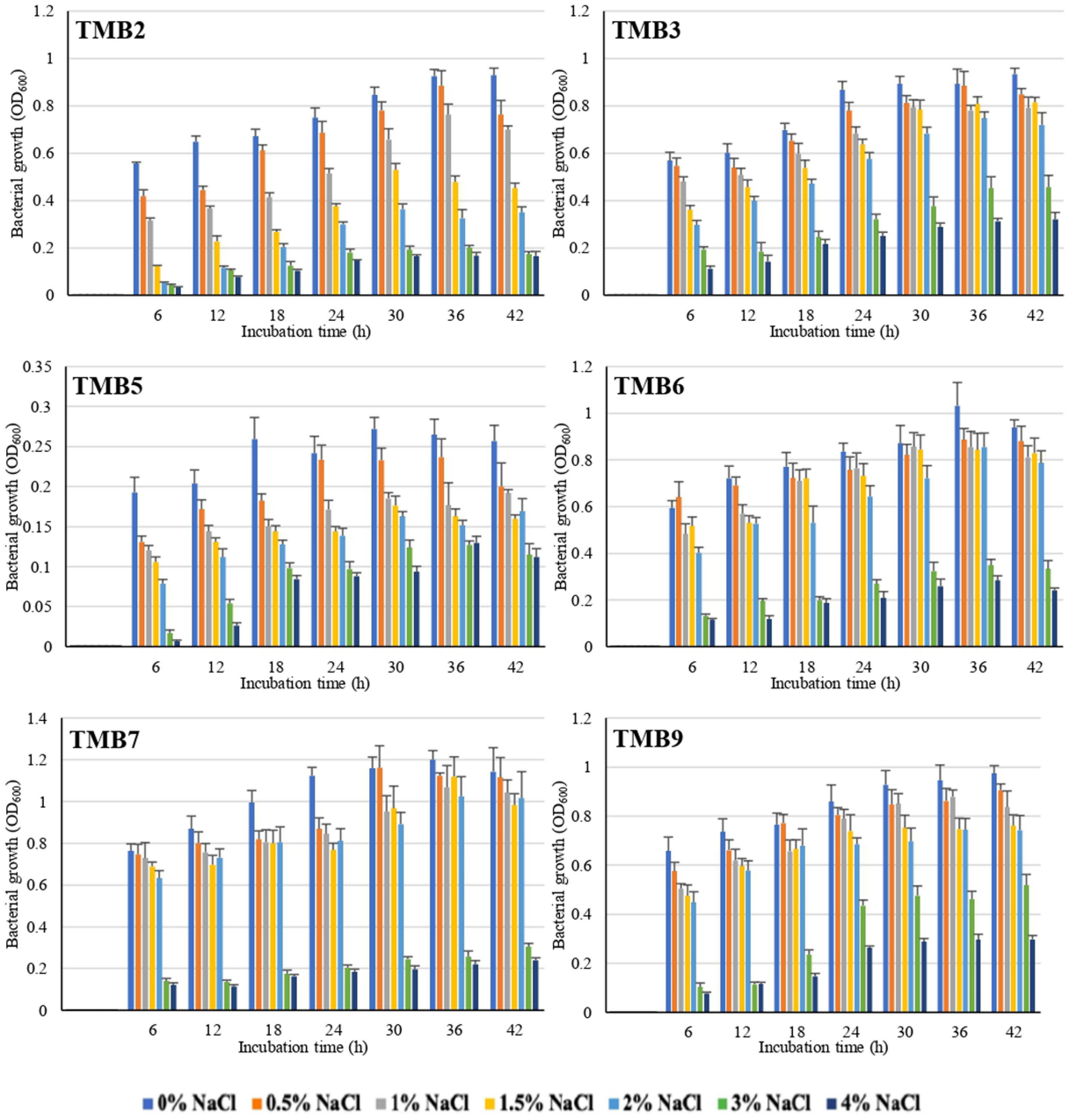

FIGURE 1
Graphical representation of mungbean root endophytic bacteria to tolerate salinity at different salt concentrations. Six bacterial isolates, TMB2, TMB3, TMB5, TMB6, TMB7 and TMB9, displayed salt-tolerance and grew well upto 2% NaCl. Growth readings of all bacterial isolates under saline conditions are mentioned in the Supplementary material.

dS m^{-1} is equal to 0.22% NaCl concentration and EC value of 25.8 dS m^{-1} is equal to 2% NaCl concentration (observation during lab general experiments). Therefore, the potential endophytic bacteria exhibited salt tolerance ability up to 2% NaCl concentration were considered potential candidates for their use as biofertilizers at salinity affected soils. 2% NaCl is the highest realistic-concentration of salt to test microbes for salinity-tolerance, as it is the maximum concentration reported at most of the salinized land worldwide. The world's well-known saline sites including Solonchaks (Russia), Halosols (China) and Salida (United States) have an EC ranging 8–15 dS m^{-1} (Egamberdieva et al., 2019). Soil and irrigation water in Pakistan generally have high soluble salt contents, which is a major limiting factor for plant growth. The value of EC in heavily salt affected soil of Pakistan at Uchhali Lake in the Salt Range region is 15.42 dS m^{-1}, which is equal to 1.2% NaCl (Hameed et al., 2009). A concentration higher than 2% salinity is very stringent to test bacterial salt-tolerance and might result in losing too many potential bacteria. Cortés-Lorenzo et al. (2015) demonstrated that higher levels of salinity inhibited the nitrification process of nitrite-oxidizing bacteria. Hong et al. (2013) also reported that higher salinity can reduce the metabolic activity of microorganisms, results in bacterial growth inhibition and cell death. As the tested bacteria of the current study demonstrated

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TABLE 2 *In vitro* testing of plant growth-promoting attributes of mungbean root endophytic bacteria.

Isolates	Phosphate Solubilization ($\mu g\ mL^{-1}$)	IAA Production ($\mu g\ mL^{-1}$)	Nitrogen fixation	Zinc solubilization efficiency (ZnO)	Zinc solubilization efficiency ($ZnCO_3$)	Cellulase activity index	Pectinase activity index	Biofilm formation (OD_{570nm})
TMB1	0	6.8 ± 0.4	+	0	0	0	0	0.11 ± 0.012
TMB2	8.1 ± 0.44	1.1 ± 0.18	+++	0	0	1.66	1.2	0.53 ± 0.049
TMB3	5.8 ± 0.32	3.3 ± 0.28	++	0	0	0	0	1.59 ± 0.043
TMB4	0	0	+	0	0	0	0	0.63 ± 0.039
TMB5	7.9 ± 0.46	1 ± 0.19	++	0	0	0	0	0.91 ± 0.021
TMB6	17.7 ± 0.62	1.4 ± 0.33	++	260%	200%	1.75	0	2.59 ± 0.041
TMB7	17.23 ± 0.22	12.1 ± 0.51	+++	237%	177%	1.5	1.4	1.83 ± 0.041
TMB8	16.9 ± 0.33	3 ± 0.18	+	0	0	0	0	1.55 ± 0.032
TMB9	13.8 ± 0.68	4.6 ± 0.27	+++	233%	140%	0	0	1.41 ± 0.052
TMB10	11.2 ± 0.6	0.3 ± 0.33	++	0	0	0	0	0.96 ± 0.02
TMB11	0	0	+	0	0	0	0	0.51 ± 0.029
TMB12	0	0	+	0	0	0	0	0
TMB13	0	2.8 ± 0.12	0	0	0	0	0	0

salinity-tolerance upto 2% NaCl concentration, the beneficial characteristics of these bacteria may remain unaffected even in the saline environment. Such bacteria are promising to be used as biofertilizers for crop production at salinity affected soil and the farmland irrigated with saline-water. It is strongly suggested that biofertilizer bacteria should be tested for salt stress tolerances before application, as most of the irrigation water and soils are affected with high concentration of salts.

Characterization of STEB for plant growth-promoting properties

Phosphate solubilization was examined in mungbean isolates under saline conditions. Out of 13 mungbean isolates, TMB2, TMB3, TMB5, TMB6, TMB7, TMB8, TMB9 and TMB10, showed phosphate solubilization ranging from 5.8–17.7 $\mu g\ mL^{-1}$. TMB6 exhibited the highest phosphate solubilization ability, whereas TMB3 exhibited the lowest phosphate solubilization ability (Table 2). Phosphate is one of the most crucial nutrients for balanced plant growth. Deficiency of phosphate in plants usually results in stunted growth of plants (Lun et al., 2018). Previously, Hakim et al. (2020) isolated endophytic bacteria from mungbean and explained the phosphate solubilizing potential of these bacteria upto 195 $\mu g\ mL^{-1}$. Recently, Belkebla et al. (2022) demonstrated that halotolerant PGPB isolated from south of Algeria exhibit phosphate solubilizing potential and improve wheat growth. Mahdi et al. (2021) also reported that halotolerant endophytic bacteria have the ability to solubilize phosphate and promote seed germination. Likewise, Mei et al. (2021) also demonstrated that endophytic bacteria have the potential to solubilize phosphate and their application resulted in increased pepper and tomato growth.

IAA was quantified by spectrophotometric pink coloration estimation method. Ten isolates, TMB1, TMB2, TMB3, TMB5, TMB6, TMB7, TMB8, TMB9, TMB10 and TMB13, showed IAA production ranging from 0.3–12.1 $\mu g\ mL^{-1}$ at 2% NaCl supplementation. TMB7 showed the highest production of IAA, whereas TMB10 showed the lowest production of IAA (Table 2). IAA is a phytohormone also produced by many bacteria, which is involved in cell division, cell enlargement and root elongation (Bhutani et al., 2018b). Our results are in agreement with Widowati and Sukiman (2019), who reported IAA production up to 12.28 $\mu g\ mL^{-1}$ in the endophytic bacteria of mungbean. Saleem et al. (2021) also demonstrated the mitigating efficiency of IAA production from salt tolerant bacteria isolated from cotton. Jabborova et al. (2020) also reported that endophytic bacteria have ability to produce IAA. Recently, Desai et al. (2023) explained the ability of salt-tolerant PGPB isolated from mungbean to produce IAA under salt stress. IAA production is one of the very important features for the screening of plant beneficial bacteria.

Nitrogen fixation ability of mungbean root endophytic bacteria was tested by growing bacteria on NFM agar plates. All isolates showed nitrogen fixation ability except TMB13 under saline conditions. TMB2, TMB7 and TMB9 showed highest ability (Table 2). Nitrogen is the most important element for plant growth and development. Bacteria produce nitrogenase enzyme to fix the atmospheric nitrogen (Gu et al., 2018). Favero et al. (2021a) isolated the nodule endophytic bacteria from the mungbean, which showed nodule formation and nitrogen fixation capability. *Bradyrhizobium* sp. exhibited the maximum potential for nodulation and nitrogen fixation. Tang et al. (2020) also reported that endophytic bacteria have the potential to fix biological nitrogen in tropical forest soil. Zhang et al. (2022) also identified that endophytic bacteria isolated from cassava roots exhibit nitrogen fixation ability. Potential root-associated bacteria can fix atmospheric nitrogen and alleviate nutrient stress in plants.

Zinc is an essential micronutrient involved in several cellular processes including metabolism, mitochondrial activity mitosis and cell development. It mainly participates in the redox reactions and works as a catalyst for enzymes (Ditta et al., 2022). Out of 13 root endophytic bacteria of mungbean, only three isolates, TMB6, TMB7 and TMB9, were able to solubilize zinc on tris minimal media

supplemented with zinc oxide and zinc carbonate under saline conditions. TMB6 showed higher solubilization efficiency of 260% in zinc oxide and 200% in zinc carbonate media (Table 2). Previously, Singh et al. (2020) explained the zinc solubilizing ability of *Burkholderia arboris* and demonstrated the positive role of its inoculation in mungbean cultivation. Zinc solubilizing potential of bacteria was reported by several studies, which play crucial roles in soil fertility (Rani et al., 2022; Verma et al., 2022). Similarly, Ali et al. (2022) also demonstrated that endophytic bacteria have the potential to solubilize zinc and their combination with synthetic fertilizer significantly increased the plant growth compared to the sole application of chemical fertilizer.

Cellulase and pectinase activity of mungbean rhizobacteria was observed on NaCl supplemented plates. Out of 13 isolates, only TMB2, TMB6 and TMB7 showed cellulase activity, where TMB6 showed highest activity with 1.75 index. Only two isolates, TMB2 and TMB7, exhibited pectinase activity (Table 2). Cellulase and pectinase belongs to the family of hydrolytic enzymes. Hydrolytic enzymes play a pivotal role in the decomposition of dead organic matter present in the soil and provide nutrients to plants (Reetha et al., 2014). Recently, Reddy et al. (2022) reported PGPB have the ability to produce hydrolytic enzymes. Bhutani et al. (2021) also isolated cellulase and pectinase producing bacteria from the mungbean endosphere, which showed plant growth-promoting (PGP) potential. Dogan and Taskin (2021) also demonstrated that endophytic bacteria isolated from *Poaceae* plant displayed cellulase and pectinase production ability. Borah et al. (2019) also reported that endophytic bacteria from tea plant exhibit cellulase and pectinase activity. Cellulase and pectinase might enable bacteria to invade the roots and nodules of host plant. Futuristic comprehensive studies should be designed to explore the role of hydrolytic enzymes in root/nodule invasion and plant growth promotion by developing cellulase and pectinase negative mutants or using other cutting-edge techniques.

Biofilm formation activity was examined in microtiter plate assay. All bacterial isolates except TMB12 and TMB13 showed biofilm formation ranging 0.11–2.59 at OD_{570nm}. TMB6 showed the highest efficiency of biofilm formation, while TMB1 showed the lowest efficiency of biofilm formation (Table 2). Several PGPB can effectively interact with the plants root zone and form biofilm on its surface, which protects plants against environmental stresses (Ansari and Ahmad, 2018). Previously, Yasmeen et al. (2020) isolated halotolerant bacteria from saline soil and demonstrated their biofilm formation ability under salt stress. Alaa (2018) also reported that *Pseudomonas anguilliseptica* have biofilm formation potential under different levels of salts. Generally, efficient biofilm forming bacteria perform their inherent functions effectively, even in the challenging environment (Tariq et al., 2014).

Antibiosis activity of isolates was checked by growing pair of bacteria together in an overlay plate assay. Bacterial isolates, TMB1, TMB2, TMB3, TMB5, TMB6, TMB7, TMB8 and TMB9, displayed maximum compatibility to grow together. TMB7 demonstrated the highest compatibility with all isolates except TMB13 (Figure 2). Kumawat et al. (2021) demonstrated that *Rhizobium* sp. and *Enterococcus mundtii* have growth compatibility. When these bacteria applied in consortia on mungbean the growth parameters of mungbean were increased as compared to single inoculation. Latha et al. (2009) also isolated *P. fluorescens* (Pf1 and Py15) and *B. subtilis* (Bs16) from tomato and demonstrated that all of three bacterial strains are compatible to grow together. Similarly, Ashraf et al. (2019) isolated four plant growth-promoting rhizobacteria (PGPR) isolates from wheat rhizosphere and checked their antimicrobial activity against three bacterial strains *Vibrio cholera*, *Enterobacter aerogenes,* and *Klebsiella pneumoniae.* Only one isolate showed antimicrobial activity against *K. pneumoniae* while others were compatible to each other. Compatible bacteria do not inhibit the growth of each other and perform effectively in consortium to promote plant growth.

Phylogenetic identification of efficient STEB

Amplification of *16S rRNA* gene using fD1 and rD1 primers produced approximately 1,500 bp DNA band as shown in Figure 3. After sequencing and assembling, DNA sequence contigs of more than 1,400 nt were generated. Sequences of *16S rRNA* showed maximum similarity of more than 98% with the different sequences available in nucleotide databases and identified TMB2 as *Rhizobium pusense*, TMB3 as *Agrobacterium leguminum*, TMB5 as *Achromobacter denitrificans*, TMB6 as *Pseudomonas extremorientalis*, TMB7 as *Bradyrhizobium japonicum* and TMB9 as *Serratia quinivorans*. Sequences were deposited in NCBI GenBank under the accession numbers OP935921–OP935926 (Table 3). Phylogenetic tree of these sequences was constructed with 42 authentic sequences belonging to 6 identified genera using maximum likelihood method with 1,000 bootstrap value and *Methanoregula boonei* was used as outgroup. All the sequences were grouped into 3 clades belonging to common ancestor. TMB7 was placed in clade 1, TMB6, TMB5

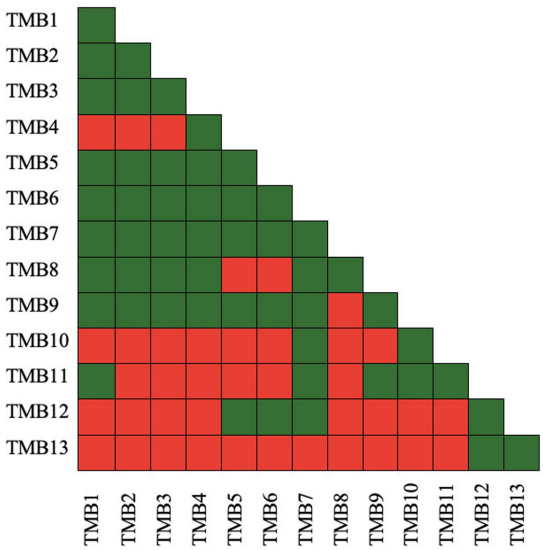

FIGURE 2
Antibiosis activity of mungbean root endophytic bacteria. Green color presents bacterial compatibility to grow together, while red color presents inhibitory interaction between bacteria. TMB7 demonstrated growth compatibility with most of the bacterial isolates, while TMB13 demonstrated growth inhibitory interaction with most of the bacterial isolates.

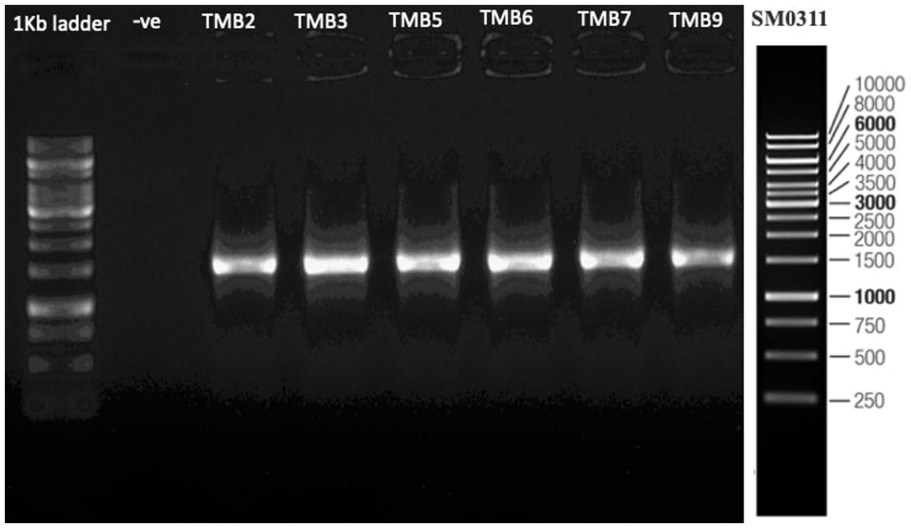

FIGURE 3
16S rRNA gene amplification of potential bacterial isolates. Amplification of DNA bands of 1,500 bp were produced, which were confirmed by comparing with 1 kb DNA ladder.

TABLE 3 Taxonomic identification of potential salinity-tolerant endophytic bacteria.

Bacterial isolates	Taxonomic identification	Percentage identity	Accession numbers
TMB2	*Rhizobium pusense*	98.81%	OP935921
TMB3	*Agrobacterium leguminum*	99.79%	OP935922
TMB5	*Achromobacter denitrificans*	99.17%	OP935923
TMB6	*Pseudomonas extremorientalis*	98.93%	OP935924
TMB7	*Bradyrhizobium japonicum*	100%	OP935925
TMB9	*Serratia quinivorans*	98.30%	OP935926

and TMB9 in clade 2 and TMB2 and TMB3 in clade 3 shown in Figure 4. A color coded pairwise identity matrix was also created, in which each colored cell represents the percentage identity of two sequences. The identity percentages between the selected sequences were ranging 80–100 (Figure 5).

Existence of *Rhizobium pusense* and *Bradyrhizobium japonicum* in mungbean root has been reported in the literature. *Rhizobium pusense* colonize mungbean roots and improve plant growth by producing phytohormones (Chaudhary et al., 2021). Similarly, Nguyen et al. (2022) demonstrated the occurrence of *Rhizobium pusense* in rice and increased its growth and yield upon inoculation. Members of genus *Bradyrhizobium* dominantly exist in the roots and nodules of mungbean and soybean. Favero et al. (2021b) isolated *Bradyrhizobium japonicum* from mungbean nodules and demonstrated the positive effect on yield and growth of mungbean plant. Yasmeen et al. (2012) also explained that occurrence of *Bradyrhizobium japonicum* in mungbean. Similarly, Chhetri et al. (2019) isolated *Bradyrhizobium japonicum* from root nodules of soybean. Generally, bacteria belonging to *Rhizobia* are well-known for nitrogen fixation, which ultimately increases crop yield (Anjum et al., 2006).

In this study, we reported the occurrence of *Agrobacterium leguminum, Achromobacter denitrificans, Pseudomonas extremorientalis* and *Serratia quinivorans* in the roots of mungbean for the first time. Recently, Castellano-Hinojosa et al. (2021) isolated *A. leguminum* from the *Phaseolus vulgaris* nodules and claimed it as a novel species based on the data obtained from colony morphology, sequence analysis, phylogenetic analysis and taxonomic characterization. Previously, Sultana et al. (2020) isolated *A. denitrificans* from the rice plant, which showed PGP properties under salt stress. Wang et al. (2019) isolated *P. extremorientalis* from the rhizosphere of pear plant. Kaur et al. (2022) reported the existence of *P. extremorientalis* in the endophytic region of wheat. *P. extremorientalis* improved plant growth under salt stress by reducing harmful effects of salt (Egamberdieva et al., 2016). Recently, researchers have revealed the existence of *S. quinivorans* in the oak, *Petroselinum crispum* and *Picrorhiza kurroa* (Kumar et al., 2021; Reis et al., 2021; Chlebek et al., 2022). Novel plant-bacterial associations might be due to the changes in environmental conditions.

Biofertilizers potential of STEB under controlled-conditions

Potential isolates including TMB2, TMB3, TMB5, TMB6, TMB7, TMB9 and consortia were tested for PGP properties under

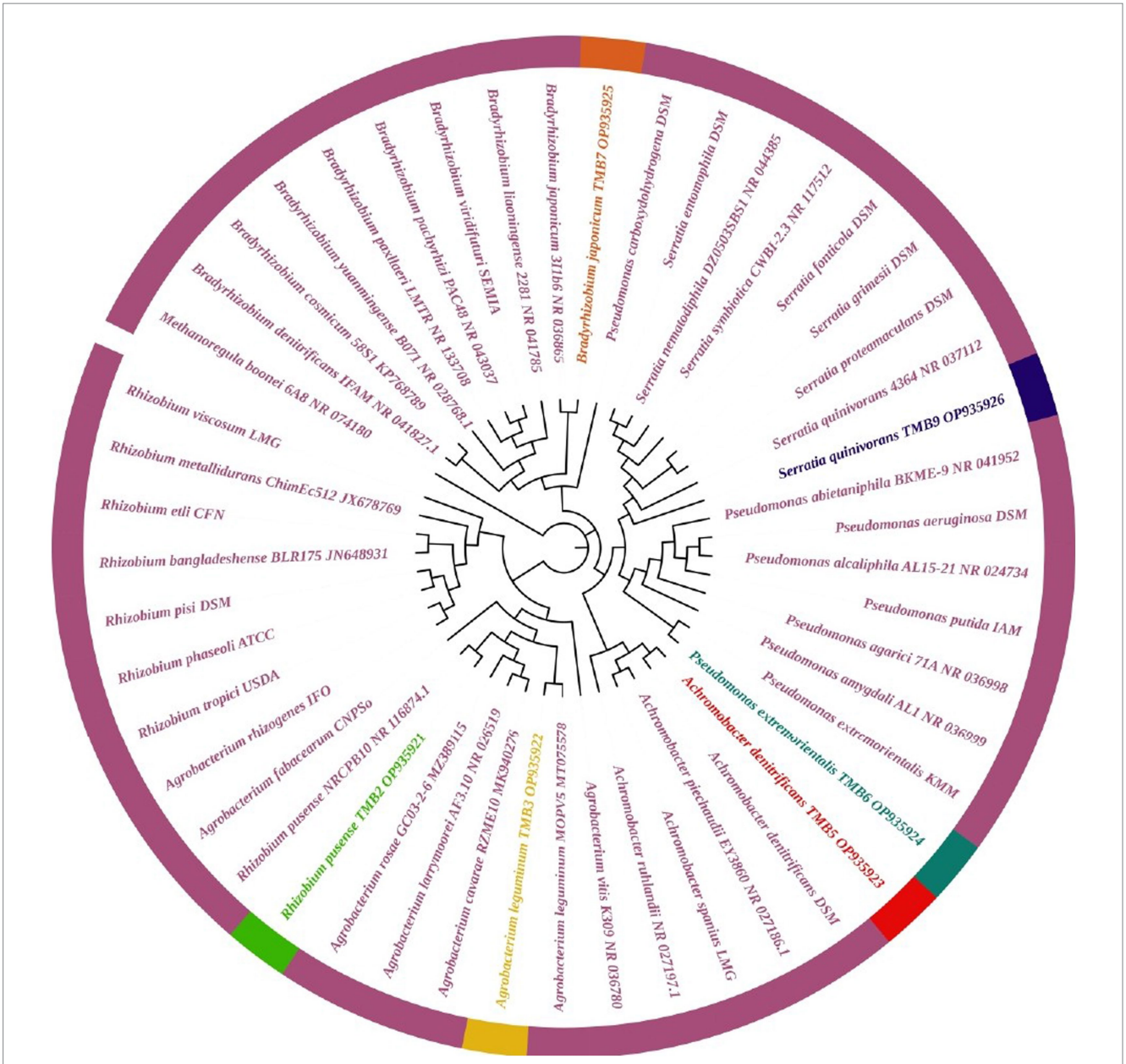

FIGURE 4
Phylogenetic tree of mungbean root endophytic bacteria. All the sequences were grouped into 3 clades. TMB2 positioned in the neighborhood of *Rhizobium pusense*, TMB3 in *Agrobacterium leguminum*, TMB5 in *Achromobacter denitrificans*, TMB6 in *Pseudomonas extremorientalis*, TMB7 in *Bradyrhizobium japonicum* and TMB9 in *Serratia quinivorans*.

controlled-conditions experiment (Supplementary Figure S1). After 6 weeks of inoculation, agronomical parameters were calculated and statistically analyzed (Table 4). Inoculation of bacterial isolates, *P. extremorientalis* TMB6, *B. japonicum* TMB7 and *S. quinivorans* TMB9, showed maximum potential in improving plant growth parameters. TMB2, TMB6 and TMB7 showed a significant increase in root length compared to control. All isolates exhibited a significant increase in shoot length compared to control except consortia. TMB6 and TMB7 showed a significant increase in plant fresh weight. *P. extremorientalis* TMB6, *B. japonicum* TMB7 and *S. quinivorans* TMB9 were most efficient and showed a significant increase in plant dry weight by 52, 61 and 45%, respectively, compared to control. Nodulation was observed by the inoculation of TMB2, TMB7 and consortia. Inoculation of *B. japonicum* TMB7 showed maximum potential to increase plant growth parameters, i.e., root length (59%), shoot length (45%), fresh weight (67%) and dry weight (61%) among all isolates. Consortia did not show any positive effect on plant growth. Biochemical attributes, i.e., proline content, glycine betaine and total soluble proteins were increased by all treatments of root endophytic bacteria under salt stress as shown in Figure 6. Inoculation of TMB7 showed significant potential to increase proline contents by 77%, glycine betaine by 78% and total soluble proteins by 64% compared to control.

PGPB have the ability to enhance plant biochemical attributes such as proline, glycine betaine and total soluble proteins under salt stress to overcome the effects of salinity on plant growth. Proline and glycine betaine play an important role as osmoprotectants and osmoregulatory elements to reduce the harmful effects of salinity by

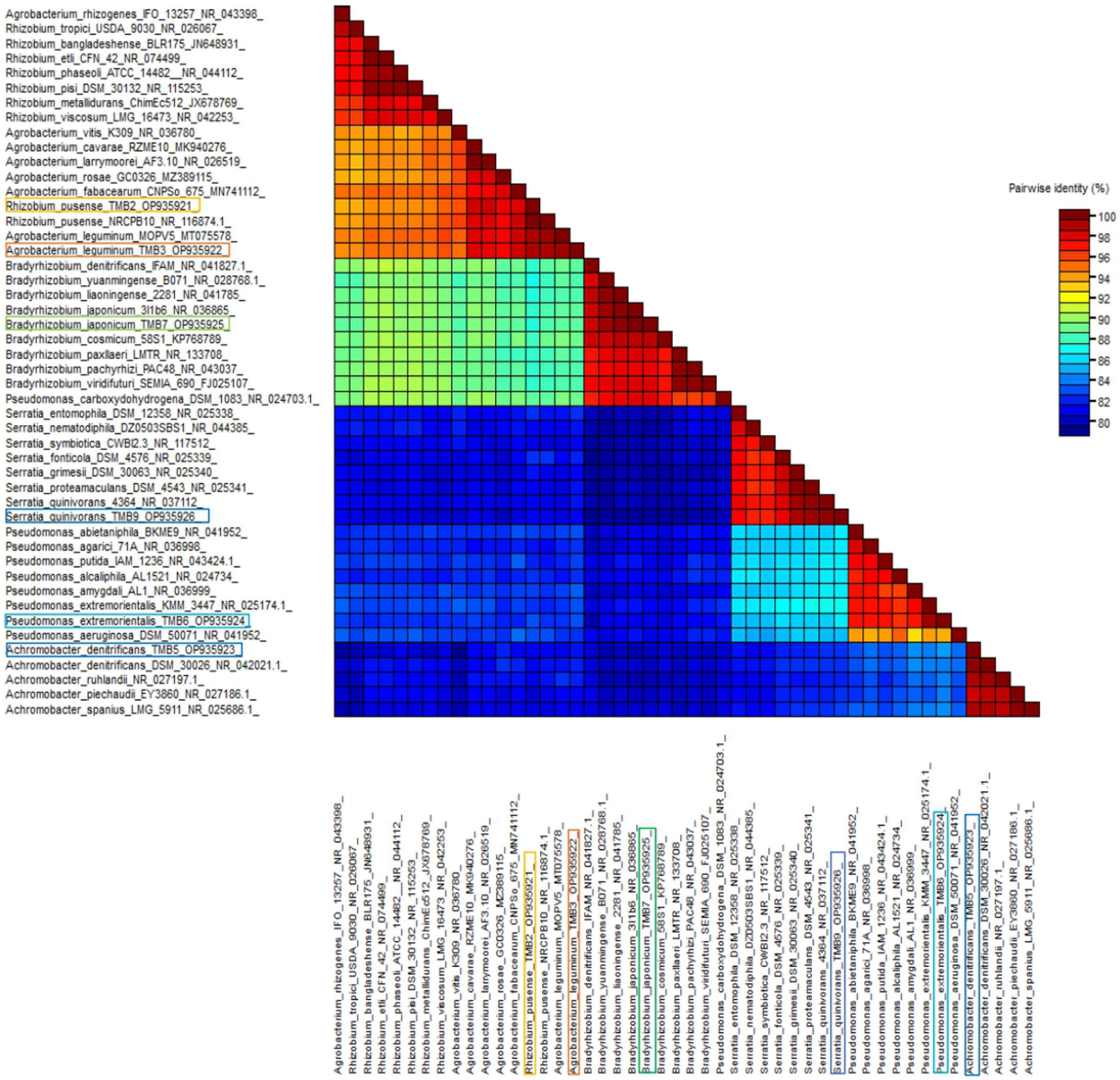

FIGURE 5
Pairwise identity chart of mungbean root endophytic bacteria. The identity percentage of different bacterial sequences ranged 80–100.

TABLE 4 Effect of potential salinity-tolerant endophytic bacteria on mungbean growth under controlled-conditions.

Treatment	Root length (cm)	Shoot length (cm)	Plant fresh weight (mg)	Plant dry weight (mg)	Number of nodules per plant
Control	21 ± 1.7^{cd}	30.8 ± 1.3^{b}	737 ± 10^{e}	82.5 ± 8^{cd}	0
TMB2	29.5 ± 1.32^{a}	42.8 ± 1.6^{a}	950 ± 30^{d}	110 ± 20^{abc}	26 ± 0.8^{b}
TMB3	29.3 ± 1.4^{ab}	42 ± 2.0^{a}	920 ± 60^{d}	90 ± 8^{bcd}	0
TMB5	22 ± 1.5^{cd}	41.5 ± 1.7^{a}	970 ± 50^{cd}	105 ± 20^{abcd}	0
TMB6	31.5 ± 1.3^{a}	43 ± 1.1^{a}	$1{,}160 \pm 40^{ab}$	125 ± 6^{ab}	0
TMB7	33.3 ± 1.8^{a}	44.8 ± 0.9^{a}	$1{,}230 \pm 20^{a}$	132.5 ± 8^{a}	31.3 ± 0.5^{a}
TMB9	24.8 ± 1.1^{bc}	42.5 ± 0.6^{a}	$1{,}080 \pm 40^{bc}$	120 ± 12^{ab}	0
Consortia	19 ± 2.1^{d}	32.3 ± 0.9^{b}	640 ± 20^{e}	72.5 ± 6^{c}	19.5 ± 1^{c}
LSD (0.05)	4.53	3.9	117	37	1.4
ANOVA	***	***	***	*	***

Each value represents mean ($n = 4$) ± standard error. Values followed by the different letters in same column indicate significant difference and followed by same letters are not significantly different. *Represents significance and *** represents high significance. LSD, least significant difference.

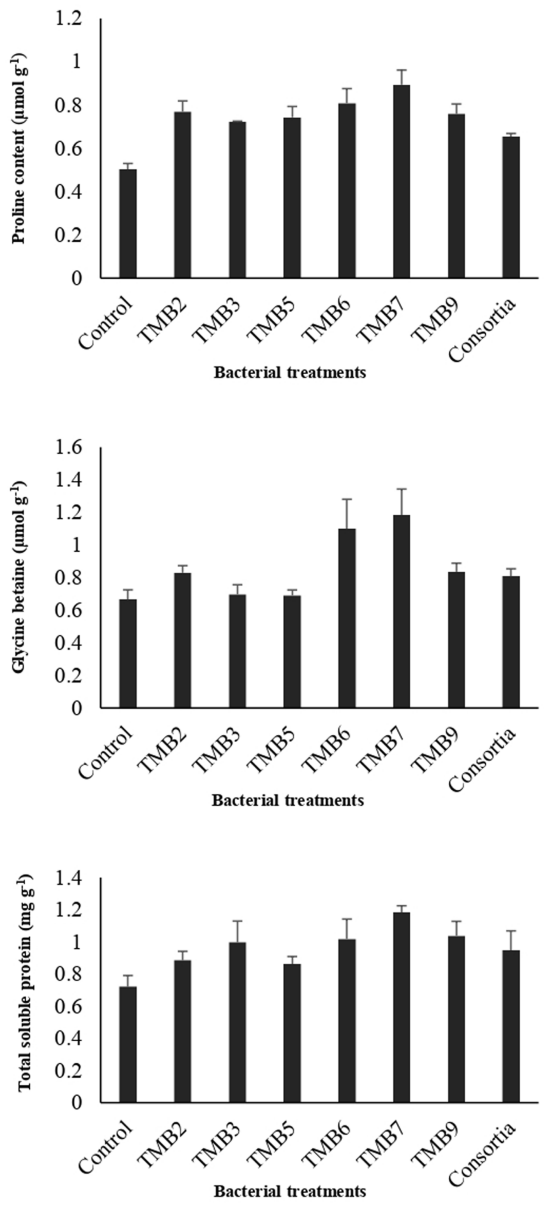

FIGURE 6
Effect of potential salt-tolerant endophytic bacteria (STEB) on biochemical contents of mungbean under salt stress. Concentrations of proline, glycine betaine and total soluble proteins were determined. Inoculation of TMB7 showed significant potential to increase proline contents by 77%, glycine betaine by 78% and total soluble proteins by 64% compared to control.

boosting the defense mechanism against oxidative damage under salt stress conditions (Diagne et al., 2020). Our results are in agreement with the previous studies which reported that the inoculations of bacteria enhanced legumes plant biochemical properties such as proline and glycine betaine content under salt stress (Ashraf and Bashir, 2003). Irshad et al. (2021) demonstrated that inoculation of *Rhizobium* sp. enhanced salt tolerance in *medicago truncatula* by increasing glycine betaine, proline, total soluble proteins and solutes contents. Mushtaq et al. (2021) also revealed that inoculation of *Rhizobium* enhanced total soluble proteins and proline contents in *Cicer arietinum* to alleviate salt stress.

Kaur et al. (2022) described that the inoculation of *P. extremorientalis* on peal millet increased plant growth parameters which are in agreement with our results. Devi et al. (2022) also demonstrated that inoculation of *P. extremorientalis* increased the plant growth parameters such as fresh weight, dry weight, shoot length and root length of chili. Similarly, Kiruthika and Arunkumar (2021) also demonstrated that inoculation of *B. japonicum* increased fresh and dry weight of mungbean. Miljaković et al. (2022) also reported that inoculation of *B. japonicum* improved growth parameters of soybean. Similarly, Zveushe et al. (2023) demonstrated that *Bradyrhizobium japonicum* enhanced plant growth parameters of soybean under salt stress. Our results are in agreement with Kumar et al. (2021), who demonstrated the potential role of *S. quinivorans* inoculation to increase the growth of *Picrorhiza kurroa* under control condition experiments. In this study, consortia did not perform well for plant growth promotion. Our results are in disagreement with Mogal et al. (2022) who reported that consortia of rhizobial bacteria have positive effects on plant growth parameters of mungbean. Previously, Consentino et al. (2022) described that inoculation of consortia did not improve growth parameters of lettuce, compared to the inoculation of pure bacterial culture. Poor performance of bacterial consortia can be attributed to the antagonism which may exist among the different bacteria of consortia. Single inoculations performed better for mungbean growth promotion and the extent of growth improvement corresponds to the bacterial ability to produce plant growth-promoting substances.

Conclusion

Out of thirteen root endophytic bacteria, six isolates, TMB2, TMB3, TMB5, TMB6, TMB7 and TMB9, were able to tolerate salinity up to 2% NaCl and have the *in vitro* potential to produce plant growth-promoting substances under salt stress conditions. Phylogenetic analysis revealed the novel association of *Agrobacterium leguminum, Achromobacter denitrificans, Pseudomonas extremorientalis* and *Serratia quinivorans* with roots of mungbean. Inoculation of bacterial isolates, *Pseudomonas extremorientalis* TMB6, *Bradyrhizobium japonicum* TMB7 and *Serratia quinivorans* TMB9, showed maximum potential in improving plant growth and development under salt stress conditions. These potential salt-tolerant endophytic bacteria can be used as biofertilizer after field-testing for better production of mungbean crop at salt-affected lands.

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

MT and SZ conceived the project and designed the study. SZ, MT, AA, and MA collected the samples and performed the experiments. SZ, MT, MA, MZ, and MS performed data analysis. SZ, MT, MA, MZ, and TY wrote the manuscript. SZ, MT, TY, HZ, and MA applied statistics and critically reviewed manuscript. All authors contributed to the article and approved the submitted version.

Funding

The current work was funded by Higher Education Commission (HEC) Pakistan under the NRPU research project no. 8278/Punjab/NRPU/R&D/HEC/2017 awarded to MT.

Acknowledgments

Authors are highly thankful to Satya Kirri, Graduate Student, Department of Biological Chemistry, University of California, Irvine, USA, for proofreading and improving the write-up of manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1149004/full#supplementary-material

SUPPLEMENTARY FIGURE
Effect of inoculation of potential salt-tolerant endophytic bacteria (STEB) on mungbean growth under controlled-conditions. Inoculation of bacterial isolates, *P. extremorientalis* TMB6, *B. japonicum* TMB7 and *S. quinivorans* TMB9, showed maximum potential in improving plant growth parameters. Photographed at 4-week stage.

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